Fabrication of Zein Nanoparticle-Biopolymer Complexes to Deliver Essential Oils in Aqueous Dispersions

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ABSTRACT

Zein, alcohol-soluble corn proteins, easily precipitate as nanoparticles after dispersing a stock zein solution with a polar solvent into water. The process can be used to nanoencapsulate essential oils (EOs). However, the stability of hydrophobic zein nanoparticles is a challenge for their application in aqueous food systems. The goal of this dissertation reseach was to stabilize zein nanoparticles in aqueous dispersions by forming complexes with water-soluble biopolymers. The stabilization of zein nanoparticles was first studied using sodium caseinate by dispersing hot 50% aqueous ethanol solution with both polymers in water. The nanoparticles contained κ-casein and zein, and the spray-dried nanoparticles were easily re-dispersed in water with good stability at pH 7.0 during storage. Before advancing to encapsulate EOCs, the distribution of free EO components (EOCs) in milk was studied in relation to their antimicrobial activity. It was found that, when the concentration of EOCs in the milk serum was above the minimum bactericidal concentration (MBCs) for *Listeria monocytogenes* Scott A (established in tryptic soy broth), complete inhibition was observed in milk. Conversely, when zein/casein complexes were used to co-encapsulate eugenol and thymol, the bactericidal effect against *Escherichia coli* O157:H7 and bacteriostatic effect against *L. monocytogenes* were observed at concentrations of EOCs in the milk serum well-below their corresponding MBCs. This suggested a maintenance of, and possible synergistic activity, of EOCs after nanoencapsulation. The spray-dried nanocapsules with co-encapsulated thymol and eugenol were easily hydrated in water as stable dispersions with particles smaller than 200 nm. Because zein/casein nanocomplexes were stable only at around neutral pH, gum arabic (GA) was also studied as another biopolymer to form

complexes with zein nanoparticles. Zein was pre-dissolved in propylene glycol, with peppermint oil as an EO model, and dispersed in water to form nanoparticles. The subsequent addition of GA formed nanocomplexes smaller than 200 nm and stable at pH 3.0-8.0. A gradual release of peppermint oil from freeze-dried samples was observed at pH 2.0-8.0, with a faster release at lower pHs. Therefore, water-soluble biopolymers such as caseins and GA can stabilize zein nanoparticles to enable the delivery of bioactive compounds in aqueous food dispersions.

Key words: zein nanoparticles, sodium caseinate, gum arabic, nanocomplexes, essential oils, dispersion stability

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Chapter 1. Introduction and literature review

1.1 Introduction

Essential oils (EOs) are important products that are valued for their flavor and aroma for a long history. Currently, the use of EOs as natural antimicrobial agents to replace traditional antimicrobials is increasingly investigated. However, the low solubility of EOs in water limits their application in aqueous systems. This calls for appropriate delivery systems such as o/w emulsions prepared with generally-recognized-as-safe (GRAS) biopolymers.

Zein is a group of GRAS and very hydrophobic proteins derived from maize and is considered as a good candidate for encapsulating hydrophobic EOs. As already studied, EOs encapsulated in zein nanoparticles after spray drying and freeze drying maintained their antimicrobial activity (Li et al., 2013a; Wu et al., 2012a; Xiao et al., 2011b; Xiao et al., 2011c). However, hydrophobic zein nanoparticles in aqueous dispersions, especially at pH 7.0, can precipitate. Technologies are needed to stabilize zein nanoparticles at a wide pH range to enable their application in various food products.

1.2 An overview of essential oils

1.2.1 Source and use of EOs

EOs are natural plant products with various biological properties. EOs are the aromatic volatile liquids or semi-liquids obtained from plant or plant parts such as flower, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, roots and resins (Burt, 2004; Saad et al., 2013). Due to their volatility, they are also called volatile or ethereal oils (Burt, 2004). EOs are secondary metabolites that play roles as antibacterial, antiviral, antifungal, and insecticidal agents for plants and also against herbivores for plant itself (Bakkali et al., 2008). EOs are traditionally used as spices and seasonings in culinary and

processed foods. Orange, cornmint and peppermint oils are the top three flavor EOs widely consumed yearly (Chizzola, 2013). EOs and EO components (EOCs) are also used as fragrances in cosmetics products such as skin creams, lip balms, shampoos, soaps and perfumes (Chizzola, 2013). Recently, EOs are gaining interest as antimicrobial agents to be applied in food as an additional hurdle post-processing to prolong the shelf life, due to their natural sources which is attractive to consumers.

1.2.2 Methods used to extract EOs from plants and plant parts

EOs are present in various parts of plants and are mainly accumulated in secretory cells, cavities, canals, epidermic cells or glandular trichomes due to their lipophilicity (Bakkali et al., 2008). To obtain EOs, several methods have been applied, e.g., expression (cold pressure), solvent extraction, enfleurage, fermentation, simultaneous distillation–extraction (SDE), microwave distillation and supercritical fluid extraction (SFE), and water/steam distillation (Damian and Damian, 1995; Lucchesi et al., 2004; Saad et al., 2013). While SDE, SFE, and other methods have a higher yield of oils with a larger variety of components than water/steam distillation methods (Guan et al., 2007; Ozel and Kaymaz, 2004; Paibon et al., 2011), EOs from citrus fruits can only be obtained via water/steam distillation or the cold pressure method according to the International Organization for Standardization (ISO 9235, 1997) (Turek and Stintzing, 2013). This is because other methods may lead to the extraction of many compounds that are not usual EO compounds, e.g., wax and chlorophyll (Guan et al., 2007). In laboratories, the water distillation (hydrodistillation) method with a Clevenger-type apparatus is usually adapted to extract EOs (Lucchesi et al., 2004; Vardar-Unlu et al., 2003).

1.2.3 Compositions of EOs

EOs are complex mixtures which can contain hundreds of components. The composition of EOs varies with the material and method used in extraction. For example, the EO hydrodistilled from the roots, stem and leaves, and flower of *Cuminum cyminum* L. contained bornyl acetate (23%), α-terpinene (34%) and γ-terpinene (51%), respectively, as the major component (Bettaieb et al., 2010). The plant harvested at different times of year also results in different oil yield and distribution of major components. For example, Greek oregano harvested in July had the highest oil yield, and thymol content changed from 149 mg/100 g in April to 1124 mg/100 g in July (Jerković et al., 2001).

Structurally, components in the EOs can be classified into major groups including monoterpenes (C_{10}), sesquiterpene hydrocarbons (C_{15}), and phenylpropanoids (Saad et al., 2013). All these compounds are low-molecular-weight molecules (Bakkali et al., 2008) and therefore they have high vapor pressure. The structure and biosynthesis pathways of monoterpenes and sesquiterpenes was reviewed in a recent paper (Chizzola, 2013).

1.2.3.1 Monoterpenes

Isoprene is a five carbon (C_5) base unit for both monoterpenes and sesquiterpenes (Bakkali et al., 2008; Chizzola, 2013). Monoterpenes have a basic molecular formula of $C_{10}H_{16}$, while sesquiterpenes are $C_{15}H_{24}$ (Hüsnü et al., 2007). Based on the skeleton, the monoterpenes could be categorized as acyclic, monocyclic, bicyclic and tricyclic. Diverse structures can be derived from the very basic carbon skeleton after isomerization rearrangement or oxygenation to alcohols, aldehydes, ketones or esters. Terpenes with

oxygen are also called terpenoids (Hüsnü et al., 2007). Representative structures of some monoterpenes, such as *p*-cymene, limonene, linalool, thymol, and carvacrol are listed in Figure 1-1. Monoterpenes usually constitute 90% of EOs (Bakkali et al., 2008).

1.2.3.2 Sesquiterpenes

Similar to monoterpenes, sesquiterpenes can be classified into acyclic, monocyclic, bicyclic, tricyclic, and nor-sesquiterpenes. Cyclic structures are the most diverse (Hüsnü et al., 2007). Nor-sesquiterpenes have one or more fewer carbon atoms but still have the structure of sesquiterpenes (Chizzola, 2013) and therefore are still included in sesquiterpenes. Examples of plants containing sesquiterpenes are peppermint, orange, lavender, rosemary, and agarwood (Bakkali et al., 2008; Naef, 2011). The structures of representative sesquiterpenes, including guaiane and β-caryophyllene are presented in Figure 1-2.

1.2.3.3 Phenylpropanoids

Phenylpropanoids contain one or more C6–C3 fragments, and the C6 unit is a benzene ring (Hüsnü et al., 2007). Common phenylpropanoids include anethole from anise and fennel (Karapinar and Esen Aktuğ, 1987), estragole from basil oil (Bagamboula et al., 2004), eugenol from clove bud oil (Karapinar and Esen Aktuğ, 1987), cinnamaldehyde from cinnamon bark and leave oil (Singh et al., 2007) and vanillin from vanilla bean (Jadhav et al., 2009) (Figure 1-3).

1.2.4 Biological activity of EOs

1.2.4.1 Antimicrobial activity

Contamination by microorganisms, including bacteria and fungi, of food is a major risk to consumer food safety. Viable microorganisms could cause severe infections

or intoxications to humans. The use of EOs or EOCs as antimicrobial agents are gaining interest because they have natural sources and may be classified as organic. Recently, many studies on composition and antimicrobial activity of EOs from various sources have been published. More than 70 EOs and their components have been listed as GRAS additive by FDA (http://www.libertynatural.com/info/eoinfo/FDA_EO_GRAS.htm). A few preservatives containing EOs are already commercially available. For example, 'DMC Base Natural' is a food preservative comprising 50% essential oils from rosemary, sage and citrus and 50% glycerol, which has been used in Spain (MendozaYepes et al., 1997).

Antimicrobial activities of EOs have been demonstrated against a wide variety of microorganisms, including Gram-positive and Gram-negative bacteria as well as fungi (Burt, 2004). Basil and thyme EOs and their major components inhibit the growth of *Shigella sonnei* and *S. flexneri* in both agar medium and food model matrix lettuce (Bagamboula et al., 2004). Clove bud oil has been found to exhibit activities to bacteria *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Campylobacter jejuni*, *Salmonella* Enteritidis, *Escherichia coli*, *Staphylococcus aureus* and the fungi *Candida albicans* and *Trichophyton mentagrophytes* (Chaieb et al., 2007). Oregano essential oil is effective against *Pseudomonas aeruginosa* and *E. coli* (Bozin et al., 2006). Tea tree oil has been used for almost 100 years in Australia, and it shows a broad spectrum activity to more than 27 strains of bacteria, including *Acinetobacter baumannii*, *Bacillus cereus*, *S. aureus* (methicillin resistant), and 24 strains of fungi, such as *Aspergillus flavus*, *C. albicans*, *Cryptococcus neoformans* (Carson et al., 2006).

Besides complete EOs, some components from EOs are highly active. Thymol and carvacrol, major components in EOs derived from thyme and oregano, have phenol structures and are very active against a wide spectrum of microorganisms, including pathogens *Listeria* spp., *Shigella* spp. and *Escherichia* spp. (Burt, 2004; Koroch et al., 2007). Eugenol derived from clove oil, also a phenolic compound, demonstrated less potent activity than thymol and carvacrol, while cinnamaldehyde derived from cinnamon bark oil with the aldehyde group displayed similar effect to thymol and carvacrol against *E. coli* (Pei et al., 2009). Pulegone, fenchone, α-thujone and camphor, which are ketones, were reported to have moderate antimicrobial activities (Koroch et al., 2007). Menthol and linalool were also reported to have moderate activity against bacteria, such as *S. aureus, E. coli,* periodontopathic and cariogenic bacteria (Park et al., 2012; Trombetta et al., 2005). Minor components in EOs are usually not discussed but their activity should not be ignored. This happened to the antibacterial activity of sage EO being contributed by the synergistic effect from its minor components (Marino et al., 2001), due to the major components cannot explain the difference. Generally, the antimicrobial activity of EOCs with different functional groups follows the decreasing order of phenols > aldehydes > ketones > alcohols > esters > hydrocarbons (Koroch et al., 2007).

The modes of action of EOs or EOCs to microorganisms have been hypothesized to include degradation of the cell wall, interaction with membrane proteins, lysis or disruption of the cytoplasmic membrane, increasing the cell permeability, leakage of cell contents such as protein or ATP, and inhibition of ATPase (Chizzola, 2013; Hyldgaard et al., 2012b; Saad et al., 2013). It is likely that most or all of these modes contribute to the antimicrobial activity, tut a complete and clear mechanism has not been elucidated.

1.2.4.2 Anti-viral activity

The antiviral activity of EOs has been investigated less than their antibacterial activities. To study antiviral activity, one of the most commonly studied human pathogens, herpes simplex virus (HSV, type 1 or type 2), is often used because of its prevalence (Wei and Shibamoto, 2010b). Studies also have demonstrated the great potential of EOs against viruses. EOs from anise, hyssop, thyme, ginger, camomile and sandalwood were screened for their inhibitory effect against HSV-2 in vitro on RC-37 cells using a plaque reduction assay (Koch et al., 2008). The inhibitory concentrations (IC_{50}) were determined to be 0.016, 0.0075, 0.007, 0.004, 0.003 and 0.0015% for anise oil, hyssop oil, thyme oil, ginger oil, camomile oil and sandalwood oil, respectively. A study by Astani and colleagues (2009) showed that 10 out of 11 tested monoterpenes, including α-terpinene, γ-terpinene,α-pinene, β-pinene, α-terpineol, terpinene-4-ol, limonene, thymol, *p*-cymene, citral and 1,8-cineol, showed high antiviral activity against free HSV. At a noncytotoxic concentration, except for 1,8-cineole, all the other components reduced plaque formation by 80–100 % (Astani et al., 2009). The authors also found that the hydrocarbon monoterpenes displayed better inactivation activity than the oxygenated ones (Astani et al., 2009). Schnitzler *et al.* (2001) examined the antiviral activity of eucalyptus oil against HSV-1 and HSV-2 in vitro on RC-37 cells using a plaque reduction assay. The IC_{50} value of the oil was 0.009% for the HSV-1 and 0.008% for the HSV-2. Recently, Usachev *et al.* (2013) investigated the antiviral activity of tea tree oil and eucalyptus oil aerosols in range of concentrations against enveloped Influenza A virus and non-enveloped *E. coli* phage M13. Results showed that the exposure of tested

oils for 5-15 min could inactive the two viruses by 95%, which indicated the strong antiviral action of these two EOs (Usachev et al., 2013).

1.2.4.3 Antioxidant activity

Oxidation damages various biological substances and subsequently causes many diseases, including cancer, liver disease, Alzheimer's disease, aging, and inflammation (Shaaban et al., 2012). Oxidation of food lipid resulted in off-flavor, reducing the nutritional value, probably developing atherosclerosis for consumers (Jacobsen, 1999). Monoterpenes and sesquiterpenes, major EOCs, have strong antioxidant activity (Graßmann, 2005) and, therefore, most EOs are expected to have some antioxidant activity.

From a structure perspective, EOCs with a phenol group should have good antioxidant activity since almost all phenols can function as antioxidants of lipid peroxidation (Shahidi et al., 1992). In one study, among the 25 EOs tested, thyme oil exhibited the greatest antioxidant activity, followed by clove leaf, cinnamon leaf, basil, eucalyptus, and chamomile oils (Wei and Shibamoto, 2010a). This was attributed to the high content of thymol in thyme oil (23%) and eugenol (77%) in clove leaf oil (Wei and Shibamoto, 2010a). At higher concentrations, the antioxidant activities of thymol and carvacrol were close to that of α-tocopherol, which is a strong free radical scavenger (Koroch et al., 2007). For tea tree oil, which has been commercialized in cosmetics, the main antioxidant component is terpinen-4-ol (Lee et al., 2013). Another commercialized oil is rosemary, which also displays antioxidant activity and has been used for its anti-aging property in addition to fragrance in cosmetics (Cronin and Draelos, 2010; Erkan et al., 2008).

1.2.4.4 Anti-carcinogenic activity

Due to their antioxidant activity, EOs are believed to be directly anti-carcinogenic because of their radical scavenging properties (Bakkali et al., 2008). Most of anti-carcinogenic compounds, especially these synthetic ones, such as cisplatine, docetaxel and carmustine are also toxic to normao cells as well as those cancerous cells, thereof producing serious side effects after being taken into human body (Remesh et al., 2012; Thanki et al., 2013). In contrast, due to their natural source, EOs are attracting much interest in pharmaceutics. Monoterpenes have been found to inhibit the proliferation of cancer cells (Koroch et al., 2007). In one study using five monoterpenes including thymol, carveol, carvone, eugenol and isopulegol, carvacrol was found to be the most cytotoxic monoterpene against five tumor cell lines (Jaafari et al., 2012). The monoterpenes stopped cell cycle at different stage. Carvacrol and carveol stopped the cell cycle progression in the S phase, which is the DNA synthesis replicates phase, while thymol and isopulegol stopped it in the G0/G1 phase, which is the initial step for the cell division (Jaafari et al., 2012). No effect on cell cycle was observed for carvone or eugenol. Moreover, all of them showed synergistic effect with two conventional anti-tumor drugs (Jaafari et al., 2012). *D*-limonene, which is rich in citrus oil, has been shown to inhibit angiogenesis and metastasis of human colon cancer cells (Murthy et al., 2012). Perillyl alcohol, the oxygenated derivatives of limonene, showed better anticancer and lower toxicity than limonene (Koroch et al., 2007). It has been isolated from EOs of lavendin, peppermint, spearmint, cherries, celery seeds, and several other plants, but at low levels (Belanger, 1998).

1.2.5 Antimicrobial activity of EOs in microbiological media and food systems

To characterize the activity of EOs, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are determined using the broth dilution or agar dilution methods to evaluate the strength of their antimicrobial activity (Burt, 2004). In order to determine immediacy and duration of antimicrobial activity, growth curves can be studied over time at different temperatures (Burt, 2004).

Often the MIC/MBCs of EOs/EOCs in growth media are very low (Table 1-2) indicating excellent antimicrobial activity. However, the antimicrobial activity of EOs in microbiological media is not directly applicable when EOs are used in foods. As shown in Table 1-2, the activity of EOs/EOCs in complex food systems such as milk is significantly reduced when compared to the results in microbiological media. This requires an increased concentration of EOs/EOCs to achieve the same effectiveness as tested in tryptic soy browth (TSB). The reason why higher concentrations of EO/EOCs are needed in complicated food systems is likely their interaction with proteins, lipids and polysaccharides. When hydrophobic EO/EOCs are added to a food system, there is a competition between food components and bacteria to attract EOCs. Some amino acid residues of proteins and lipids are hydrophobic, while bacteria have hydrophilic surfaces and therefore are expected to disperse in the aqueous phase of food. According to "like dissolves like" rule, EOCs are expected to partition to a higher extent to hydrophobic proteins and lipids (Gaysinsky et al., 2005), and as a result, only a small portion of EOCs is present in the aqueous phase of foods. Furthermore, from the environmental stress aspect, bacteria will minimize these EOCs with antibacterial activities from approaching their surface because EOCs function as "enemies" (Ben-Jacob et al., 2000). The effects of

polysaccharides on the activity of EO/EOCs are less studied, but they increase the viscosity of foods and thereof may limit the diffusion of EOCs in the continuous aqueous phase to access bacteria (Ma et al., 2013).

1.3 An overview of delivery systems studied for EOs

Many EOs are only sparingly soluble in water (up to *ca*. 0.1 %) which limits their application (Weiss et al., 2009). Therefore, various delivery systems have been proposed and developed to enhance solubility, bioavailability and efficacy of EOs by limiting the interactions with the hydrophobic food components and controlling their release. For example, encapsulation can increase the concentration of antimicrobial compounds in food areas where microorganisms preferably locate, *e.g.* water-rich phases or liquid–solid interfaces (Weiss et al., 2009). Encapsulated EOs can be applied as films on solid food matrices or colloidal particles for distribution in aqueous food matrices. The latter is being reviewed in this chapter.

1.3.1 EO emulsions prepared with small-molecular surfactants

Small-molecular surfactants, such as polysorbates, in aqueous solutions above their critical micellar concentration (CMC) could self-aggregate into a defined geometry such as micelles with a hydrophobic core and a hydrophilic shell (Figure 1-4a). When EOs are mixed in an aqueous solution of surfactants in a homogenizer or microfludizer, surfactants adsorb on the newly-formed oil droplet surface and can stabilize the interface to form emulsions. The reduction of the oil/water (o/w) interfacial tension by surfactants facilitates further droplet disruption to finer particles. Based on the particle dimension, emulsions can be classified as macroemulsions and nanoemulsions. Macroemulsions

usually have a size range of 0.1-5 μm, and nanoemulsions have a size range of 20-100 nm. Both groups of emulsions are only kinetically stable (Tadros, 2013).

A third group of surfactant-oil-water mixtures is thermodynamically stable microemulsions that have a droplet size range of 5-50 nm (Tadros, 2013). In addition to a major surfactant, a co-solvent and/or a second surfactant is used to facilitate the formation of microemulsions. Multiple surfactants modify the overall hydrophile–lipophile balance (HLB) of the system. Usually a low HLB surfactant is used with a high HLB surfactant to get an appropriate HLB for an oil (Narang et al., 2007). Surfactants with HLB values >7 are predominantly hydrophilic and favor the formation of o/w emulsions, while surfactants with HLB values <7 are hydrophobic and form w/o emulsions (Walstra, 2002). Co-solvents (co-surfactants) are usually used to increase the solubility of EOs, reduce the interfacial tension, and stabilize the dispersed phase (Narang et al., 2007). Commonly used co-surfactants include ethanol and propylene glycol. Microemulsions usually have transparent appearance and are suitable for various food products, especially beverages requiring clear appearance.

The shortcoming of these kinds of delivery systems are that the surfactants are usually food-grade (such as polysorbates and span 80) but not GRAS. Their amount allowed in food is limited (http://www.fda.gov/). The World Health Organization also suggests the use of synthetic surfactants, for example a daily intake of Tween® 20 not higher than 25 mg per kg body weight (http://www.who.int/en/). Some researchers have debated on the toxicity of high doses of polysorbates tested in model animals (Csaki, 2011; Ema et al., 2008). Due to these reasons, the use of non-GRAS surfactants is a concern for consumers.

1.3.2 Liposomal vesicles

Liposomes are vesicle structures self-assembled from phospholipids in aqueous solutions, consisting of an aqueous medium surrounded by a lipid membrane (Varona et al., 2011). The vesicle structures can retain water-soluble substances in the inner aqueous phase and oil-soluble substances like EOs in the lipid bilayer membrane. Liposomes can consist of one or more lipid bilayers, suitable for entrapping hydrophilic and hydrophobic compounds, respectively (Singh et al., 2012). Liposomes of single bilayer can be further classified into small unilamellar vesicles and large unilamellar vesicles based on their dimension (Figure 1-4b) (Singh et al., 2012). Small unilamellar vesicles usually have a dimension between 20 and 100 nm, and large unilamellar vesicles are bigger than 100 nm (Sherry et al., 2013).

Several investigations have demonstrated the improvement of biological activity of EOs when encapsulated into liposomal delivery systems. *In vitro* anti-herpes activity of EO extracted from *Artemisia arborescens* L. was observed to have significantly increased after encapsulating the EO in multi-layered liposomes, corresponding to 100% vs. 22.86% reduction of viral cytopathic effects at 100 μg/ml of EO (Sinico et al., 2005). The inhibition zone of thymol and carvacrol or their combination against tested bacteria strains was increased after encapsulated in liposomes (Liolios et al., 2009). It was attributed to the better interaction of liposomes with cell membrane, which improved the cellular transport and released the active component inside the cell (Liolios et al., 2009). Bergamot EO encapsulated in liposomal vesicles also demonstrated the increased anticancer activity in vitro against human SH-SY5Y neuroblastoma cells (Celia et al.,

2013). The similar structure of liposomes with cell membrane may increase its affinity with cell membrane, which improves the target sites and mass transfer of EOs.

Even though liposomes are considered as GRAS and have been applied in the marketplace for the purpose of food fortification and as nutritional supplements, there are some obstacles of liposomes as a carrier material. The most significant ones include the high cost of food-grade raw materials for liposome preparation and poor loading capacity and encapsulation efficiency (Sagalowicz and Leser, 2010). Therefore, the application of liposomes in the food industry is limited.

1.3.3 Biopolymer-based emulsions as delivery systems

In addition to the above delivery systems, naturally derived biopolymers, i.e., polysaccharides and proteins have been widely investigated as wall materials to encapsulate EOs for evenly distribution in the aqueous phase. Wet emulsions prepared from biopolymers are also dried to prepare powdered particles for the convenience of transportation and application.

1.3.3.1 Polysaccharides

Most water-soluble polysaccharides can act as stabilizers of o/w emulsions, but only a few of them can act as emulsifiers. The most widely used polysaccharide emulsifiers in food applications are gum arabic (*Acacia senegal*), pectin, chitosan, modified starches, modified celluloses, and some galactomannans (Dickinson, 2009; Pedro et al., 2009). The emulsifying property of these polysaccharides derives from either the non-polar nature of chemical groups attached to the hydrophilic polysaccharide backbone (e.g., hydrophobically modified starch/cellulose) or the presence of a protein

component linked covalently or physically to the polysaccharide (e.g. gums and pectins) (Dickinson, 2009).

Gum arabic is a hetero-polysaccharide containing both protein and polysaccharide residues, including arabinogalactan, glycoprotein, and arabinogalactan-protein complex. The arabinogalactan-protein fraction is considered to be mostly responsible for the emulsifying properties of the gum, forming "wattle blossom" type structure on the oil surface (Dickinson, 2003). Gum arabic is the most commonly used polysaccharide to encapsulate flavor oils for their use in beverages (Akhtar and Dickinson, 2007) due to its good emulsifying property and relative low viscosity in comparison with other surface-active polysaccharides (Weinbreck et al., 2004). Studies showed that gum arabic has good capacity of emulsifying and stabilizing EOs. When centrifugation was used to study the stability, the absorbance at 400 nm of emulsions containing 6.5% orange oil and 20% gum arabic did not change over 45 min of centrifugation (Tse and Reineccius, 1995). Emulsions with 5% w/w orange oil stabilized by gum arabic had better stability against droplet aggregation in a wide range of pH values, ionic strengths, and temperatures than emulsions stabilized by β-lactoglobulin, due to strong steric repulsion of gum arabic rather than electrostatic repulsion (Qian et al., 2011). In a current study, gum arabic was used to encapsulate EOC with antimicrobial activity, and the EOC activity after encapsulation was compared with free EOC. It was found thatwhen 1% w/w clove bud oil was emulsified by 1% w/w gum arabic, the particle size increased after 7-d storage, but the MIC/MBC of the emulsion against several bacteria did not change when compared to free clove bud oil (Luo et al., 2014b). Additionally, the emulsion stabilized

by gum arabic showed better short-term reduction of *L. monocytogenes* Scott A than free clove bud oil in TSB.

Pectin is another widely studied polysaccharide. Typically, commercial pectin extracted from citrus peel or apple is not effective as emulsifying agents irrespective of the degree of esterification. However, pectin from sugar beet has a protein content (10.4%) higher than that from apple (1.6%) and citrus (3−3.3%) (Li et al., 2013b; Williams et al., 2005) and there has strong emulsifying property. The higher contents of ferulic acid and acetyl groups were also reported as the reasons for its good emulsifying property (Li et al., 2012b; Williams et al., 2005). In a study comparing emulsifying property of sugar beet pectin, soybean soluble polysaccharide, and gum arabic, o/w emulsions prepared with sugar beet pectin were found to be the most stable (Nakauma et al., 2008).

Chitosan, a linear polysaccharide, is the *N*-deacetylation product of chitin which is the second most abundant naturally occurring polysaccharide after cellulose (Pedro et al., 2009). Chitosan is composed of glucosamine and *N*-acteyl glucosamine units that are linked via ß(1-4) glycosidic bonds and are randomly or block distributed throughout the biopolymer chain, depending on the method used to derive chitosan from chitin (Pedro et al., 2009). It has three amino groups in its structure, attributing to its positive charges that lead to its antimicrobial activity (Pedro et al., 2009). Chitosan has good properties of forming films that have been widely studied to associate EOs to control their release, which are potential packing materials to prolong the shelf life of foods (Hosseini et al., 2009; Peng and Li, 2014; Zivanovic et al., 2005). Starch and cellulose are usually not amphiphilic, but grafting of various hydrophobic groups onto the polysaccharide

backbone can produce amphiphilic properties feasible for emulsifying oils (Almgren et al., 2007; Nilsson and Bergenstahl, 2006).

1.3.3.2 Proteins

Proteins have amphiphilic structures, making them good emulsifiers. Similar to polysaccharides, food proteins are considered as GRAS, biodegradable, non-antigenic, highly nutritional, abundant, and renewable (Elzoghby et al., 2012). Compared to polysaccharides, proteins are much more surface active and have faster interfacial adsorption kinetics (Li et al., 2012b). As a result, a great amount of experimental work has been carried out on creating protein-based o/w emulsions. Proteins can be animal- or plant-derived. Animal proteins with emulsifying capacity include dairy proteins (caseins and whey proteins), gelatin, albumin, and collagen. Among them, dairy proteins, especially caseins, are studied more frequently than other proteins due to their availability, relatively low cost, excellent emulsifying and self-assembly functionalities (Elzoghby et al., 2012). Well-known plant proteins include prolamins (zein) from corn and proteins from soybean (Elzoghby et al., 2012).

When proteins are used to prepare emulsions, a higher protein content typically results in a higher amount of oil loading, and smaller particles, and more stable emulsions (Bouyer et al., 2011; Sun and Gunasekaran, 2009). When 0.2% w/w xanthan gum and various amounts of whey protein isolate were used to prepare emulsions, the increase of whey protein isolate concentration decreased particle size and led to additional negative charges on oil droplet surfaces, which consequently prevented oil droplets from aggregation or coalescence due to the strengthened steric and electrostatic repulsions (Sun and Gunasekaran, 2009). In another study, emulsions with 30% almond oil

emulsified in 0.5% w/w β-lactoglobulin were macroscopically stable for 20 days, whereas those emulsified in 2.5% w/w β-lactoglobulin remained stable over 122 days (Bouyer et al., 2011). However, if the amount of whey protein exceeds the amount required to cover the surfaces of the oil droplets, increases in protein concentration may not reduce the particle size any further or may even increase particle size (Onsaard et al., 2006).

1.3.3.3 Protein-saccharide conjugates

Many protein and saccharides (are cheaper than naturally-occurring protein-polysaccharide conjugates with emulsifying properties. This has drawn interest in producing covalently-linked protein-reducing saccharide conjugates in the laboratory using the Maillard reaction by heating a solution (wet method) or powder (dry method) under controlled conditions. In the dry method, relative humidity, heating duration and temperature, and ratio and nature of protein and saccharides are variables determining the properties of produced conjugates (Chevalier et al., 2001; Liu and Zhong, 2013; Nursten, 2005). The attachment of the saccharide on proteins can decrease the protein *pI* (Liu and Zhong, 2013; Wang and Zhong, 2014) and increase its solubility under acidic conditions (Chevalier et al., 2001; Li et al., 2013c), as well as affect the gelling (Matsudomi et al., 2002) and emulsifying properties of proteins (Ho et al., 2000; Li et al., 2013c; Shah et al., 2012b).

During emulsification, the protein moiety of conjugates is anchored on the surface of oil droplets and the carbohydrate moiety protrudes into the continuous phase. This increases the thickness of interfacial layer and the steric hindrance enhances the colloidal stability when compared to the protein-based emulsion. Rice protein hydrolysates

conjugated with glucose, lactose, maltodextrin, and dextran all showed improved solubility and emulsifying property at neutral pH than hydrolysates alone (Li et al., 2013c). For whey proteins conjugated with maltodextrin (MD) with three different dextrose equivalents, the conjugate with MD of dextrose equivalent to 19 produced emulsions with the smallest particles that were even smaller than emulsions prepared with gum arabic (Akhtar and Dickinson, 2007). This indicates the potential of whey protein- maltodextrin conjugates as replacers for the expensive gum arabic in the food industry. In a series of studies, whey protein-maltodextrin conjugates were emulsified with EOs dissolved in hexane, followed by spray-drying, and the spray-dried powder showed good re-dispersibility in water and had improved antimicrobial activity (Shah et al., 2012a; Shah et al., 2013b; Shah et al., 2012b). There are several shortcomings of Maillard-type conjugates. Firstly, yellow or brown color is derived from the Maillard reaction, which may affect the color of food (Nursten, 2005). Secondly, lysine, one of the essential amino acids, is lost in reducing saccharides during the Maillard reaction (Nursten, 2005). Acrylamide, a compound causing carcinogenic diseases of humans, is also formed during the Maillard reaction (Mottram et al., 2002). Identifying reaction conditions is critical to prepare conjugates with properties appropriate for a specific food product.

1.3.3.4 Protein/polysaccharide complex

Four types of structures can be formed when proteins and polysaccharides are mixed together. When protein and polysaccharide are oppositely charged, they can form soluble complexes or coacervates via weak attractive and nonspecific interactions such as electrostatic, van der Waals, hydrophobic interactions, or hydrogen bonding between

proteins and polysaccharides (McClements, 2006).Visually, there is one-phase when soluble complexes are formed but two phases for coacervates. If there are strong repulsive forces between proteins and polysaccharides due to one or both of the biopolymers having same type of charges, they form one phase solutions with non-associated biopolymers at low concentrations or two-phase system with one biopolymer rich in each phase.

To form emulsions, soluble complexes or coacervates are preferred. The first study on protein-polysaccharide interaction was dated in 1920s, using gum arabic and gelatin which is still being used to encapsulate flavor oils (Evans et al., 2013). This has been frequently reviewed (Benichou et al., 2002; Dickinson, 2008; Evans et al., 2013; Schmitt and Turgeon, 2011). The routes to create emulsions stabilized by electrostatic polysaccharide–protein complexes can be classified into four types that were summarized by Evans et al. (2013) and are presented in Figure 1-5. Traditionally, polysaccharide and protein are premixed to form soluble complexes or coacervates, and the oil phase is added afterwards by stirring or homogenization (Fig. 1-5 I&IV). Another approach is to emulsify the oil phase first by proteins, followed by addition of polysaccharides to form another layer on proteins, also known as multiple-layered emulsions (Fig. 1-5 II&III). A variation to this procedure is to dissolve proteins and polysaccharides together at conditions that they are not associated. When the oil phase is added and homogenized, surface-active proteins adsorb on oil droplets to form primary emulsions. By adjusting pH such that proteins and polysaccharides have opposite charges, polysaccharides adsorb on droplets coated with proteins to form secondary emulsions.

No matter which procedure is used, most studied have presented the improved stability of emulsions prepared from complexes than those with proteins or polysaccharides only (Farshchi et al., 2013; Harnsilawat et al., 2006; Li et al., 2012b; Liu et al., 2012). In one study, 20% sunflower oil prepared with 1.0% w/w sodium caseinate was only stable at pH 6.0 and 6.5 against coalescence, but the supplementation of locust bean gum improved the stability of emulsions even at a pH around the *pI* of sodium caseinate (Farshchi et al., 2013). In another study, corn oil emulsified by sodium caseinate had an initial stability at pH 6 and 7 that was expanded to pH 5.0 for over 60 d after addition of 0.1-0.4% carboxymethylcellulose (Liu et al., 2012). The main reason for the better stability of emulsions stabilized by protein/polysaccharides can be attributed to the dense layer of polysaccharides coating interfacial proteins. The polysaccharide layer can also provide repulsive steric and electrostatic interactions and decrease the van-der-Waals attraction, both of which can stabilize the emulsion by inhibiting droplet aggregation (Liu et al., 2012). The increased viscosity of the continuous phase due to the thickening function of free polysaccharides is also one of the reasons lessening the creaming of emulsion (Farshchi et al., 2013). However, an excess amount of polysaccharides can result in depletion flocculation, which is one of the reasons for the instability of emulsion emulsified by protein/polysaccharides (Farshchi et al., 2013; Liu et al., 2012). Additionally, physical interactions between polysaccharide and protein are sensitive to changes in environment conditions.

1.3.3.5 Other complexes

Other kinds of complexes consisting of polysaccharide, protein, and lipid have also been found to have the improved properties in emulsion systems. When vanilla oil

was encapsulated in chitosan and gum arabic complexes at a vanilla oil:chitosan mass ratio of 2:1, the encapsulation efficiency was as high as 94.2%, and the thermal stability and release property of vanilla oil were improved after encapsulation (Yang et al., 2014). In a recent study, blending gelatin and soybean lecithin resulted in translucent thymol emulsions, while turbid emulsions were observed for treatments prepared with individual emulsifiers (Xue and Zhong, 2014). In another recent study, a combination of gum arabic and lecithin improved surface hydrophobicity and the ability to emulsify clove bud oil when compared to gum arabic and lecithin alone, but the stability of emulsions was not improved for the gum arabic/lecithin combination (Luo et al., 2014b).

1.3.4 Emulsions stabilized by colloidal particles

Emulsions prepared with surface-active particles are known as "Pickering emulsions," was originally observed by Ramsden and studied in detail by Pickering (Vignati et al., 2003). In contrast with the emulsions described above, this type of emulsion does not require amphiphilic surfactants. Colloidal particles with a contact angle <90 °can be used to prepare o/w emulsions, while w/o emulsions can be created for overall hydrophobic particles with a contact angle > 90° (Aveyard et al., 2003; Vignati et al., 2003). The most studied particles are those of silica after surface modification to prepare either w/o or o/w emulsions (Frelichowska et al., 2009). More information about this type of emulsions can be found in reviews by Aveyard et al. (2003) and Tcholakova et al. (2008).

1.4 Zein as a carrier material

1.4.1 Origin, composition, and structure of zein

Zein is the major protein of maize and makes up 35-60% of the total proteins present exclusively in the corn endosperm. In the US, there is about 300-325 million tons (270,000-363,000 Kg) of corn produced per year, which accounts for about 40-50% of the world's annual production (Cheryan, 2009). About 25% of corn is used for ethanol production. Zein can be extracted from byproducts of the milling and bioethanol industries deriving from processes of wet-milling, dry-milling, dry-grind processing, and alkaline treatment (Shukla and Cheryan, 2001). The purity of zein may vary among purification methods. Currently, the price of zein is $10-40/kg depending on purity, which is higher than petroleum-based materials ($2/kg) (Anderson and Lamsal, 2011; Shukla and Cheryan, 2001). Therefore, novel applications of zein with unique properties will increase the demand of zein, which will in turn drive the bioethanol industry to develop more cost-effective processes to extract zein. This will reduce to the price of zein and expand its use.

More than 50% of the amino acids in zein are nonpolar, including proline, leucine, alanine phenylalanine, isoleucine and valine (Shukla and Cheryan, 2001). This makes zein insoluble in water but soluble in aqueous alcohol, high concentrations of urea, alkali solutions (> pH 11), and anionic detergents (Shukla and Cheryan, 2001). Therefore, according to the Osborne classification of cereal proteins, zein is a member of the prolamine group (Lasztity, 1995; Luo and Wang, 2012, 2014). Based on solubility, amino acid sequence, and molecular weight, zein is classified into α-, β-, γ- and δ- zein (Zhang et al., 2011), with amino acid contents listed in Table 1-1 (Larkins et al., 1993).

α-Zein accounts for more than 70% of the total zein protein and is commercially available (Luo and Wang, 2014). It consists of two monomers with molecular weight of 19 (Z19) and 22 (Z22) kDa based on reducing SDS-PAGE (Zhang et al., 2011; Zhong and Ikeda, 2012). Z19 and Z22 contain 210-220 and 240-245 amino acid residues and have the true molecular weight of 23,000-24,000 and 26,500-27,000 Da, respectively (Matsushima et al., 1997b). Both Z19 and Z20 consist of three domains: *N*-terminus, repeating domain, and *C*-terminus (Shewry and Tatham, 1990). Structurally, there are a total of 10 repeating units in Z22 and 9 in Z19, and the additional repeating unit of Z22 is present at the *C*-terminal end (Shewry and Tatham, 1990). The structure model of the repeating domains of α-zein was first characterized by Argos et al. (1982) and later revised by Matsushima et al. (1997b). In both models, the repeating units align in the antiparallel manner due to hydrogen bonding at the polar region and are flanked by polar glutamine-rich turns. In the Argos' model, the polar glutamine-rich turns exist at the top and bottom of the cylindrical clusters resulting in an ellipsoidal structure (Figure 1-6a). While in this Matsushima's model, this structure is described as an elongated "prism-like" shape (Figure 1-6b).

1.4.2 Delivery films based on zein

Because of the lack of tryptophan and lysine (Table 1-1), zein has poor nutritional quality. Zein is well-established for its good film forming capacity and has been used as edible coatings in food and pharmaceutical products because zein films have low water uptake, high thermal resistance, and good mechanical properties (Elzoghby et al., 2012). When sodium caseinate films were coated by zein, the water vapor and oxygen permeabilities were significantly reduced (Yin et al., 2014). When a high intensity sweetener was loaded in films consisting of zein and shellac, the stability of the

encapsulated sweetener in chewing gum was improved during storage, and gradual and controlled release of the encapsulated sweetener was achieved during chewing (Campbell and Zibell, 1992).

The incorporation of antimicrobials in zein films has also been widely studied to prolong the shelf life of semi-solid foods. Several studies showed no impacts on film formation and activity after incorporating antimicrobials in zein films. This has been demonstrated for zein films incorporated with lysozyme or nisin prepared using both cast and heat-press methods (Padgett et al., 1998). For these films, the inhibition of bacterial growth was enhanced after supplementing with ethylenediaminetetraacetate (EDTA) resulting from the synergism of EDTA and the incorporated antimicrobial (Padgett et al., 1998). When thymol was loaded in zein films, it exhibited control release over time, and its activity was similar to the treatment with thymol directly added into the bacteria growth media (Del Nobile et al., 2008).

Plasticizers are commonly used to improve the flexibility of zein films. Fatty acids such as palmitic acid and oleic acid have been shown to maintain the film strength but improve the flexibility and water resistance of zein films significantly (Lai et al., 1997; Santosa and Padua, 1999; Wang and Padua, 2005). The incorporation of these hydrophobic plasticizers in zein films slowed down the release rate of encapsulated lysozyme (Arcan and Yemenicioglu, 2013). In another similar study, the gradually released lysozyme from zein–wax composite films resulted in significant log reduction of *Listeria monocytogenes* ATCC 7644 inoculated in cheese, while the comparable treatment without wax in the zein film only showed the bacteriostatic effect (Unalan et al., 2013).

1.4.3 Zein fibers produced by electrospinning

Nanofibers produced from natural polymers have received increasing attention due to their potential use as delivery systems of bioactive compounds or biodegradable materials. Nanofibers have an extremely high surface area to volume or weight ratio of up to 1000 m^2/g and have the potential to minimize the burst release of encapsulated compounds (Wongsasulak et al., 2013). Electrospinning is one of the most efficient, simple, cost-effective, and versatile methods to produce nanofibers (Kriegel et al., 2008). It uses an electrical field to draw a fine liquid jet, typically at the micro or nanometer scale, from liquid reservoir, and the evaporation of solvent form the liquid jet results in fiber formation. Commonly, the point electrode at a characteristic voltage provides a high electrical energy on the cuspidate spinneret, which creates a charge aggregation into the solution at the spinneret of syringe (Figure 1-7) (Li et al., 2010). The liquid is drawn by electrostatic force and the droplets are stretched to eruption, what is known as "Taylor cone", at the outlet of spinneret. It is then drawn out from spinneret as the "Liquid jet." Because of the electrostatic repulsion from the charges in the polymer and the solvent evaporating from the polymer, the liquid is drawn thinner and thinner, and finally stretching, looping and ultrathin nanofibers are obtained in the collector (Li et al., 2010). To produce zein nanofibers by electrospinning, hydrophobic bioactive compounds and zein are usually co-dissolved in 70-85% ethanol or other appropriate solvents, such as isopropanol. Followed, the solution is emerged through an electrospinning set to produce the nanofibres as described above. Zein nanofibers encapsulated with β-carotene into by electrospinning had minimum and maximum cross section diameters of 540 and 3580 nm, respectively (Fernandez et al., 2009). β-Carotene was found to be widely distributed

in zein fibers and its oxidative and light stabilities were significantly improved when compared to free β-carotene (Fernandez et al., 2009). The stability of (−)-epigallocatechin gallate was enhanced after encapsulation in zein nanofibers with an average diameter 472 ± 46 nm (Li et al., 2009). If the fiber was aged at 0% relative humidity under ambient temperature for at least 1 d, it showed >98% recovery, which was an improvement from the 82% recovery of the freshly prepared fibers (Li et al., 2009). Docosahexaenoic (DHA) encapsulated in zein nanofibers by electrospinning showed a 2.5-fold reduction in the degradation rate constant and a much higher degradation induction time than free DHA (Torres-Giner et al., 2010). Curcumin encapsulated in zein nanofibers with a mean diameter of 310 nm showed the sustained release and the improved free radical scavenging activity (Brahatheeswaran et al., 2012). A higher encapsulation efficiency (>90%) of fish oil was observed in zein nanofibers produced by electrospinning method than conventional methods, such as anti-solvent method and double-emulsification method (Moomand and Lim, 2014), and the encapsulated fish oil demonstrated a better oxidative activity than unencapsulated control (Moomand and Lim, 2014). In another study, a composite blend of zein, poly (ethylene oxide) and chitosan was used to produce electrospun fibers that demonstrated potential as a mucoadhesive delivery vehicle in the gastrointestinal tract (Wongsasulak et al., 2013). α-Tocopherol encapsulated at 20% w/w in these composite fibers additionally improved the mucoadhesivity significantly, without affecting fiber morphology (Wongsasulak et al., 2014). The encapsulated α-tocopherol was found to exhibit controlled release in simulated gastric fluid at pH 1.2 and 2 (Wongsasulak et al., 2014).

1.4.4 Zein nanoparticles produced by liquid-to-liquid dispersion method

Because of its high hydrophobicity, zein has been considered as a promising carrier material to deliver hydrophobic bioactive compounds. Liquid-to-liquid dispersion is the most studied method to produce zein nanoparticles (Zhong and Jin, 2009b). In this method, the principle is to dissolve zein in a good solvent as a stock solution and disperse the stock solution into a solvent (typically water) miscible with the solvent in the stock solution. During the dispersion process, the solvent in the stock solution sheared droplets with zein partitions into the bulk water phase and reduces to a concentration that is no-longer a good solvent of zein, resulting in the precipitation of zein to form nanoparticles (Figure.1-8). The shear stress during dispersion and concentrations of zein and solvent in the stock solution are among the factors important to the formation and dimension of zein particles (Zhong and Jin, 2009b). Water is usually used as an anti-solvent, and polar organic solvent in the stock solution has been studied for ethanol (Patel et al., 2010b), methanol (Patel et al., 2010b), isopropanol (Patel et al., 2010b), and dimethylsulfoxide (Thies, 2012). For food applications, zein is commonly dissolved in 70-80% aqueous ethanol, a GRAS solvent, and the dispersion of stock solution into water results in the zein nanoparticles with a diameter ranging from <100 nm to ~400 nm (Zhong and Jin, 2009b). Lipophilic bioactive compounds can be dissolved with zein in the stock solution and co-precipitate with zein during liquid-liquid dispersion to become encapsulated. This has been studied for curcumin (Gomez-Estaca et al., 2012; Patel et al., 2010b), EOs (Li et al., 2013a; Parris et al., 2005; Wu et al., 2012a), and fish oil (Zhong et al., 2009), among others.

Bioactive compounds encapsulated in zein particles have showed the improved bioactivity, controlled release, and improved stability under environmental stresses, and maintained or enhanced antimicrobial activity. Oregano, red thyme, and cassia oil encapsulated in zein nanoparticles were released slowly in the small intestine, and more rapid released in the large intestine due to the microbial digestion of zein in the latter (Parris et al., 2005). Fish oil encapsulated in zein by liquid-to-liquid dispersion followed by freeze drying showed the significantly reduced oxidant rate , especially when the mass ratio of zein:fish oil was above 4:1 (Zhong et al., 2009). The water solubility of thymol or carvacrol encapsulated in zein nanoparticles was enhanced, but the antioxidant and antimicrobial activity were not changed (Wu et al., 2012a). When sodium carbonate particles were dispersed with zein in 70% ethanol, hollow zein nanoparticles formed after the liquid-to-liquid dispersion because of the immediate dissolving of initially encapsulated sodium carbonate particles (Xu et al., 2011). The average particle size of these hollow zein nanoparticles was more than 60 nm smaller than solid zein nanoparticles (Xu et al., 2011). When it was used to load a drug compound, metformin, the loading capacity was improved from 10% of solid zein nanoparticles to 36.9% of hollow zein nanoparticles (Xu et al., 2011).

Currently, the combination of zein with other biopolymers to form complexes to encapsulate bioactive compounds has been gaining increasing attention. To produce complexes, zein and biopolymers can be dissolved in the same stock solution or, more often separately, before the liquid-to-liquid dispersion. Usually, zein is dissolved in aqueous ethanol and water. In the liquid-to-liquid dispersion process, zein/polymer complexes are formed mainly by hydrophobic interactions and hydrogen bonding, due to

the weak charge property of zein. Both hydrophilic and hydrophobic compounds have been encapsulated by dissolving hydrophilic compounds in the water phase and hydrophobic compounds together with zein in the stock solution. Luo et al. (2010) encapsulated hydrophilic sodium selenite in chitosan nanoparticles cross-linked by tripolyphosphate at pH <5.5. Compared to non-coating chitosan nanoparticles, chitosan nanoparticles with zein coating improved the encapsulation efficiency from 60% to 95%. The cumulative release of selenite in phosphate-buffered saline (pH 7.4) was reduced from 98% to 32% within 4 h after coating chitosan nanoparticles with zein (Luo et al., 2010). When tested in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), 94% and 50% selenite was released in SGF within 2 h for chitosan capsules without and with zein coating, and another 50% further release was observed for the zein-coated capsules in the following 4 h incubation in SIF (Luo et al., 2010). In another study by Luo et al. (2011), hydrophobic α-tocopherol was co-dissolved in 75% ethanol with zein, and the water phase with chitosan was stirred into the zein solution, producing α-tocopherol encapsulated in zein nanoparticles coated by chitosan. The chitosan coating did not change the encapsulation efficiency but lowered the controlled release rate of α-tocopherol in both PBS and simulated gastrointestinal conditions (Luo et al., 2011). The stability of the dispersion was also improved by chitosan coating that provided repulsive electrostatic and steric interactions preventing nanoparticle aggregation (Luo et al., 2011).

Zein has also been used to form beads with pectin or alginate to change the release site of encapsulated compounds. In a study (Liu et al., 2006), pectin-bovine serum albumin (BSA) solution was sheared into zein dissolved in 75% ethanol by stirring,

forming pectin/zein complexes that were cross-linked by calcium to form beads. In simulated gastrointestinal and colon conditions, pectin beads without zein released most BSA, while pectin/zein beads with calcium released most BSA in post-gastrointestinally (colon conditions) (Liu et al., 2006). Alcantara et al. (2010) co-dissolved sodium ibuprofen in 80% ethanol with zein and which was then sheared into aqueous alginate solution. Calcium was then added to form zein/alginate beads. When compared to the control without zein, the release rate of sodium ibuprofen from beads with zein was slowed under the simulated gastrointestinal conditions.

1.5 Methods of stabilizing zein particles in aqueous systems

Due to the highly hydrophobic nature, the stability of zein nanoparticles in aqueous dispersions is poor, especially at a pH close to the *pI* of zein, around pH 7 (Patel et al., 2010a). To solve this problem, strong repulsive forces must be created for zein nanoparticles to overcome attractive forces of hydrophobic interactions, hydrogen bonding, and van der Waals interactions (Bryant and Mcclements, 1998). Known repulsive forces include electrostatic interaction, steric repulsion and hydration, with the former two most dominant (Bryant and Mcclements, 1998). Alternatively, hydrophobic interactions may be weakened by modifying surface amino acid composition of zein. A two-step enzymatic hydrolysis method has been found to be able to produce water soluble zein (Mannheim and Cheryan, 1993). Zein was first partially hydrolyzed in 50-70% alcohol at pH 9.0 and 50 ℃ by alcalase. In the second step, hydrolysis was conducted in an aqueous phase with the same protease, pH and temperature. It was found that when the degree of hydrolysis was over 20, the hydrolysate was 100% soluble in

water. If dialysis membrane was used to obtain the small fraction of hydrolyzed product, a degree of hydrolysis of 12 was enough to produce water soluble zein fractions.

To introduce repulsive forces on zein particles, water soluble biopolymers adsorbing on zein nanoparticles can be studied. This has been demonstrated for sodium caseinate that stabilized zein nanoparticles at neutral acidity (Patel et al., 2010a). The *pI* of the complex shifted from 6.2 of zein to 5.0, indicating the adsorption of sodium caseinate on zein nanoparticle surface.

The powdered form of zein nanoparticles is important to practical applications because of not only the convenience of storage, transportation, and application but also the removal of organic solvent such as ethanol. This requires the hydration of powder to disperse zein nanoparticles in aqueous systems. When sodium caseinate was dissolved in the aqueous phase to prepare zein nanoparticles by liquid-to-liquid dispersion, the dispersion was quite stable at neutral pH, and the freeze dried powder was freely res-dispersible in aqueous phase (Patel et al., 2010a). The zein nanoparticle-caseinate complexes have been studied for encapsulation of several bioactive compounds (Li et al., 2013a; Patel et al., 2010b). However, these complexes are not stable at acidic pH because of the precipitation of caseins, and freeze-drying is an expensive process for food production.

1.6 Hypothesis and overview of dissertation research

The overall hypothesis of this dissertation is that the stability of zein nanoparticles in the aqueous phase can be improved through the complex formation with biopolymers that can provide electrostatic and/or steric interactions preventing particle aggregation. The biopolymers selected for the current research sodium caseiante and gum arabic.

Sodium caseinate has been found to stabilize zein nanoparticles when supplemented during the liquid-to-liquid dispersion and improved the dispersibility of freeze-dried powder at pH 7.4 (Patel et al., 2010a). Because the association and dissociation of casein is a function of pH (Huppertz et al., 2010), temperature (O'Connell et al., 2003), and aqueous ethanol concentration (O'Connell et al., 2003), we hypothesize that conditions favoring the dissociation of sodium caseinate can expose more hydrophobic sites to interact with zein and provide the improved stability of zein nanoparticles. The newly established complexes are also expected to be effective in encapsulating EOs. Gum arabic is a frequently studied emulsifier whose surface activity is provided by a small fraction of protein naturally-glycated with polysaccharide chains (Dickinson, 2003, 2009). Gum arabic has also been studied to stabilize sodium caseinate and whey protein in a wide pH range (Weinbreck et al., 2004; Ye et al., 2012; Ye et al., 2006). We hypothesize that gum arabic is able to form complexes with zein nanoparticles by hydrophobic interactions to provide stability in a wide range of acidity.

Based on these hypotheses, the research in chapter 2 focused on effects of pH adjustment and heating aqueous ethanol dispersion with sodium caseinate and zein on nanoparticles prepared by the liquid-to-liquid dispersion method. Zein and sodium caseinate were dissolved in 50% aqueous ethanol, adjusted to pH 8.0, and heated at 90 ℃, followed by dispersion into water. The subsequently prepared dispersions were spray-dried, and the dispersions hydrated from spray-dried powder were characterized. Before application to encapsulate EOs, Chapter 3 was conducted to understand the distribution of EOCs in milk as impacted by preparation conditions of pre-dissolving in 95% ethanol or simple mixing. The distribution of EOs in the milk serum was correlated to the

antimicrobial activity of EOCs observed in milk and microbial growth media in order to provide a more rational design of delivery systems encapsulating EOs. In Chapter 4, zein/sodium caseinate complexes in Chapter 2 were used to co-encapsulate EOC of eugenol and thymol. The physical properties of capsules prepared at different pH conditions were compared, such as particles size, morphology, and release profile, as well as the antimicrobial activity. Finally, Chapter 5 was dedicated to study zein nanoparticle-gum arabic complexes with stability at a wider range of pH. Furthermore, propylene glycol (non-flammable and GRAS) was used as a replacement for ethanol to make it more suitable in industrial production. Because gum arabic is a common choice to emulsify flavor oils, peppermint oil was chosen as the EO for encapsulation in zein-gum arabic complexes. In Chapters 3 and 5, the composition of zein nanoparticle-biopolymer complexes was studied to provide a better understanding of the structure and physical bases of stability improvements.

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Appendix

Table 1-1. Numbers of amino acid residues in α-, β-, γ-, and δ-zein (Larkins et al., 1993).

Amino Acids	α-zein	β-zein	γ-zein	δ-zein
Ala	34	18	10	7
Arg	4	7	5	0
Asn	13	2	0	3
Asp	0	4	0	1
Cys	1	6	14	5
Gln	31	28	30	15
Glu	1	5	2	0
Gly	2	12	13	4
His	3	4	16	3
Ile	11	1	4	3
Leu	42	15	19	15
Lys	0	0	0	0
Met	5	11	2	29
Phe	8	0	2	5
Pro	22	13	51	20
Ser	18	11	4	8
Thr	7	5	9	5
Trp	0	1	0	0
Tyr	6	16	4	1
Val	17	4	15	5
Total residues	225	163	200	129

Table 1-2. Selected MICs and MBCs of essential oils (EOs) or essential oil components (EOCs) tested *in vitro* against food borne pathogens.

EOs/EOCs	Bacteria species	Media	MIC (g/L)	MBC (g/L)
Thymol	*Escherichia coli* O157:H7	TSB	0.3 (Shah et al., 2013a); 0.0938 (Ma et al., 2013)	0.5(Shah et al., 2013a); 0.0938 (Ma et al., 2013)
	Listeria monocytogenes	TSB	0.3-0.5(Shah et al., 2013a); 0.1875(Ma et al., 2013); 0.2 (Chen et al., 2014a); 0.2 (Xue et al., 2013)	0.5-0.75(Shah et al., 2013a); 0.375(Ma et al., 2013); 0.3 (Chen et al., 2014a); 0.045 (Xue et al., 2013)
		2% reduced-fat milk	3.0 (Ma et al., 2013)	
	Staphylococcus aureus	TSB	0.5 (Shah et al., 2013a)	0.75 (Shah et al., 2013a)
	Salmonella Typhimurium	TSB	0.5 (Shah et al., 2013a)	0.75 (Shah et al., 2013a)
	Salmonella Enteritidis	TSB	0.1875 (Ma et al., 2013)	0.1875 (Ma et al., 2013)
Eugenol	*Escherichia coli* O157:H7	TSB	1.0 (Shah et al., 2013b);0.75(Ma et al., 2013)	1.5 (Shah et al., 2013b);0.75 (Ma et al., 2013)
		Skim milk	3.5 (Shah et al., 2013b)	4.5 (Shah et al., 2013b)
		2% reduced-fat milk	4.5 (Shah et al., 2013b); 3.0 (Ma et al., 2013)	5.5 (Shah et al., 2013b)
		Whole milk	5.5 (Shah et al., 2013b)	6.5 (Shah et al., 2013b)
	Listeria monocytogenes	TSB	1.5 (Shah et al., 2013b);0.75 (Ma et al., 2013); 0.8 (Chen et al., 2014a)	1.75 (Shah et al., 2013b);1.5 (Ma et al., 2013); 1.3 (Chen et al., 2014a)
		Skim milk	3.5 (Shah et al., 2013b)	6.5 (Shah et al., 2013b)

Table 1-2 continued

EOs/EOCs	Bacteria species	Media	MIC (g/L)	MBC (g/L)
		2% reduced-fat milk	4.5 (Shah et al., 2013b);	6.5 (Shah et al., 2013b)
		Whole milk	6.5 (Shah et al., 2013b)	6.5 (Shah et al., 2013b)
	Listeria innocua	TSB	2.0 (Hill et al., 2013)	>2.0 (Hill et al., 2013)
	Salmonella Typhimurium	TSB	>1.0 (Hill et al., 2013)	>1.0 (Hill et al., 2013)
	Salmonella Enteritidis	TSB	0.75 (Ma et al., 2013)	0.75 (Ma et al., 2013)
Carvacrol	*Listeria monocytogenes*	TSB	0.2 (Chen et al., 2014a)	0.3 (Chen et al., 2014a)
	Escherichia coli O157:H7	TSB	0.5 (Kim et al., 1995b)	0.5 (Kim et al., 1995b)
	Salmonella Typhimurium	TSB	0.5 (Kim et al., 1995b)	0.5 (Kim et al., 1995b)
Cinnamaldehyde	*Listeria monocytogenes*	TSB	0.2 (Chen et al., 2014a)	1.4 (Chen et al., 2014a)
	Listeria innocua	TSB	0.5 (Hill et al., 2013)	2.0 (Hill et al., 2013)
	Salmonella Typhimurium	TSB	0.4 (Hill et al., 2013)	>1.0 (Hill et al., 2013)
Cinnamon leaf oil	*Escherichia coli* O157:H7	TSB	0.75 (Ma et al., 2013)	0.75 (Ma et al., 2013)
	Listeria monocytogenes	TSB	0.75 (Ma et al., 2013)	1.5 (Ma et al., 2013)
		2% reduced-fat milk	3.0 (Ma et al., 2013)	
	Salmonella Enteritidis	TSB	0.75 (Ma et al., 2013)	0.75 (Ma et al., 2013)

Table 1-2 continued

EOs/EOCs	Bacteria species	Media	MIC (g/L)	MBC (g/L)
	Staphylococcus aureus	TSB		3.2 (Prabuseenivasan et al., 2006)
	Bacillus subtilis	TSB		>1.6 (Prabuseenivasan et al., 2006)
Thyme oil	*Escherichia coli* O157:H7	TSB	3.0 (Solomakos et al., 2008a)	6.0 (Solomakos et al., 2008a)
		minced beef mea	6.0 (Solomakos et al., 2008a)	>6.0 (Solomakos et al., 2008a)
	Listeria monocytogenes	TSB	3.0 (Solomakos et al., 2008b)	6.0 (Solomakos et al., 2008b)
		minced beef mea	3.0-6.0 (Solomakos et al., 2008b)	>6.0 (Solomakos et al., 2008b)
Cinnamon bark extract	*Salmonella* Typhimurium	minced beef mea	0.4 (Hill et al., 2013)	1.0 (Hill et al., 2013)
	Listeria innocua	TSB	0.5 (Hill et al., 2013)	2.0 (Hill et al., 2013)
Clove bud extract	*Salmonella* Typhimurium	TSB	>1.0 (Hill et al., 2013)	>1.0 (Hill et al., 2013)
	Listeria innocua	TSB	2.0 (Hill et al., 2013)	>2.0 (Hill et al., 2013)
Oregano oil	*Escherichia coli*	TSB	0.9 (Mathlouthi et al., 2012)	1.12 (Mathlouthi et al., 2012)
	Salmonella Indiana	TSB	0.9 (Mathlouthi et al., 2012)	1.12 (Mathlouthi et al., 2012)
	Listeria innocua	TSB	0.9 (Mathlouthi et al., 2012)	1.12 (Mathlouthi et al., 2012)
	Staphylococcus aureus	TSB	0.9 (Mathlouthi et al., 2012)	1.12 (Mathlouthi et al., 2012)
	Bacillus subtilis	TSB	2.25 (Mathlouthi et al., 2012)	2.25 (Mathlouthi et al., 2012)
Clove bud oil	*Staphylococcus aureus*	TSB	0.25 (Upadhyay et al., 2010)	2.0 (Upadhyay et al., 2010); >6.4 (Prabuseenivasan et al., 2006)
	Bacillus cereus	TSB	2.0 (Upadhyay et al., 2010)	4.0 (Upadhyay et al., 2010)
	Bacillus subtilis	TSB		>3.2 (Prabuseenivasan et al., 2006)
	Escherichia coli	TSB	0.5 (Upadhyay et al., 2010)	1.0 (Upadhyay et al., 2010); >1.6 (Prabuseenivasan et al., 2006)

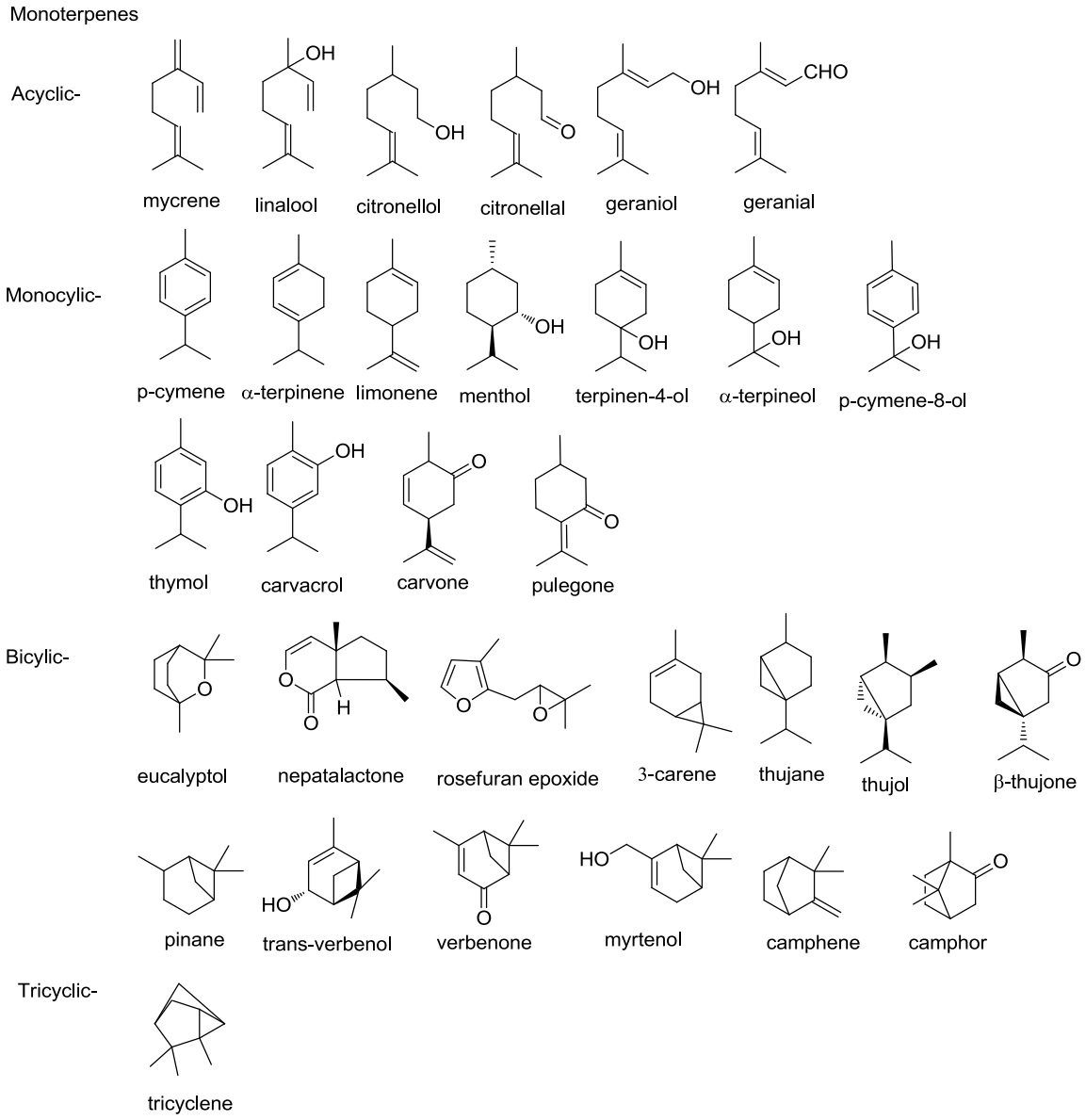

Figure 1-1. Different types of monoterpenes found in essential oils (Chizzola, 2013; Hüsnü et al., 2007).

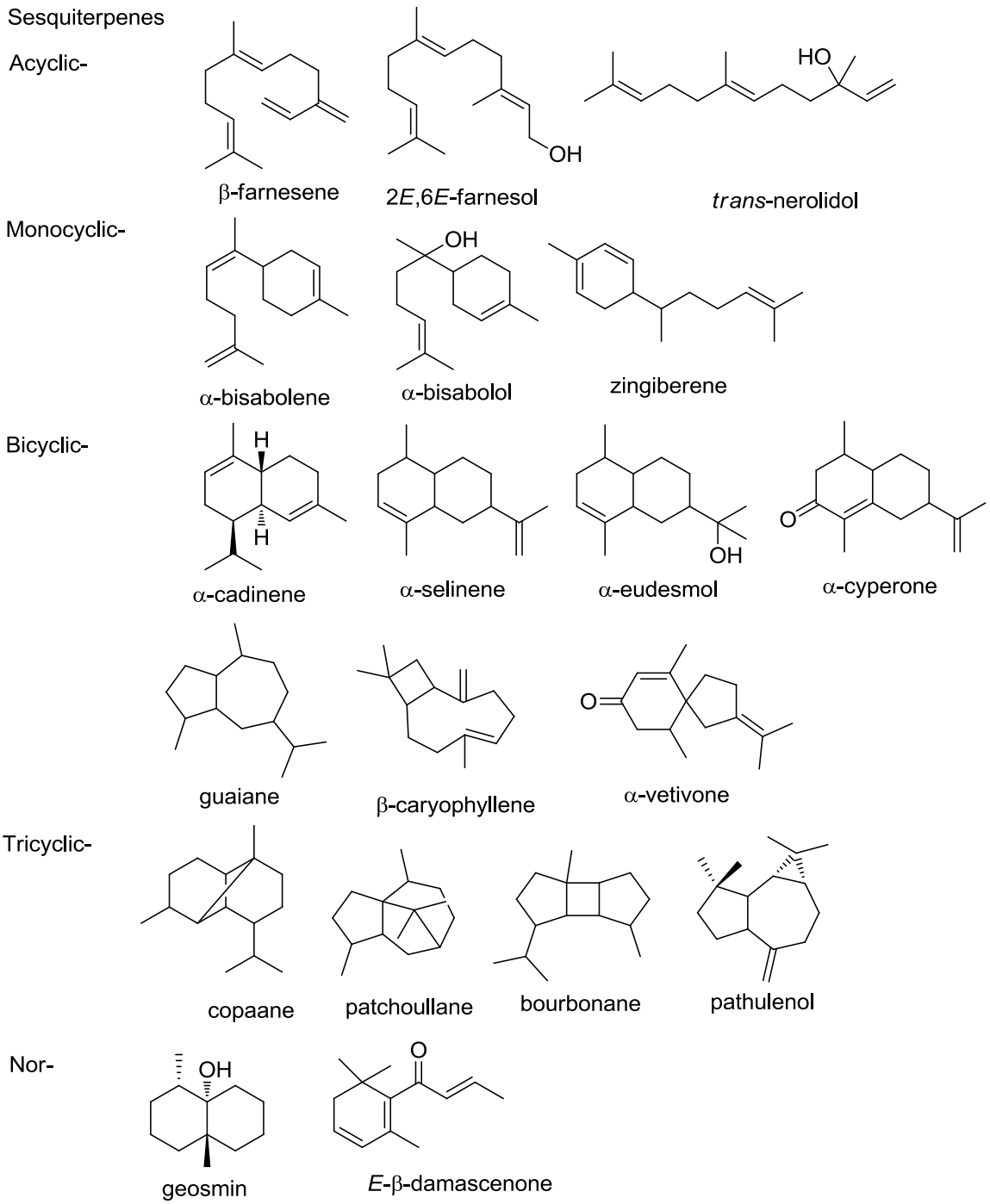

Figure 1-2. Different types of sesquiterpenes found in essential oils (Chizzola, 2013; Hüsnü et al., 2007).

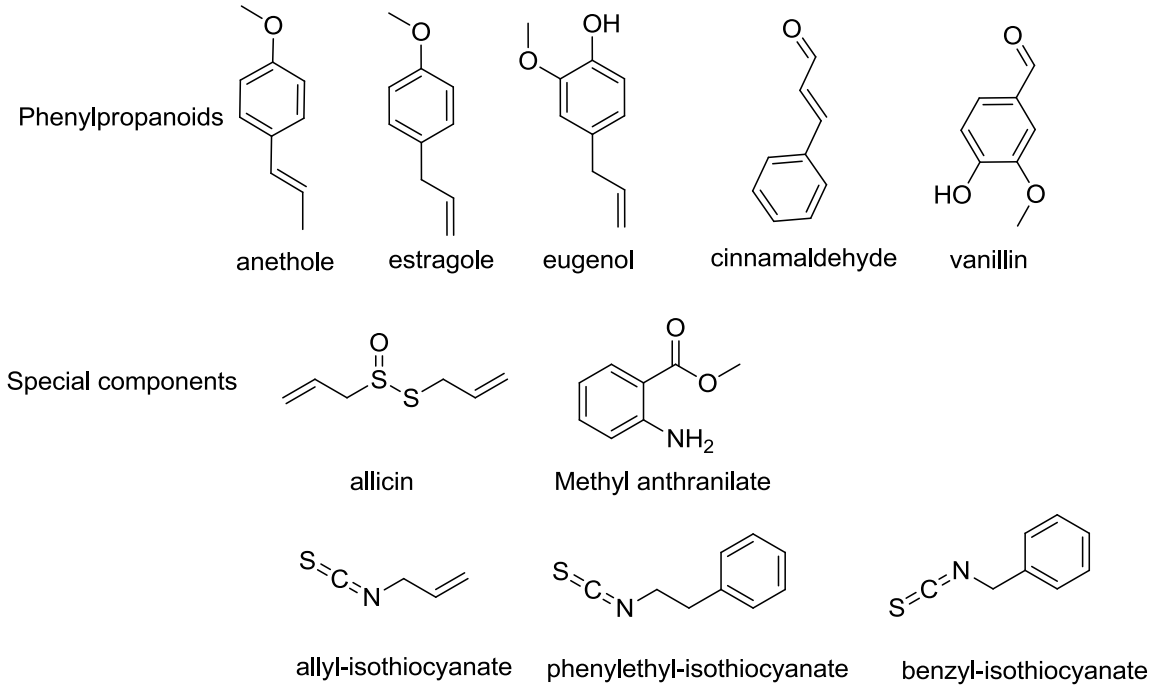

Figure 1-3. Phenylpropanoids and sulfur-, nitrogen containing components in essential oils (Hüsnü et al., 2007; Hyldgaard et al., 2012b).

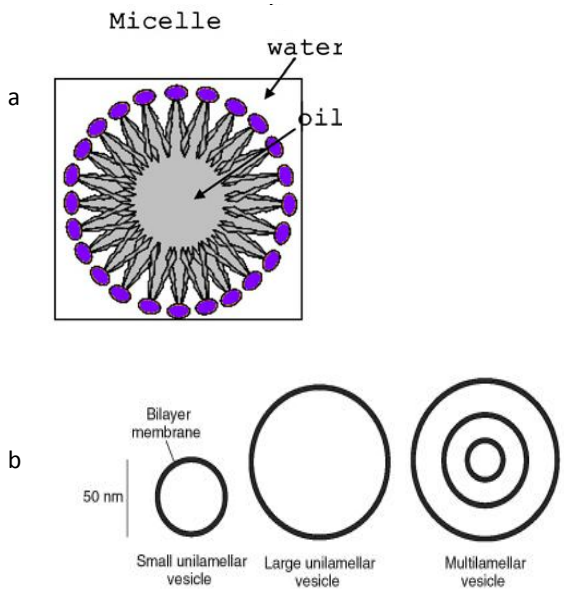

Figure 1-4. Schematic representation of (a) oils dissolved in surfactant micelles or emulsion droplets (Narang et al., 2007) and (b) vesicle structures of liposomes (Singh et al., 2012).

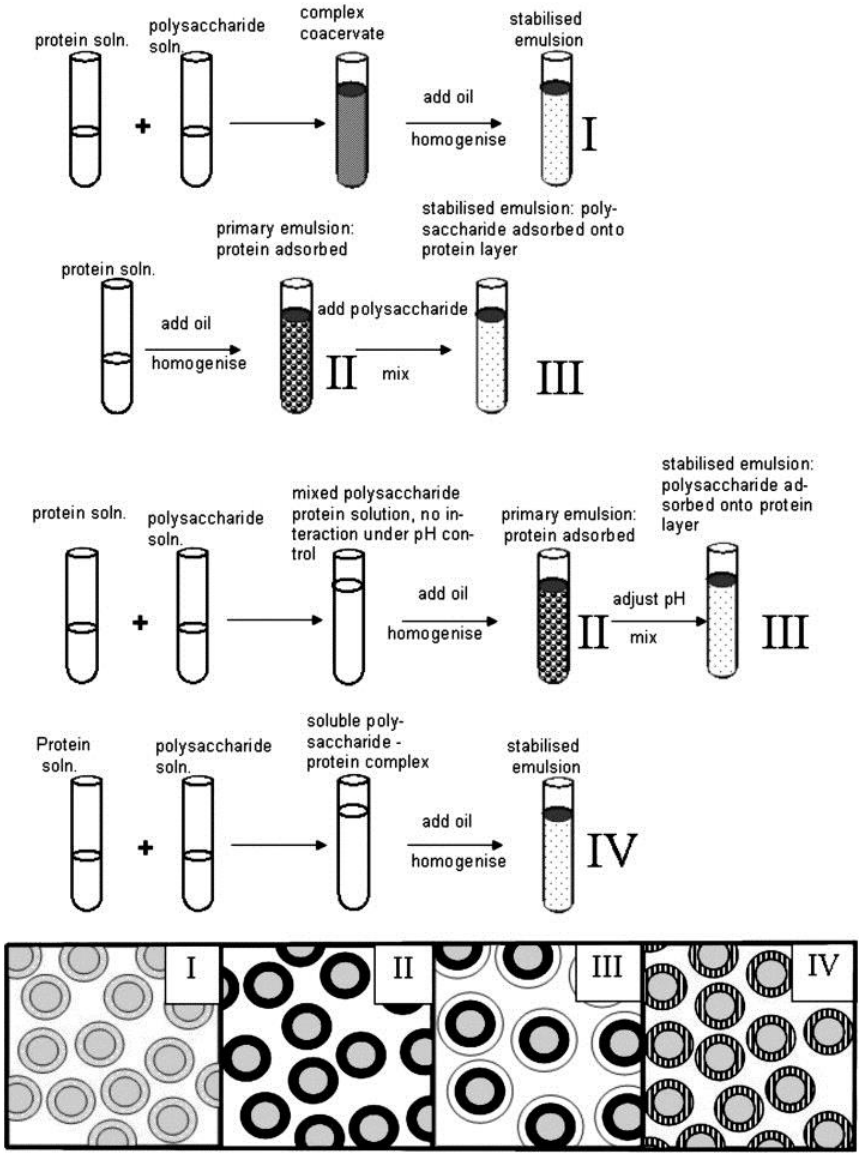

Figure 1-5. Schematic illustration of four different methods for preparing emulsions stabilized by electrostatic polysaccharide–protein complexes. Inner oil droplet is protected by adsorption of (I) complex coacervate, (II) protein layer only, (III) protein layer with an adsorbed polysaccharide layer or (IV) soluble complex (Evans et al., 2013).

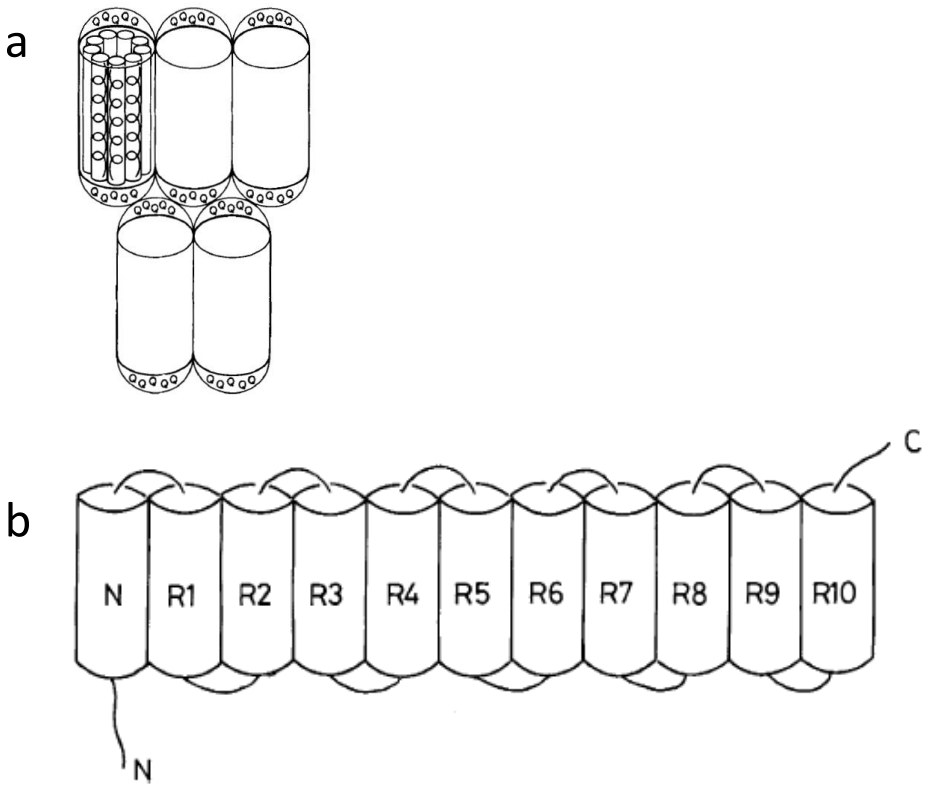

Figure 1-6. The structure model of α-zein proposed by (a) Agros (Argos et al., 1982) and (b) Matsushima (Matsushima et al., 1997b).

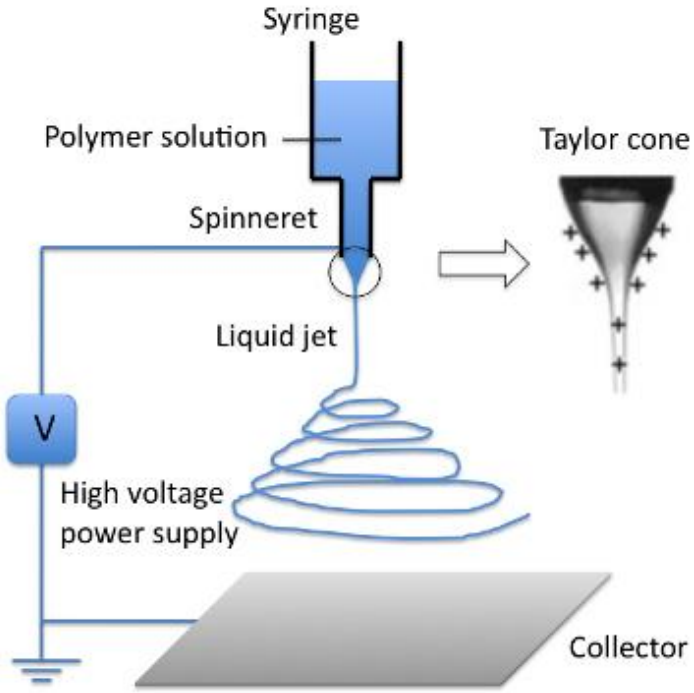

Figure 1-7. The principle of electrospinning (Li et al., 2010)

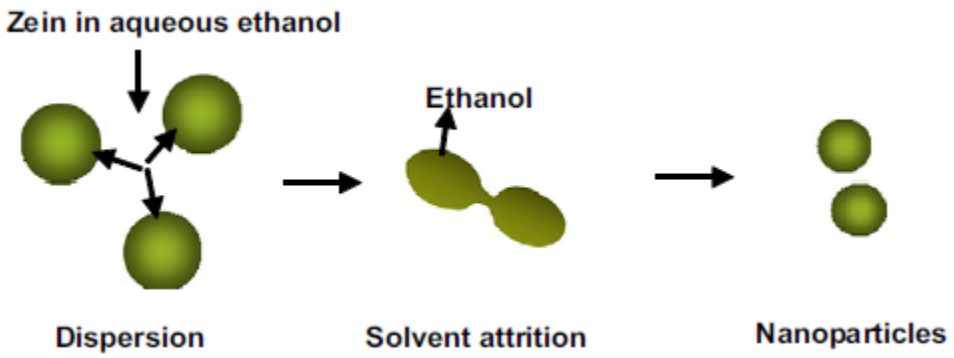

Figure 1-8. Principle of the liquid-to-liquid dispersion method to produce zein nanoparticles (Zhong and Jin, 2009b).

Chapter 2. Processes improving the dispersibility of spray-dried zein nanoparticles using sodium caseinate

A version of this chapter was originally published by Huaiqiong Chen and Qixin Zhong: Huaiqiong Chen, and Qixin Zhong. 2014. Processes improving the dispersibility of spray-dried zein nanoparticles using sodium caseinate. *Food Hydrocolloids*. 35: 358–366. My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

2.1 Abstract

Zein has been studied as a versatile food biopolymer to fabricate nanoparticles for encapsulating a large variety of bioactive compounds. Being water-insoluble prolamines, dispersing zein nanoparticles in aqueous systems is a challenge. Recently, sodium caseinate (NaCas) was observed to have improved the dispersibility of freeze-dried zein nanoparticles but not for spray-dried samples. In this paper, three different approaches were studied to produce spray-dried zein nanoparticles precipitated by dispersing aqueous ethanol solutions of zein into the aqueous phase. The control (S2) was produced by dispersing zein solution into NaCas dispersion without pH adjustment. The other two approaches involved conditions dissociating NaCas: adjusting NaCas dispersion to pH 11.0 (S5) or dissolving NaCas in heated zein solution before dispersing to a pH 8.0 buffer (S4). After hydrating the spray-dried powder, dispersions demonstrated varying turbidity and precipitation stability during storage. Entire NaCas was observed to have adsorbed on S2 zein nanoparticles, corresponding to bigger particles, higher turbidity and lower stability of dispersions than those of S4 and S5. Conversely, only κ-casein was on zein nanoparticles of S4 and S5, corresponding to a higher zein:casein mass ratio and higher surface hydrophobicity than that of S2. The best dispersibility was observed for S4 at pH 7.0 and 0–300 mM NaCl, with the smallest hydrodynamic diameter (~125 nm), lowest turbidity, and without precipitation during 15-day refrigerated storage. Compositional analyses suggested that κ-casein in S4 was a part of zein nanoparticle matrices and was not detached by increased ionic strength during storage. Conversely, caseins detached from zein nanoparticles of S2 and S5, causing particle aggregation and precipitation. Additionally, the approach in S4 utilized less ethanol (50% v/v vs. 80% in the other two approaches) to dissolve zein. Our work is significant in fabricating delivery systems of bioactive compounds utilizing zein as a carrier biopolymer.

Keywords: spray drying, zein nanoparticles, sodium caseinate, dispersibility, surface hydrophobicity, ionic strength

2.2 Introduction

Recently, there have been increasing interests in utilizing food biopolymers to fabricate delivery systems of bioactive compounds because of their non-toxicity, biocompatibility and biodegradability (Chen et al., 2006). Some of these delivery systems have demonstrated sustained or targeted release of the encapsulated bioactive components (Benjamin et al., 2012; Muller et al., 2011; Xiao et al., 2011a; Xiao et al., 2011b). Zein is a group of alcohol-soluble protein (prolamines) extracted from maize (*zea mays*) kernel (Hu et al., 2012) and has been studied as a carrier biopolymer to achieve gradual release of the encapsulated compound (Bobokalonov et al., 2012; Hu et al., 2012; Xiao et al., 2011b; Zhong and Jin, 2009a).

Zein is insoluble in water but is soluble in about 55-90% v/v aqueous ethanol (Zhong and Jin, 2009b). This solubility property has been studied in the liquid-liquid dispersion technique where an aqueous ethanol solution of zein is dispersed into water to precipitate zein to form nanoparticles (Zhong and Jin, 2009b). By co-dissolving in the aqueous ethanol solution, lipophilic bioactive compounds can co-precipitate with zein during liquid-liquid dispersion, which enables the encapsulation of a variety of bioactive compounds in nanocapsules to obtained gradual release (Wu et al., 2012b; Zhong and Jin, 2009a; Zhong et al., 2009). However, because zein has abundant non-polar amino acid residues such as leucine, proline, alanine and phenylalanine, the dispersibility of zein nanoparticles in aqueous systems is a challenge. Furthermore, zein is typically dissolved in 70-80% aqueous ethanol before preparation of nanoparticles using liquid-liquid dispersion (Zhong and Jin, 2009b), and it is desirable in the industrial production to reduce the usage of ethanol without compromising characteristics of zein nanoparticles. To favor industrial applications, it is also desirable to prepare the powdered form of zein nanoparticles, preferably by spray-drying that is less expensive and more scalable than

freeze drying (Desobry et al., 1997). Spray-dried powder also has more uniform particles and better flowability than that from freeze-drying, which favors the industrial production (Seville et al., 2002).

In a recent study, sodium caseinate (NaCas) was supplemented in water during liquid-liquid dispersion to stabilize zein nanoparticles, and the zein nanoparticles before and after freeze drying demonstrated much improved dispersibility and stability at pH 7.4 than the treatment without NaCas (Patel et al., 2010a). This is a significant finding because the neutral acidity is nearby the isoelectric point (pI) of zein, around pH 6.2 (Patel et al., 2010a). However, as detailed below, this approach was infeasible to disperse zein nanoparticles after spray drying.

We hypothesize that the application of dissociated NaCas in liquid-liquid dispersion can improve the dispersibility of spray-dried zein nanoparticles. NaCas is produced from fresh or pasteurized skim milk by precipitation near the pI of caseins (pH 4.6), followed by neutralizing the recovered precipitate using sodium hydroxide for spray drying (Neirynck et al., 2007). NaCas is more soluble than casein micelles directly separated from bovine milk (Kalichevsky et al., 1993) and is widely used in the food industry due to its versatile functional properties, such as emulsifying and stabilizing capacities (Corzo-Martinez et al., 2010). Like casein micelles, NaCas is composed of α_{s1}-, α_{s1}-, β-, and κ-caseins in weight proportions of approximately 4:1:4:1(Post et al., 2012) . Because of the similarity in composition, conditions impacting casein micelle structures likely can be used to engineer the structure of NaCas. One such example is the dissociation of casein micelles in more than 30% ethanol at 60 °C, resulting in a translucent dispersion (O'Connell et al., 2003). The dissociated caseins can reform to nanoparticles at a reduced ethanol content and/or lowered temperature. Another example is the disruption of casein micelles when the pH is increased to 10.0, and the reformed casein particles after acidification

back to pH 6.6 had a smaller particle size and a lower zeta-potential than the original casein micelles (Huppertz et al., 2010).

In this work, the major objective was to test the above hypothesis by incorporating the two conditions, i.e., hot aqueous ethanol and alkaline pH, in preparation of zein nanoparticles by liquid-liquid dispersion. Following spray drying, the obtained powder was re-dispersed in water and characterized for dispersibility and physicochemical properties important to dispersibility.

2.3 Materials and methods

2.3.1 Materials

Purified α-zein and ethanol (200 proof) were purchased from Acros Organics (Morris Plains, NJ). The NaCas was a product of Pfaltz & Bauer (Waterbury, CT). The α-, β-, and κ-caseins from bovine milk, with a purity of 70%, 98% and 70%, respectively, according to the product brochure, were procured from Sigma-Aldrich Corp. (St. Louis, MO). Potassium bromide (KBr) was a product from Fisher Scientific Inc. (Pittsburgh, Pa.)

2.3.2 Preparation of spray-dried zein nanoparticles

Ethanol concentration impacts zein nanoparticle properties produced by liquid-liquid dispersion (Zhong and Jin, 2009b) but was not studies in the present work. Three approaches were studied to fabricate zein nanoparticles, each at one ethanol concentration (Figure 2-1). In Approach A, 2.0 g zein was dissolved in 100.0 mL of 80% v/v aqueous ethanol, followed by dispersing into 250.0 mL deionized water with or without 2.0 g sodium caseinate while being agitated at 10,000 rpm for 2 min by a high-speed homogenizer (model Cyclone I.Q.^{2}, The VirTis Company, Inc., Gardiner, NY). The dispersion was spray-dried using a bench-top spray dryer (model B-290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) with the parameters from

the reference (Xiao et al., 2011b), which were a feed rate of 15%, an aspirator setting of 100%, and an inlet temperature of 105 °C. The samples were named as S1 for the treatment without NaCas and S2 for the one with NaCas. Preparation conditions of S1 and S2 were similar to those in the literature except the powdered sample was spray-dried instead of freeze-dried (Patel et al., 2010a). In the approach B, 2.0 g zein and 0 or 2.0 g NaCas were mixed with 100.0 mL of 50% v/v aqueous ethanol containing 25 mM sodium phosphate. Following adjustment of pH to 8.0 and incubating in a 90 °C water bath for 30 min, the hot solution was dispersed into 400.0 mL of 25 mM sodium phosphate buffer previously adjusted to pH 8.0, followed by spray drying. The obtained samples were named as S3 and S4 for the treatment without or with NaCas, respectively. Sample S5 was prepared using approach C that was similar to approach A with NaCas, except that the aqueous NaCas dispersion was adjusted to pH 11.0 before dispersing the zein solution. The dispersion was adjusted back to pH 8.0 and spray-dried. Additionally, three samples containing NaCas only without zein were prepared through the three approaches A, B, and C, which were named as N1, N2 and N3, respectively. All spray-dried samples were stored at -20 °C until use. Two replicates of spray-dried samples were prepared for each approach.

2.3.3 Proximate analysis of nanoparticle composition

The pH 7.0 dispersions hydrated with spray-dried powder at 10.0 mg/mL were separated into different fractions according to Figure 2-2. Fraction A referred to the supernatant after centrifuging 1.0 mL dispersion at 6700*g* for 20 min (model MiniSpin® plus, Eppendorf, Westbury, NY), theoretically containing free casein and un-precipitated zein nanoparticles. After decanting the supernatant, the Fraction B, the sediment from centrifugation, presumably with mostly zein nanoparticles and possibly adsorbed casein, was mixed with 1.0 mL of 90% v/v aqueous ethanol that is a solvent for zein but not NaCas. After mixing using an end-to-end

shaker (Thermo Scientific Inc., Waltham, MA, USA) overnight, Fraction B was separated into two fractions using centrifugation as above. After transferring the supernatant (Fraction C), the sediment was dissolved with 0.05 N NaOH (~pH 11.0), referred as Fraction D.

The total protein concentrations of the dispersion before fractionation, Fractions A and C were quantified using the bicinchoninic acid (BCA) method. The protein content in Fraction D was calculated from the mass balance. The BCA reagent A was from Pierce Biotechnology (Rockford, IL), while reagent B (4% w/v cupric sulfate) was freshly prepared. Bovine serum albumin (2 mg/mL, Pierce Biotechnology, Rockford, IL) was used as a reference to construct a standard curve. Each sample was tested for at least four times.

The Fractions A, C, and D were also analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with the instrument and procedures detailed previously (Zhong and Ikeda, 2012). All reagents were from Bio-Rad Laboratories, Inc. (Hercules, CA). The protein solution was diluted in 5 volumes of a SDS-PAGE loading buffer. The 15% Tris-HCl gels (Ready Gel, 12 wells) were used in electrophoresis for about 45 min at a constant voltage of 200 V. Afterwards, the gel was stained with Coomassie blue R-250.

2.3.4 Characterization of dispersions prepared from spray-dried powder

2.3.4.1 Sample preparation

Spray-dried powder was hydrated at 0.1% w/v (1 mg/mL) in deionized water at room temperature (21 °C) for at least 3 h. The dispersions were dissolved with 0, 75, 150, and 300 mM NaCl and adjusted to pH 7.0 using 1.0 N HCl or 1.0 N NaOH.

2.3.4.2 Stability against gravitational precipitation during storage

The pH 7.0 dispersions with various NaCl concentrations were dissolved with 0.05% w/w sodium azide to prevent microbial growth. Following storage at 4 °C in a refrigerator for up to 15 d, the occurrence of precipitation was monitored by visual inspection and photographing.

2.3.4.3 Particle size and zeta-potential measurement

The particle size distribution and zeta potential of the above dispersions were measured by dynamic light scattering (DLS) using a Delsa Nano C particle size/zeta potential analyzer (Beckman Coulter, Fullerton, CA). Samples were diluted 50-100 times using deionized water and readjusted to the target pH. The viscosity of all the three samples was 1mPa s, based on preliminary tests of viscosities of dispersions prepared with 1 mg/mL powder and thereby the effect of viscosity on particle size distribution was neglected. The particle size distributions were used to calculate the volume–length mean particle diameter ($d_{4,3}$) using Eq. (1). Each spray-dried batch was measured two times. Data from four measurements were used to calculate mean and standard deviation. All measurements were carried out at 25 °C.

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad (1)$$

where n_i is the number of particles corresponding to diameter d_i.

2.3.4.4 Transmission electron microscopy (TEM)

The size and morphology of nanoparticles were analyzed using a LV-EM5 low voltage bench-top transmission electron microscope (Delong America, Inc., Montreal, Quebec, Canada). The dispersions at pH 7.0 were prepared at 1 mg/mL in distilled water, and three drops of dispersions were placed on a 200-mesh carbon-coated copper grid (SPI supplies, Structure Probe,

Inc., West Chester, PA). Afterwards, the samples were dried at 750 mmHg under pressure and 50 °C for two days. Images were acquired at various magnifications and 25 kV.

2.3.4.5 Surface hydrophobicity

Surface hydrophobicity of samples was determined using the fluorescent probe 1-anilino-8-naphthalenesulfonate (ANS) based on the literature method (Tang et al., 2009). The 1 mg/mL dispersions were tested with and without centrifugation at 6700*g* for 20 min (model MiniSpin Personal, Eppendorf, Westbury, NY) to remove free NaCas. The precipitate was washed three times using distilled water, re-dispersed in 10 mM phosphate buffer (pH 7.0), and serially diluted using the same phosphate buffer. The 8 mM ANS solution was prepared in the same phosphate buffer. Ten μL of the ANS solution was mixed with 2.5 mL of the diluted dispersion, and the intrinsic fluorescence intensity was measured using a RF-1501 Fluor spectrophotometer (Shimadzu Corp., Tokyo, Japan). An excitation wavelength of 365 nm was applied, and the emission spectra were recorded in the range between 450 and 500 nm. All fluorescence spectra were corrected for background scattering using the phosphate buffer as a reference. The initial slope of fluorescence intensity at emission wavelength of 484 nm versus protein concentration (mg/mL) plots was used as an index of protein surface hydrophobicity (H_o). Each sample was measured three times.

2.3.5 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to study chemical structure characteristics of spray-dried nanoparticles using a Nicolet Nexus 670 IR spectrometer (Thermo Nicolet Corp., Madison, MI) which was equipped with a Germanium attenuated total reflection (ATP) accessory. Ten mg of powdered sample was mixed with 100 mg of potassium bromide (KBr) powder, and the mixture was pressed into a disk for spectrum recording. The spectra were acquired at 400-4000 cm^{-1} in 64

scans with the resolution of 4 cm^{-1}. All signals were collected against a background spectrum recorded from the KBr powder only.

2.3.6 Statistical analysis

Means and standard deviations were calculated from replicates. Data were analyzed using Origin 8.0 software (OriginLab Corp., Northampton, MA), Microsoft Excel 2010 (Microsoft Corp., Seattle, WA), and SAS 9.3 (SAS Institute, Cary, NC).

2.4 Results and discussion

The dispersions reconstituted from treatments without NaCas (S1 and S3) precipitated after incubating at room temperature for a few minutes (not shown), as expected (Patel et al., 2010a). The rest of this paper is then focused on properties of S2, S4, and S5 and their reconstituted dispersions at neutral acidity.

2.4.1 FTIR

FTIR is a technique that provides information in protein secondary structure and conformation (Bonnier et al., 2008). Figure 2-3 shows FTIR spectra of S2, S4, and S5 as compared to those of zein, NaCas, and a simple mixture of zein and NaCas. FTIR spectra of S2, S4 and S5 were similar to those of zein, NaCas and simple mixture of zein and NaCas, indicating there was no chemical reactions between zein and NaCas under the studied conditions.

Slight differences of some characteristic peaks were observed, summarized in Table 2-1, indicating differences in the secondary structure among S2, S4 and S5. The amide B band, which typically occurs near 3100 cm^{-1}, is commonly recognized as the hydrogen stretching band (Lal and Nafie, 1982). The peak shift of S2 and S4 means a greater extent of C-H stretching. The amide I band of S2, S4 and S5 was observed at the same wavenumber of 1659 cm^{-1}, which was

higher than the wavenumber of zein, NaCas or their simple mixture. The amide I band involves C=O stretching vibrations coupled to N-H bending vibrations, C-N stretching and C-C-N deformation (De Zea Bermudez et al., 1999). Because the α-helix structure can be assigned to 1645-1659 cm^{-1} (Li et al., 2013e), the shift of the amide I band indicates the slight change of α-helix structure in S2, S4 and S5 after the treatments. The amide II band of S2, S4 and S5 was observed at 1531, 1537 and 1531 cm^{-1}, respectively. When compared to the unprocessed proteins, the peak shift from that of NaCas was observed only for S4, which indicates that N-H of S4 interacted more strongly with adjacent α-helix than S2 and S5 (Li et al., 2013e). The shift of the other amide II band, from 1444 cm^{-1} to 1450 cm^{-1}, indicates the structure change in *β*-sheets of S2, S4 and S5 (Zhang and Zhong, 2012).

2.4.2 Proximate composition of nanoparticles

To understand nanoparticle composition, dispersions were separated to four fractions according to Figure 2-2. To study the impacts of processing on solubility of NaCas, samples N1, N2, and N3 were prepared and the amount of precipitated casein after centrifugation at 6700*g* for 20 min was quantified to be about 5% (Table 2-2), and there was no difference among the three treatments ($P > 0.05$). After correction for the precipitated casein in Fraction B, the mass ratio of zein:casein in the precipitate, referred as the composition of zein nanoparticles, was calculated and listed in Table 2-3. The mass ratio of zein:casein in S2 was 1.4:1, while that of S4 and S5 was similar (4.3:1). This indicates a greater amount of casein adsorbing to S2 zein nanoparticles.

The fractions were further analyzed by SDS-PAGE (Figure 2-4). The Fraction D, caseins separated from zein nanoparticles, showed α-, β- and κ-caseins for S2, while only κ-casein and almost no α- and β-caseins were observed for S4 and S5. The SDS-PAG results indicate that NaCas adsorbed on zein nanoparticles as a whole for S2, while NaCas at the studied conditions

in Approaches B (S4) and C (S5) was dissociated, which enabled the selective adsorption of κ-casein onto zein nanoparticles. As a result, the thinner κ-casein layer corresponded to a higher zein:casein mass ratio in S4 and S5 (Table 2-3). It has been reported that α-casein has two hydrophobic domains, while β- and κ-casein contain only one hydrophilic domain and one hydrophobic domain (Abate et al., 2004). The κ-casein is much more hydrophilic than α- and β-caseins because it is a glycoprotein containing an acidic (charged) carbohydrate section (Vaclavik and Christian, 2003). When NaCas and individual caseins were studied at the n-tetradecane/water interface at neutral pH, the interfacial shear viscosity followed the order of κ-casein> NaCas > α_{s1}-casein> β-casein, with the magnitude of κ-casein being more than one decade higher than that of NaCas (Dickinson, 1998). The predominance of κ-casein on zein nanoparticles of S4 and S5 is in agreement with the strongest adsorption ability onto a hydrophobic surface.

2.4.3 Particle size and morphology

The $d_{4,3}$ of S2, S4 and S5 is listed in Table 2-4 before and after removing NaCas by centrifugation. The $d_{4,3}$ range of 122-136 nm is consistent with the results reported by Patel *et. al* (2010a). The $d_{4,3}$ of S2 was significantly bigger than those of S4 and S5, particularly after centrifugation (Table 2-4), corresponding to the highest turbidity of dispersions (Figure 2-6A). The particle size of all the three samples before and after centrifugation did not show any difference. Patel *et al.* (2010a) also reported no difference in the dimension of zein nanoparticles prepared with and without NaCas.

The TEM images showed that zein nanoparticles were spherical at all precipitation conditions (Figure 2-5). The mean particle diameter of S2, S4 and S5 from TEM was estimated from the pixel-size conversion factor auto-generated for each sample by the instrument. It was

90, 80 and 81 nm for S2, S4 and S5, respectively, which agrees the trend observed in DLS (Table 2-4). The smaller particle diameter observed in TEM than in DLS likely because DLS measures hydrodynamic diameter while particles are dried for TEM, as previously discussed for the differences in the data from DLS and SEM (Zhong and Jin, 2009b).

The TEM images showed the aggregation of S3 - zein nanoparticles without NaCas (Figure 2-5A), while those with NaCas were present as single nanoparticles (Figure 2-5B-D). When compared to S2 and S5, the S4 sample showed numerous structures that appeared as flexible polymer chains, likely because NaCas was dissociated to the highest extent at the adopted conditions (heating in 50% aqueous ethanol at 90 °C for 30 min) and remained dissociated after spray drying and hydration. The NaCas itself processed by Approach B had the smallest particle size (Table 2-2), which proves that NaCas was dissociated to a greater extent in S4. It also appeared that the dissociated caseins interacted with S4 zein nanoparticles strongly. As a result, the dissociated caseins adsorbed on zein nanoparticle surfaces enabled the good hydration ability and provided repulsive electrostatic and steric interaction forces to prevent the aggregation, which has resulted in the lowest turbidity and the best storage stability among the treatments, presented below.

To further study hydration property differences suggested by TEM, powders of S2, S4, and S5 were hydrated in deionized water at pH 7.0 up to 6 h. After centrifugation at 6700*g* for 20 min, the percentages of protein in the supernatant were estimated. As shown in Table 2-5, S4 and S5 also had a significantly higher percentage of hydrated protein than S2. This group of tests confirms that conditions dissociating casein micelles had similar effects on NaCas, which improved the hydration of powder and reduced the amount of casein on zein nanoparticles for S4 and S5 treatments.

2.4.4 Zeta-potential

The zeta-potential of S2, S4 and S5 at pH 7.0 before and after centrifugation is shown in Table 2-4. Before centrifugation, S2 had the highest zeta-potential magnitude. After centrifugation to remove free NaCas, the zeta-potential of all samples decreased and had no significant difference. The pI of α_{s1}-, α_{s2}-, β-, and κ-casein was 4.94, 5.23, 5.14 and 5.90, respectively, with net charges of -21.9, -17.1, -13.3, and -2.0 at pH 6.6, respectively (O'Connell and Flynn, 2007). Since S2 zein nanoparticles had all four types of caseins adsorbed at a larger mass than those of S4 and S5 that had mostly κ-casein, similar zeta-potentials of the three treatments after centrifugation, i.e., zein nanoparticle-associated structures, suggest that κ-casein on S2 zein nanoparticles is the major contributor to the zeta-potential characteristics. This may be caused by the surface structure of S2 zein nanoparticles being analogous to that of casein micelles, with the charged glycomacropeptide of κ-casein protruding to the water phase and the other half interacting with α- and β-caseins and zein. The hypothesized surface structure however is to be determined experimentally in the future.

2.4.5 Surface hydrophobicity

The H_o values of S2, S4, and S5 are shown in Table 2-4. Before centrifugation, S4 had the lowest surface hydrophobicity, while S2 and S5 had similar H_o. Caseins on zein nanoparticles and in the continuous phase determine the binding property with ANS and thus the measured H_o. The significantly lower H_o ($P < 0.05$) in S4 likely was caused by a higher extent of dissociated NaCas that is hydrophilic. The reduction of H_o was also reported for casein micelles after treatment in hot aqueous ethanol (O'Connell et al., 2001). After centrifugation, S2 had the lowest H_o, while there was no significant difference ($P > 0.05$) between S4 and S5. As in Table 2-2, 95% of casein not binding zein nanoparticles is expected to be removed after centrifugation, and

the H_0 measured was the zein-casein complex. The results in Table 2-4 are in agreement with the complex composition in Table 2-3 that shows a greater amount of casein on the surface of zein nanoparticles in S2 and no difference in S4 and S5.

2.4.6 Dispersion stability

The dispersion stability at pH 7.0 and 0-300 mM NaCl is shown in Figure 2-6. For fresh dispersions of S2 and S5, samples appeared to be visually more turbid at a higher NaCl concentration, while the difference was not apparent for S4. After 15-day storage at 4 °C, no precipitation was observed for S4, while S2 and S5 precipitated severely, corresponding to a clear serum phase (Figure 2-6A). The visual appearance at day 0 was in agreement with Abs_{600} (Figure 2-6B). As for particle size, a bigger $d_{4,3}$ was observed at a higher NaCl concentration, suggesting aggregation between zein nanoparticles due to weakened electrostatic repulsion. The $d_{4,3}$ of S4 and S5 was similar at day 0, which agrees with no difference in particle diameter studied in TEM (Figure 2-5). The difference in turbidity (Figure 2-6B) and DLS (Figure 2-6C) may be due to the fact that the dispersions were diluted by 100 times during DLS, which dissociates some flocculated nanoparticles (Benjamin et al., 2012).

To further study the structure of nanoparticles, the precipitated particles of S2 and S5 dispersions after 15-day storage and that of (non-precipitating) S4 after centrifugation were determined for the mass ratio of zein:casein as above, summarized in Table 2-6. For S2, the zein:casein ratio increased significantly after storage (Tables 2-6 vs. 2-2) and was higher at a higher NaCl concentration, indicating the loss of caseins during storage. For S5, the treatment with 0 mM NaCl had similar zein:casein ratio before and after storage but those of treatments with 75-300 mM NaCl increased significantly. Conversely, the zein:casein ratio of S4 remained similar to that without storage (Tables 2-6 vs. 2-2) and was similar at all NaCl concentrations.

When the precipitated particles were analyzed for SDS-PAGE (Figure 2-7), S2 only had κ-casein remaining on zein nanoparticles after storage, while S4 and S5 also had κ-casein only.

2.4.7 Interpretation of structural differences leading to variations in dispersion stability

Summarizing the above findings, the structures of zein nanoparticles have substantial differences due to variations in precipitation conditions. For S2, different types of caseins in NaCas dispersion form complexes that adsorb onto precipitated zein nanoparticles as a whole. During storage, α and β-caseins detach from S2 zein nanoparticles due to weaker interactions than κ-casein, which is again in coincidence with the stronger interfacial shear viscosity of κ-casein than that of α- and β-caseins (Dickinson, 1998). Interactions between zein nanoparticles and κ-casein likely involve both hydrophobic interactions and electrostatic attraction. Electrostatic attraction can occur between oppositely-charged amino acid patches on separate proteins, even when both have net negative charges (Dickinson, 2003), and is weakened at an increased ionic strength to cause the partial detachment of κ-casein during storage. For S4 and S5, only more surface active κ-casein is associated with zein nanoparticles. In S5, κ-casein adsorbs on zein nanoparticles and the detachment due to weakened electrostatic attraction causes the instability during storage. For S4, it is possible that κ-casein co-precipitates with zein during liquid-liquid dispersion and the hydrophobic fraction is trapped in zein nanoparticle matrices. The ionic strength thus has minimized impacts on the detachment of κ-casein. The κ-casein fraction on zein nanoparticles of S4, with possibly strengthened interactions with caseins in the continuous phase (Figure 2-5), provides stronger repulsive steric and electrostatic interactions than that of S2 and S5 and thus enables better storage stability.

2.5 Conclusions

The present study showed that spray-dried zein nanoparticles produced at conditions dissociating NaCas (S4 and S5) improved the hydration of powder and dispersibility of zein nanoparticles. The dissociated casein was present at a smaller quantity on zein nanoparticles than NaCas, corresponding to higher surface hydrophobicity. By co-precipitating dissociated NaCas and zein, the formed nanoparticle complex was smaller, reduced turbidity of dispersion, and was stable against precipitation at pH 7.0 and 0-300 mM NaCl. The Approach B also reduced the amount of ethanol in producing zein nanoparticles. The findings are important for the application of zein nanoparticles as delivery systems of bioactive compounds.

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Appendix

Table 2-1. FTIR spectra peak locations and assignments for zein, NaCas, a simple mixture of NaCas and zein, S2, S4 and S5 nanoparticles.

Peaks	Structure	Peak wavenumber (cm^{-1})						Assignments
		zein	NaCas+zein	NaCas	S2	S4	S5	
p1	Amide B	3059	3061	3064	3070	3066	3064	C–H stretching (Lal and Nafie, 1982)
p2		2960	2960	2960	2960	2960	2960	CH_3 asymmetric (Abe and Krimm, 1972)
p3		2931	2931	2933	2933	2933	2935	CH_2 asymmetric (Abe & Krimm, 1972)
p4		2873	2873	2875	2873	2873	2875	CH_2 stretching (Dong et al., 2005)
p5	Amide I	1653	1643	1643	1659	1659	1659	C=O stretch/hydrogen bond coupled with COO- (Li, et al., 2013)
p6	Amide II	1533	1531	1531	1531	1537	1531	NH bend coupled with CN stretch (Li et al., 2013e)
p7		1448	1444	1444	1450	1450	1450	CH_2 bend (Kamnev et al., 2002)

Table 2-2. The particle size and precipitation percentage of reconstituted NaCas at pH 7.0, after processing as in Figure 2-1. *

Sample	$d_{4,3}$(nm) (pH=7.0)	Protein concentration (mg/mL)			Precipitation percentage (%)
		Precipitate	Supernatant	Total	
N1	166 ± 4[b]	5.78 ± 0.19	0.32 ± 0.04	6.11 ± 0.20	5.21 ± 0.54[a]
N2	137 ± 6[d]	3.13 ± 0.14	0.17 ± 0.03	3.30 ± 0.11	5.21 ± 1.03[a]
N3	155 ± 6[c]	5.43 ± 0.19	0.16 ± 0.13	5.59 ± 0.05	4.24 ± 0.16[a]
NaCas	184 ± 11[a]	-	-	-	-

* Different lower-case letters next to numbers represent significant difference ($P < 0.05$) for the mean in the same column; "-" means "not tested"; precipitation stability was tested after centrifugation at 6700*g* for 20 min.

Table 2-3. The protein concentrations in fractions separated from spray dried powders according to the scheme in Figure 2-2.

Sample	Protein concentration (mg/mL)				Estimated mass ratio of zein:casein in nanoparticles *
	Fraction A (casein+zein)	Fraction C (zein)	Fraction D (casein)	Total	
S2	2.36 ±0.249	2.10 ±0.249	1.59 ±0.531	6.06 ±0.202	1.4:1
S4	2.21 ±0.237	1.46 ±0.147	0.36 ±0.205	4.04 ±0.227	4.3:1
S5	3.62 ±0.269	2.11 ±0.407	0.41 ±0.509	6.24 ±0.407	4.3:1

* Casein mass was corrected using the precipitation percentage in Table 2-2.

Table 2-4. The $d_{4,3}$, zeta-potential and surface hydrophobicity (H_o) of dispersions reconstituted from spray-dried samples in distilled water and adjusted to pH 7.0.*

		S2	S4	S5
$d_{4,3}$ (nm)	Before centrifugation	136 ± 4^{a}	123 ± 8^{b}	128 ± 7^{ab}
	After centrifugation	136 ± 3^{a}	122 ± 6^{b}	123 ± 4^{b}
Zeta potential (mV)	Before centrifugation	-44 ± 3^{a}	-39 ± 1^{b}	-37 ± 3^{b}
	After centrifugation	-34 ± 3^{c}	-33 ± 1^{c}	-36 ± 2^{c}
H_o	Before centrifugation($\times 10^3$)	9.6 ± 0.5^{c}	6.7 ± 0.0^{d}	9.9 ± 0.6^{c}
	After centrifugation($\times 10^4$)	1.4 ± 0.2^{b}	2.1 ± 0.1^{a}	2.1 ± 0.3^{a}

* Numbers are mean ± standard deviation from two spray-dried replicates, each tested twice (n = 4). Different lower-case letters next to numbers represent significant difference ($P < 0.05$) in mean for each parameter.

Table 2-5. Percentages of proteins dissolved * after hydration for different durations.

Sample	Hydration duration (h)		
	1	4	6
S2	41 ± 5%[b]	40 ± 4%[b]	41 ± 5%[b]
S4	63 ± 7%[a]	63 ± 5%[a]	64 ± 8%[a]
S5	62 ± 1%[a]	61 ± 2%[a]	61 ± 1%[a]

* Samples were centrifugated at 6700*g* for 20 min to collect the supernantant for BCA assay. Percentages were calculated with respect to the protein concentration before centrifugation. Numbers are mean ± standard deviation from two spray-dried replicates, each tested twice (n = 4). Different lower-case letters next to numbers represent significant difference ($P < 0.05$) in mean for each parameter.

Table 2-6. The estimated zein:casein mass ratio of precipitated S2, S4*, and S5 zein nanoparticles after storage at 4 ℃ for 15 d at pH 7.0 and 0-300 mM NaCl.

Sample	NaCl concentration (mM)			
	0	75	150	300
S2	4.0:1	5.0:1	5.4:1	5.8:1
S4	4.6:1	4.7:1	4.3:1	4.5:1
S5	4.6:1	5.7:1	8.1:1	11.5:1

* S4 did not show precipitation and was centrifugated at 6700*g* for 20 min to collect particles for analysis.

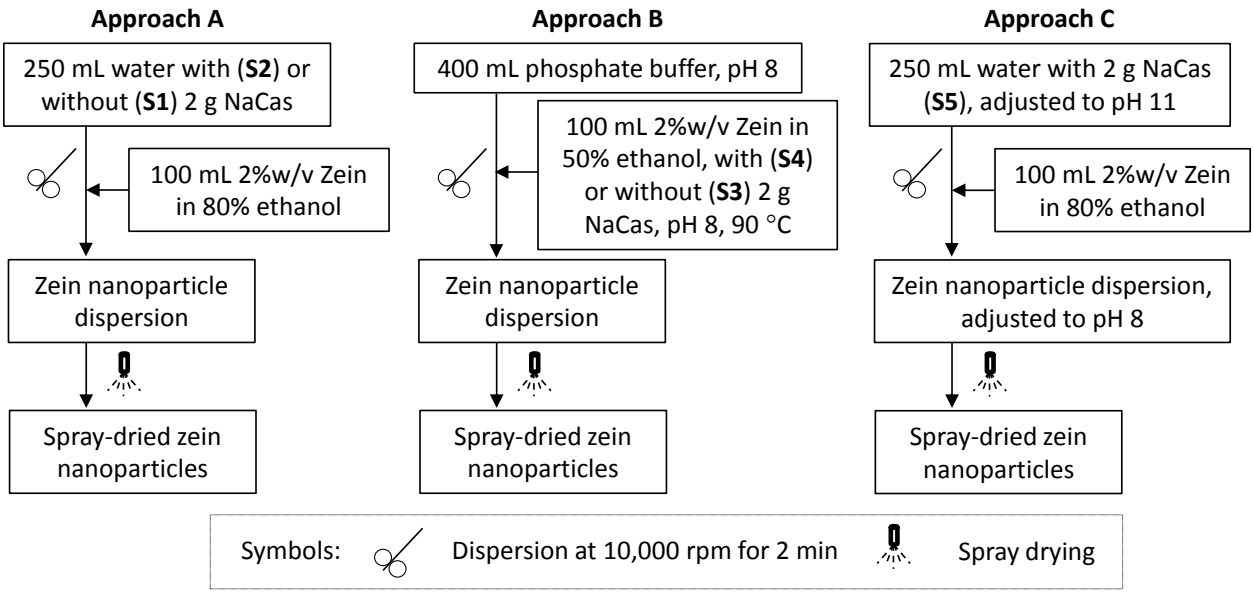

Figure 2-1. Processes of producing spray-dried nanoparticles using approaches A, B, and C.

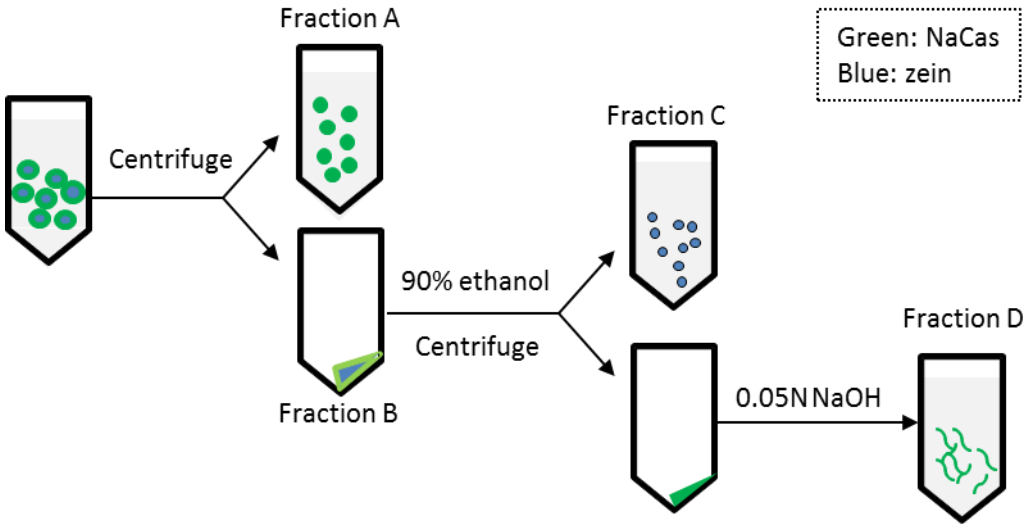

Figure 2-2. The scheme of separating S2, S4 and S5 into four fractions.

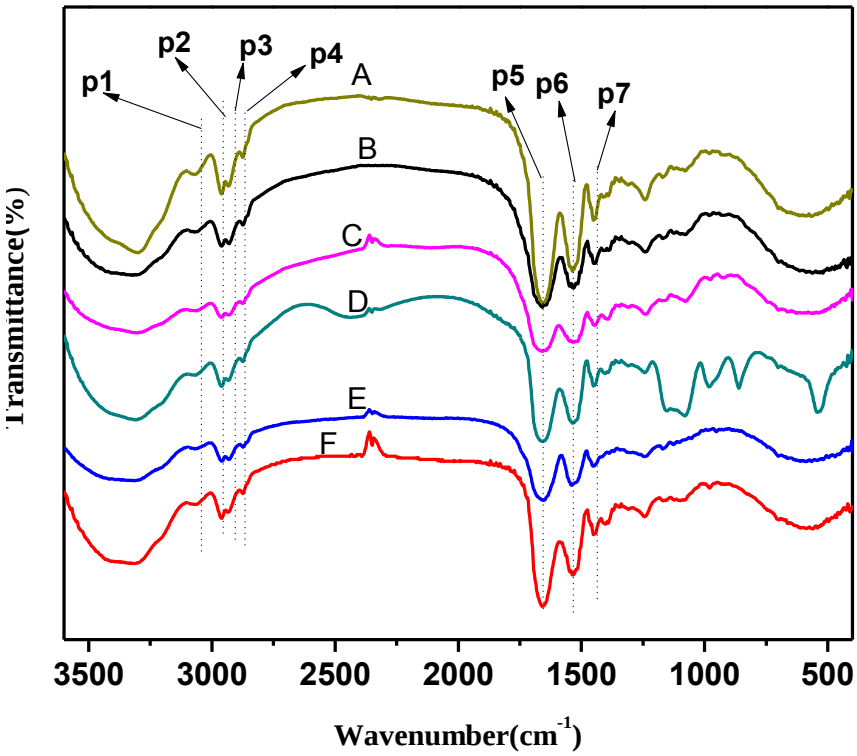

Figure 2-3. FTIR spectra of powdered sodium caseinate (C), zein (E), a simple mixture of zein and NaCas powder (B), and samples S2 (A), S4 (D), and S5 (F).

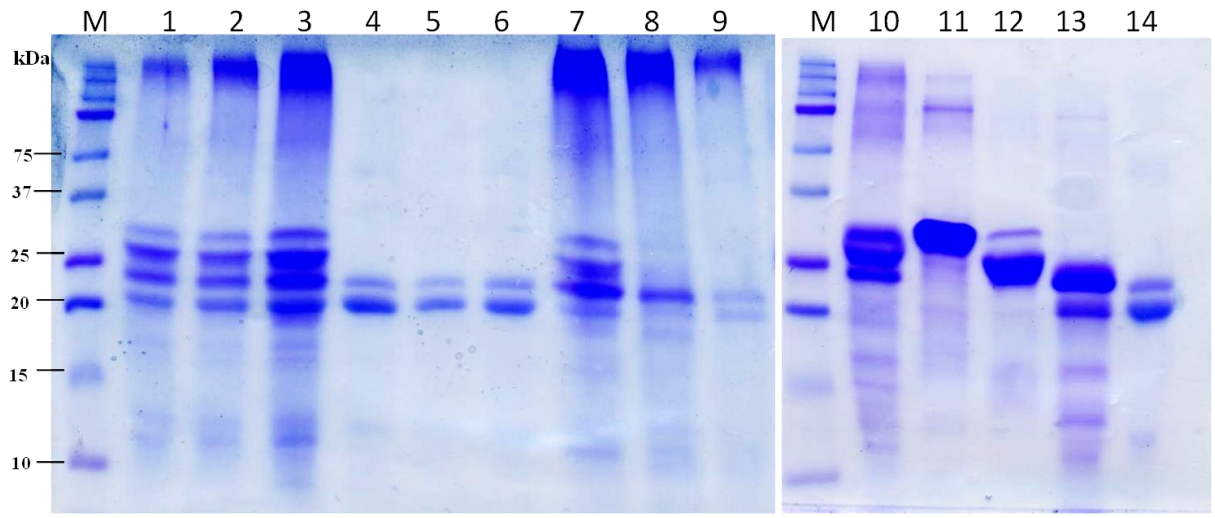

Figure 2-4. SDS-PAGE results of fractions separated from S2, S4 and S5 according to the procedures in Figure 2-2. Lane M: protein molecular weight marker. Lanes 1, 2, and 3 are the Fraction A from S2, S4, and S5, respectively. Lanes 4, 5, and 6 are the Fraction C from S2, S4, and S5, respectively. Lanes 7, 8 and 9 are the Fraction D from S2, S4, and S5, respectively. Lanes 10, 11, 12, 13 and 14 are unprocessed sodium caseinate, α-casein, β-casein, κ-casein, and zein, respectively.

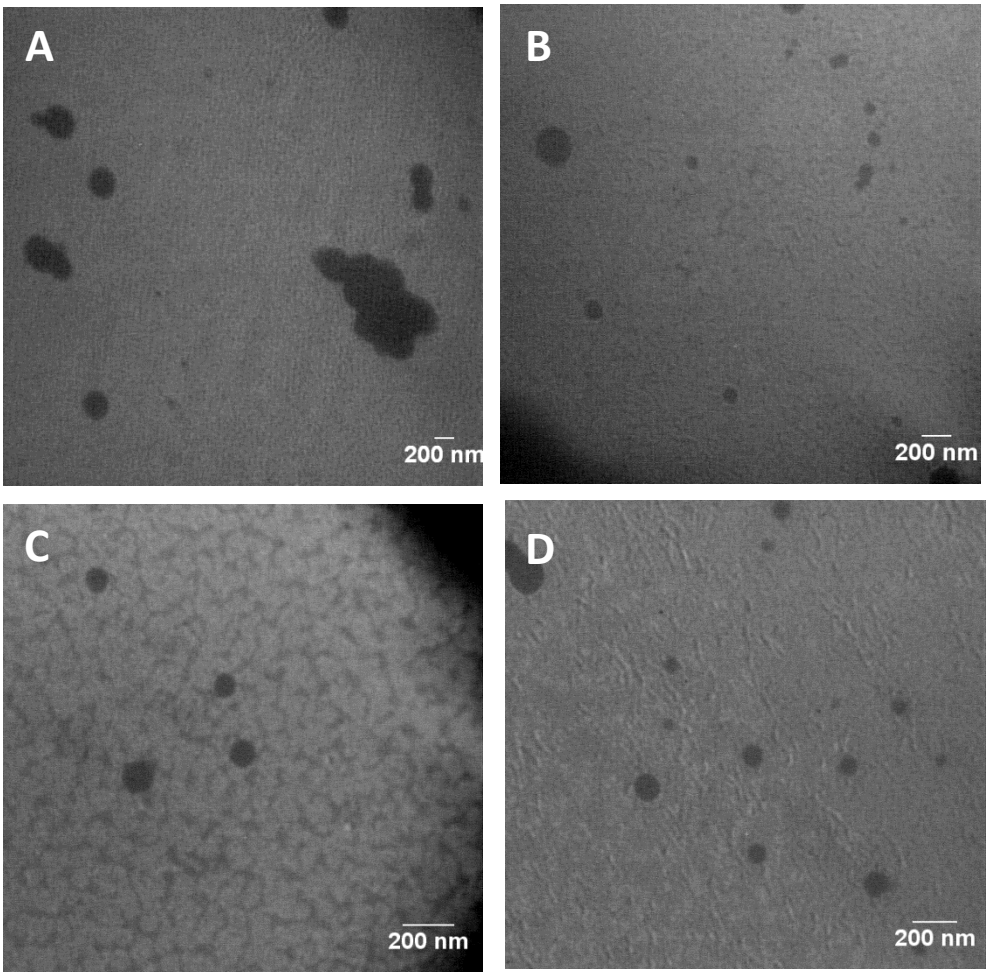

Figure 2-5. TEM images of zein nanoparticles at pH 7.0: (A) S3, (B) S2, (C) S4, and (D) S5.

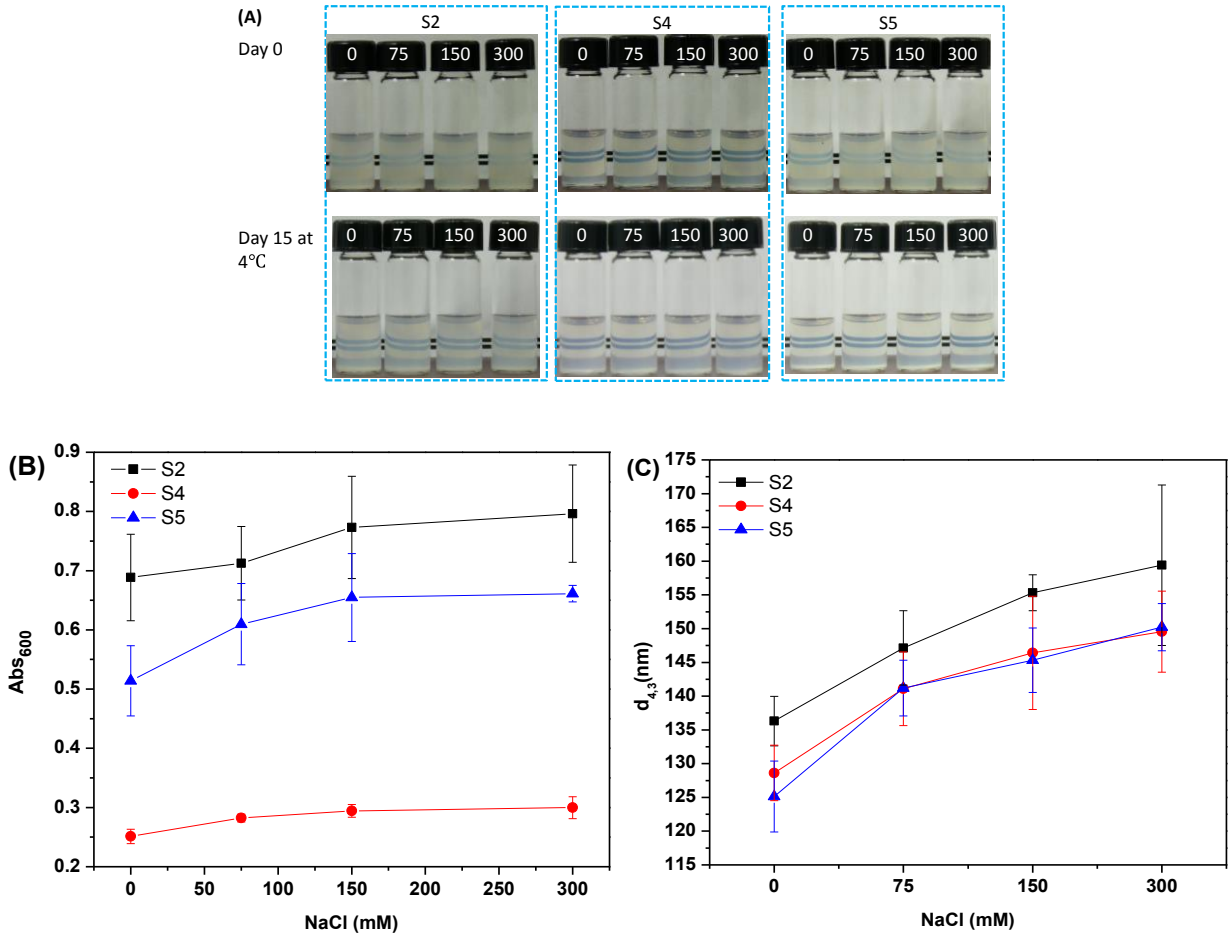

Figure 2-6. Impacts of 0-300 mM NaCl on dispersibility of nanoparticles at pH 7.0: (A) Visual appearance before and after 15-day storage at 4 °C, and (B) absorbance at 600 nm (Abs_{600}) and (C) $d_{4,3}$ of freshly prepared dispersions. Numbers on vial caps in (A) are NaCl concentrations in mM. Error bars are standard deviations from two spray-dried replicates, each tested twice (n = 4).

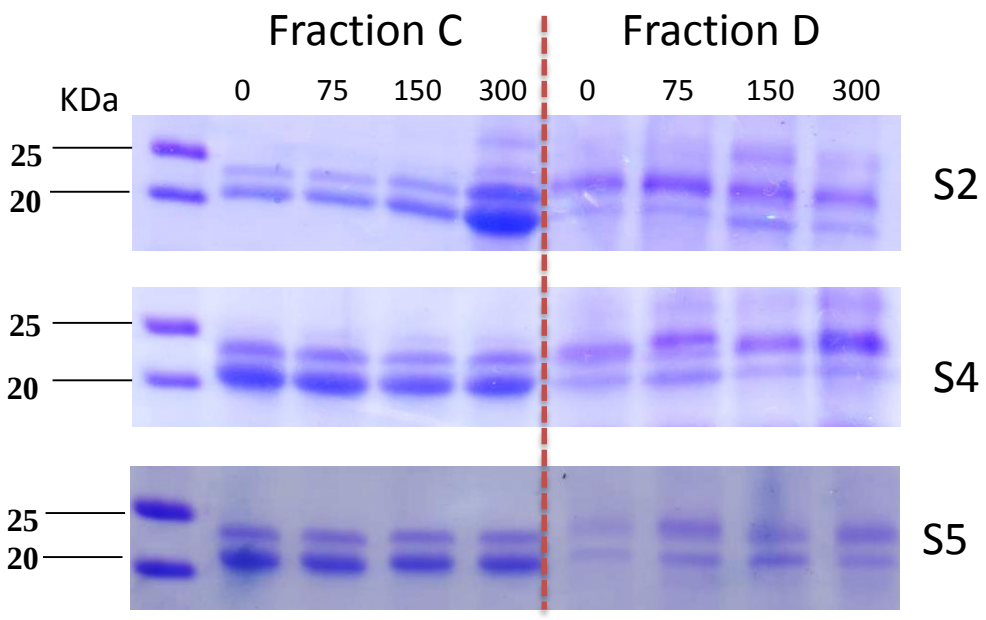

Figure 2-7. SDS-PAGE results of precipitated fractions from S2 and S5 after storage at 4 °C for 15 d. No precipitation was observed for S4 after storage, which was centrifuged at 6700*g* for 20 min to obtain the precipitated fractions. Fractions C and D were then obtained from precipitates following conditions in Figure 2-2 for SDS-PAGE. Numbers above lanes (0, 75, 150, and 300) indicate the concentration of NaCl (mM).

Chapter 3. Impacts of sample preparation methods on solubility and anti-listerial characteristics of essential oil components in milk

A version of this chapter was originally published by Huaiqiong Chen, P. Michael Davidson and Qixin Zhong:

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My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

3.1 Abstract

Essential oil components (EOCs) have limited water solubility and are used at much higher concentrations in complex food matrices than in growth media to inhibit pathogens. However, the correlation between solubility and activity has not been studied. The objective of this work was to characterize the solubility of EOCs in solvents and milk and correlate solubility with antilisterial activity. The solubilities of four EOCs, thymol, carvacrol, eugenol, and *trans*-cinnamaldehyde, in water was significantly increased in the presence of 5% (v/v) ethanol. In milk, the solubility of EOCs was lower than in water, with lower solubility in higher-fat milk. EOCs applied to milk as stock solutions (in 95% aqueous ethanol) enabled quicker dissolution and higher solubility in milk serum than other methods of mixing, such as end to end, and greater reductions of *Listeria monocytogenes* Scott A after 0 and 24 h. When the EOC concentration detected in milk serum was above the minimum bactericidal concentration, complete inhibition of L. monocytogenes in tryptic soy broth resulted. Therefore, the antilisterial properties in milk could be correlated with the solubility by comparison to the minimum inhibitory or bactericidal concentrations of EOCs. While the EOCs applied using ethanol generally had solubility and activity characteristics superior to those of other mixing methods, ethanol is not used to a great extent in nonfermented foods. Therefore, mixing methods without an organic solvent may be more readily adaptable to enhancing the distribution of EOCs in complex food systems.

Key words: essential oil components, solubility, anti-listerial activity, preparation methods, 95% ethanol, gentle mixing

3.2 Introduction

Plant essential oils (EOs) or EO components (EOCs) are gaining intensive interest as naturally-occurring food preservatives due to their broad spectrum of activity against foodborne pathogens and generally-recognized-as-safe (GRAS) regulatory status (Burt, 2004). It is well known that EOs/EOCs perform well in antimicrobial assays conducted using microbial growth media, also called "*in vitro*" tests, but their effectiveness is much reduced in complex food matrices with compounds binding EOs/EOCs. EOs/EOCs are lipophilic and have limited solubility in water (Table 3-1), mostly below 2 g/L at around 20°C. The levels of EOCs used in *in vitro* tests, with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) being the most frequently compared parameters, are usually within the solubility limit (Nazer et al., 2005; Nostro et al., 2004; Pol and Smid, 1999). The level of EOs/EOCs needed to achieve inhibitory and bactericidal activities in foods is highly dependent on composition. Typically, food matrices with hydrophobic food components, like proteins and lipids can cause significant reductions in antimicrobial activities of EOs/EOCs (Kühn et al., 2006). Despite numerous studies on antimicrobial activities of EOs/EOCs and speculation about food matrix interferences (MendozaYepes et al., 1997; Shah et al., 2012a; Ultee and Smid, 2001), quantification of solubility of EOCs in food systems and correlation to antimicrobial activities in food matrices have not been attempted.

In order to improve the distribution of EOs/EOCs in food matrices and reduce interference by food components, various delivery systems such as emulsions (Weiss et al., 2011; Weiss et al., 2006) and biopolymer capsules (Fernandes et al., 2008; Shah et al., 2012a; Shah et al., 2013b; Si et al., 2006) have been studied to enhance the antimicrobial activity and reduce the usage level. Commonly, the efficacy of delivery systems is compared to same

concentrations of free (unencapsulated) EOs/EOCs. Tests in food matrices typically require the use of EOs/EOCs above the solubility limit. Various methods have been used to prepare free EOs/EOCs and thus, comparisons of various studies is a challenge. In most studies, organic solvents such as dimethyl sulfoxide (Cao et al., 2009), methanol (Sokmen et al., 2004a; Sokmen et al., 2004b), ethylene glycol (Lahlou, 2004), and ethanol (Helander et al., 1998; Mourey and Canillac, 2002; Rivas et al., 2010) are used to prepare stock solutions that are diluted in a test medium to obtain the overall test concentration. The use of water-miscible organic solvents changes the polarity of the aqueous phase when the stock solution is blended with a liquid medium. However, food matrices are typically free of organic-solvents. The solubility of EOs/EOCs can also be altered by using surfactants. The polysorbate family of surfactants including Tween® 20 or Tween® 80 (Carson and Riley, 1995; Cox et al., 2000; Kim et al., 1995a) can be used by mixing water, surfactant and EOs/EOCs using a vortex mixer. Vortex mixing is a low-shear process that creates an oil-in-water emulsion that is studied as a category of delivery systems, as discussed previously, and changes distribution properties of EOs/EOCs. Characterization of the solubility characteristics of EOs/EOCs, prepared using various methods in various matrices and correlation of solubility characteristics with antimicrobial activities may thus enable the comparison of studies using free antimicrobials. Such information also provides a rational basis to select free antimicrobials in studying delivery systems of antimicrobials.

The first objective of the present study was to characterize the solubility of four commonly-studied EOCs, thymol, carvacrol, eugenol and *trans*-cinnamaldehyde, in various solvents as a result of different preparation methods. The second objective was to compare antimicrobial activity of EOCs prepared with or without ethanol as a solvent, using *Listeria monocytogenes* Scott A as a model bacterium due to its significance to microbiological safety of

dairy products (Mussa et al., 1998). The third object was to qualitatively correlate antimicrobial activity with solubility by comparing to the MICs/MBCs of EOCs under the corresponding solvent conditions.

3.3 Materials and methods

3.3.1 Materials

Thymol (99% purity) and ethanol (100% and 95%) were products from Acros Organics (Morris Plains, NJ). Eugenol (>98% purity), *trans*-cinnamaldehyde (99% purity) and carvacrol (99% purity) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). High-performance liquid chromatography (HPLC)grade water and methanol (>99% purity) were purchased from Fisher Scientific (Pittsburgh, PA). Ultrahigh temperature-processed skim milk, 2% fat reduced milk and full (3.3%) fat milk (Simple Truth Organic^{TM}, San Diego, CA) were purchased from a local grocery store.

3.3.2 Solubility of EOCs in water, TSB, 2% v/v ethanol and 5% v/v ethanol

The solvents studied were water and low concentrations (2 and 5% v/v) of aqueous ethanol. To determine the solubility of EOCs in different solvents, 5 g/L of an EOC was mixed with each solvent and stirred using a stir plate (Fisher Scientific) at room temperature (21°C) for at least 24 h to reach solubilization equilibrium. The mixture was filtered through a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Fisher Scientific) and 1 mL of the permeate was used for the HPLC analysis (see below). The solubility in tryptic soy broth (TSB) was tested similarly. Each sample was analyzed in triplicate.

3.3.3 Solubility of EOCs in milk prepared with different methods

Three methods were compared for mixing EOCs with milk at room temperature (21°C). In the end-to-end shaker method (ETEM), an EOC was weighed directly into glass tubes according to the mass expected from the overall concentration (5 g/L for thymol and carvacrol, 7 g/L for eugenol, and 9 g/L for *trans*-cinnamaldehyde), 9 mL milk plus 1mL water was added, and the tubes were capped and attached on an end-to-end shaker (Thermo Scientific, Hudson, NH) for 0, 5, 15, and 30 min, or 24 h. In the vortex method (VM), the capped tubes with milk and EOC were agitated with a vortex mixer at the lowest speed for different durations that for the ETEM. In the ethanol pre-dissolving method (EPM), stock solutions of EOCs were prepared in 10 mL volumetric flasks at a concentration of 100 g/L for thymol and carvacrol, 140 g/L for eugenol, and 180 g/L for *trans*-cinnamaldehyde in 95% aqueous ethanol. The 500 μL of the stock solution was added into 8.5 mL milk with 1mL water and mixed by vortex for ~10 s to obtain the same EOC final concentrations as ETEM and VM. Samples were assayed shortly after mixing (0 h) and after 24 h.

The above-mentioned milk samples were acidified to pH 4.6 to precipitate caseins. After centrifugation at 4564 × *g* for 5 min (Sorvall RC-5B plus, Sorvall, Newtown, CT) at 20 ℃, the supernatant was filtered through a 0.45 μm PVDF membrane (Fisher Scientific) to obtain the permeate for HPLC analysis. The permeate as prepared is referred as milk serum hereafter.

3.3.4 HPLC apparatus and quantification conditions

A reversed phase HPLC system (1200 series, Agilent Technologies, Waldbronn, Germany) was used to quantify EOCs. The system consisted of a quaternary pump module, a degasser, an auto-sampler, a temperature-controlled column chamber, and an Agilent diode array detector. Chromatograms were recorded and integrated by the 1200 LC Chromatography Data

System. All experiments were performed on an Agilent ZORBAX Eclipse Plus C18 HPLC column (5 μm; 150 mm by 4.6 mm; Agilent, Palo Alto, CA), protected by a ZORBAX Eclipse Plus C-18 guard column (4.6 by 12.5 mm; 5 μm). The sample injection volume was 10 μL, and the detection wavelength was 274 nm. A binary solvent mixture of water (solvent A) and methanol (solvent B) at different proportions was used as the mobile phase. The optimized elution conditions were a linear gradient from 20% B to 80% B within 20 min, an isocratic step with 80% B for 5 min, and a linear gradient from 80% B to 20% B in 5 min. The flow rate was 0.5 mL/min, and the column chamber was controlled at 25 ℃.

External standards were used to establish calibration curves. EOCs were dissolved in methanol at 2 g/L in 10mL flask volumetric flasks to be transparent solutions and were filtered through the 0.45 μm PVDF membrane before dilution in methanol to the concentration range in Table 3-2. At least five concentrations within the range were used to generate a calibration curve correlating the peak area (*A*) and EOC concentration (*C*). To test the recovery of EOCs in milk serum samples, a known amount of each EOC was spiked in the serum prepared as above from full fat milk without EOC at an overall concentration of 0.05, 0.1, and 0.2 g/L for thymol, eugenol and carvacrol and 0.005, 0.01, and 0.02 g/L for trans- cinnamaldehyde. The samples were then filtered as described above for quantification using HPLC. The recovery percentage was calculated based on the amount of EOC estimated using the calibration curve with respect to the amount of EOC spiked in the milk serum.

3.3.5 Culture preparation

The *L. monocytogenes* strain Scott A culture was obtained from the culture collection of the Department of Food Science and Technology at the University of Tennessee (Knoxville, TN). The stock culture, stored in glycerol at -20°C, was transferred in TSB at 32 ℃ for 2

consecutive days. The *L. monocytogenes* was grown for 18 h at 32 ℃ before dilution to ~7.0 log CFU/mL in TSB as the working culture.

3.3.6 Microplate dilution assay to determine MICs and MBCs of EOCs against L. monocytogenes

A broth dilution method with modification was used to determine the MICs and MBCs of EOCs against *L. monocytogenes* (Basile et al., 2006; Dung et al., 2008). Each EOC was prepared at a stock concentration of 100 g/L in 95% ethanol in 10 mL volumetric flasks. The working solution with 4 g/L of each EOC was prepared by diluting the 100 g/L stock solution in TSB. The 4 g/L working solution was further diluted in TSB to an EOC concentration of 0.1-4 g/L, with 0.2 g/L increments. To study the impacts of ethanol, the 4 g/L working solution was diluted from the 100 g/L stock solution with ethanol and TSB to an ethanol concentration of 5% v/v or 10% v/v. TSB was mixed with ethanol to an ethanol concentration of 5% v/v or 10% v/v, which was then used to dilute the 4 g/L working solution to obtain 0.1- 4 g/L EOC in 5% v/v or 10% v/v aqueous ethanol. The final EOC solution was added at 120 µL in each well of the sterile 96-well plate. A total 120 µL of the working culture with 6 log CFU/mL *L. monocytogenes* was added to each well, corresponding to overall EOC concentrations of 0.05-2 g/L. Plates were incubated at 32 ℃ or 21 ℃ for 24 h. The MIC was the lowest concentration that did not show any visual growth (Basile et al., 2006; Dung et al., 2008). Using the results of the MIC assay, 20 µL of each culture broth from the wells with EOC concentrations equal to or higher than the MIC was transferred onto tryptic soy agar (TSA) plates and incubated for another 48 h at 32 ℃ or 21 ℃. The lowest concentration of each EOC corresponding to the absence of *L. monocytogenes* growth on TSA was treated as the MBC. Fresh working cultures were grown separately in triplicate, and each EOC concentration was tested four times for each working culture.

3.3.7 Anti-listerial activity of EOCs in milk

The ETEM and EPM were chosen to compare differences of anti-listerial properties in milk as affected by the preparation method. *L. monocytogenes* working culture (1 mL), prepared to ~7.0 log CFU/mL in TSB as described above, was added to milk to obtain an overall population of around 6 log CFU/mL. In the ETEM, 9 mL milk was mixed with 1 mL of the working culture, while 8.5 mL milk was mixed with 1 mL of the working culture and 0.5 mL of the EOC stock solution (in 95%) in the EPM. The negative control was the mixture of milk and working culture, while the ethanol control had an additional 500 μL of 95% or 70% ethanol, to test the growth of *L. monocytogenes* in a low overall concentration of ethanol (~4.75% v/v and ~3.5% v/v). *L. monocytogenes* was enumerated by the pour plate method on TSA plates before (treated as 0 h time point) and after 24 h incubation at room temperature (21°C), and the log reduction was determined. For the 0 h time point, the working culture was mixed into milk by vortex mixing as the last step and the plating on TSA was conducted immediately afterwards. Each treatment was repeated in three independent replicates, with fresh working culture grown separately in each replication (n = 3). The qualitative correlation of anti-listerial activity and EOC concentration was done by comparing the EOC concentration in milk serum to the MIC/MBC of EOCs at the corresponding solvent conditions.

3.3.8 Statistical analysis

All experiments were conducted in triplicate and data were expressed as the mean ± standard deviation. Statistical significance (ANOVA) was determined using SAS software (version 9.3, SAS Institute, Cary, NC). Differences between pairs of means were analyzed using a post hoc Tukey test at a significance level of 0.05.

3.4 Results

3.4.1 Validation of the HPLC assay for quantifying EOC solubility in solvents and milk

The calibration curves of the HPLC assay for the four EOCs are presented in Table 3-2. The linear range for thymol, carvacrol and eugenol was from 0 to 1 g/L while the upper limit of *trans*-cinnamaldehyde was 0.1 g/L. The R^2 values of all calibration curves were greater than 0.999. The chromatograph of the serum from full fat milk did not show milk component peaks appearing at the elution time range (19-26 min) of EOCs (chromatographs not shown). The recovery percentage was higher than 95% for all the samples tested, indicating the applicability of the calibration curves to the samples prepared from milk. Therefore, the established HPLC conditions can be used to quantify the solubility of EOCs in both solvents and milk.

3.4.2 Solubility of EOCs in water, TSB, 2% v/v ethanol and 5% v/v ethanol

The solubility of thymol, carvacrol, eugenol and *trans*-cinnamaldehyde in water at room temperature (~21℃) was 0.48, 0.45, 1.35 and 1.41 g/L, respectively (Table 3-2). The solubility data tested at the studied conditions show some differences from the literature that also varied significantly (Table 3-1). Generally, the solubility of these four EOCs was not different in water and TSB ($P > 0.05$) and increased with the presence of low levels of ethanol, with the 5% ethanol treatment being significantly (~20 to 30%) higher than those in water.

3.4.3 Amounts of EOCs dissolved in milk using different mixing methods

The ETEM, VM, and EPM using 95% ethanol were first compared for the thymol concentration in the serum of full fat milk. Mixing 5 g/L of thymol with milk using ETEM and VM for up to 30 min resulted in significant increases in the thymol concentration while the increase was insignificant ($P > 0.05$) after mixing for 24 h (Figure 3-1). Less than 0.15 g/L thymol was detected in the serum, and was not different ($P > 0.05$) betwen the two agitation

methods. The thymol concentration in milk serum using the EPM was similar after different mixing durations (0.18 g/L at 0 h versus 0.17 g/L at 24 h), but was higher ($P < 0.05$) than that using the ETEM and VM. The 20% greater thymol concentration in the serum prepared using the EPM when compared to the ETEM/VM is similar to the results with water and 5% ethanol (Table 3-2). Even though a low speed was used, VM caused visible structural changes in the milk after long mixing times (Figure 3-2), which contrasted with no visible changes in the milk using the ETEM. Additionally, the shear force of VM can create emulsions of EOs/EOCs if samples contain emulsifiers (e.g., surface-active proteins in milk) which would not be characteristic of "free" EOCs. Therefore, the ETEM method, which is easier to use to reach the solubility (in 30 min) than VM, is recommended to maintain solubility properties of EOCs.

The four EOCs were then studied in the skim, 2% fat and 3.3% fat milk products prepared by mixing for 30 min using ETEM and the short-time vortexing using EPM. The concentration of EOCs in the milk serum was determined after 0 and 24 h (Figure 3-3) and was found to be affected by both the mixing method (ETEM or EPM) and the milk fat composition. The EOC concentration in the milk serum decreased with an increase in fat content ($P < 0.05$). The ETEM duration did not show any significant effects on carvacrol and eugenol solubility (Figure 3-3 B&C) but the concentration of *trans*-cinnamaldehyde in skim milk decreased after 24 h (Figure 3-3D). The EOC concentration in the milk serum prepared by the EPM was consistently higher than that with the corresponding ETEM treatment, and the effect of incubation time was insignificant ($P>0.05$) (Figure 3-3).

3.4.4 MICs and MBCs of EOCs in TSB and correlation with solubility

The MICs and MBCs of EOCs in TSB with different amounts of ethanol at 32 ℃ and 21 ℃ are listed in Table 3-3. For ethanol alone, the MIC was 10% v/v and 5% v/v at 32 ℃ and

21 ℃, respectively, while the MBC was 20% v/v at both temperatures. The MICs of thymol and carvacrol were 0.2 g/L at both 32 and 21 ℃, except the 5% ethanol treatment at 21 ℃, which had a MIC below the lowest EOC concentration tested due to sufficient inhibition (MIC) by 5% ethanol alone. The MBCs of thymol and carvacrol was 0.3 g/L at all tested conditions. Conversely, the combination of ethanol with eugenol and *trans*-cinnamaldehyde showed enhanced anti-listerial properties. With the exception of the MIC of *trans*-cinnamaldehyde (0.2 g/L) at 32 ℃, reduced MICs and MBCs were observed at a higher level of ethanol. Overall, the MBCs of the four EOCs did not differ between 32 ℃ and 21 ℃ but the MIC was lower at 21°C for *trans*-cinnamaldehyde and eugenol.

Increased solubility of EOCs in the presence of low concentrations of ethanol had different effects on the MIC and MBC depending on the type of EOC. For thymol and carvacrol, the MBCs (0.3 g/L, Table 3-3) at 21°C were 69.8% and 73.2% of the solubility in TSB (Table 3-2), respectively. Because the MBC is well-below the solubility, the same MIC and MBC at the three ethanol concentrations tested were observed (Table 3-3). Conversely, the MBCs of eugenol and *trans*-cinnamaldehyde with the lowest ethanol concentration (Table 3-3) at 21°C were 91.5% and 92.7% of their solubility in TSB (Table 3-2), respectively, and the increased solubility by low concentrations of ethanol reduced the MIC and MBC.

3.4.5 Anti-listerial activity of EOCs in milk and correlation to solubility

The log reductions of *L. monocytogenes* in the three milk samples after treatment by EOCs using the ETEM and EPM are presented in Tables 3-4 to 3-6. When 500 μL of 70% and 90% aqueous ethanol was mixed with 8.5 mL milk and 1 mL culture, the overall concentrations of ethanol corresponded to around 3.5% v/v and 4.75% v/v, respectively. Growth of *L. monocytogenes* was observed in both 3.5% v/v and 4.75% v/v ethanol controls but was slightly

lower than that of the negative control ($P < 0.05$). As 5% v/v ethanol was near the MIC at 21°C (Table 3-3), this agrees with the slight inhibition of growth of the microorganisms (Tables 3-4 to 3-6).

In skim milk (Table 3-4), 5 g/L thymol and carvacrol inactivated *L. monocytogenes* to below the detection limit with ETEM or EPM, and no recovery was observed after 24 h. The thymol concentrations in the aqueous phase (Figure 3-3) at 0 and 24 h were both higher than the MBC, 0.3 g/L (Table 3-3). For the 2.5 g/L thymol treatment, the difference between ETEM and EPM was apparent shortly after mixing with only a 0.3 log CFU/mL reduction for the former and below the detection limit for the latter. For the 2.5 g/L carvacrol treatment in skim milk, the *L. monocytogenes* was not detected in any treatment.

For *trans*-cinnamaldehyde at 4.5 or 9 g/L in skim milk (Table 3-4), a *ca.* 1 log CFU/mL reduction occurred after 24 h in the ETEM compared with a >5 log CFU/mL for the EPM. The differences between the ETEM and the EPM were also observed for eugenol treatments tested at 7 and 3.5 g/L. With 7 g/L eugenol, *L. monocytogenes* was initially reduced to below the detection limit. Recovery was observed for the ETEM treatment after 24 h, but not the EPM treatment. For the ETEM, the 3.5 g/L eugenol treatment did not cause any apparent reduction of *L. monocytogenes* initially and only had a 2.8 log CFU/mL reduction after 24 h. The 3.5 g/L eugenol treatment prepared with EPM corresponded to 4.7 log CFU/mL reduction after 0 h, and *L. monocytogenes* was completely inactivated after 24 h. The differences were in agreement with the solubility characteristics shown in Figure 3-3C, i.e., a reduction of eugenol concentration after 24 h for the ETEM (although not statistically significant) compared to a slight increase in the EPM. For *trans*-cinnamaldehyde, the viable *L. monocytogenes* population was not below the detection limit. As shown in Figure 3-3D, the detected concentration of *trans*-cinnamaldehyde in

the milk serum was around 1.4 g/L at time 0 h and 1.2 g/L at 24 h for the ETEM, both of which were equal to or less than the MBC at <1.3% ethanol (1.4 g/L, Table 3-3). Conversely, the *trans*-cinnamaldehyde concentration in the milk serum was about 1.6 g/L in the EPM at both 0 and 24 h (Figure 3-3D) which was higher than the MBC (0.9 g/L, Table 3-3), resulting in complete inactivation.

In 2% reduced fat milk (Table 3-5), the inactivation of *L. monocytogenes* was reduced compared to skim milk at the same concentrations of EOCs (Table 3-4). For each EOC, even though treatments with the lower concentrations showed no or insignificant (except for 4.5 g/L *trans*-cinnamaldehyde) inactivation of *L. monocytogenes* after 24 h, there was still inhibition compared to the control of *L. monocytogenes* in EOC treatments prepared with the EPM. At higher EOC concentrations, the ETEM treatments with 5 g/L thymol and 5 g/L carvacrol reduced *L. monocytogenes* by ~1.5 log CFU/mL after 24 h, which was lower than EPM, which showed complete inactivation (>5.3 CFU/mL) after 24 h ($P < 0.05$). The solubility of thymol and carvacrol in 2% reduced-fat milk was 0.18 g/L and 0.2 g/L when prepared with the ETEM (Figure 3-3), both of which are around the MIC but lower than the MBC (Table 3-3). When thymol and carvacrol were dissolved via the EPM, the solubilities of these two EOCs were around 0.25 g/L (Figure 3-3). Even though this was slightly lower than the MBC (0.3 g/L), *L. monocytogenes* was inactivated to below the detection limit. For eugenol, the solubilities in both the ETEM and the EPM (Figure 3-3C) were lower than the MBC (Table 3-3), but the higher solubility in the EPM resulted in better log reduction at 24 h ($P < 0.05$). *trans*-Cinnamaldehyde in 2% reduced-fat milk showed a trend similar to that in skim milk. *trans*-Cinnamaldehyde (9 g/L) prepared with the EPM reduced *L. monocytogenes* to below the detection limit after 24 h, while the same concentration in the ETEM only caused a 1-log-unit reduction of *L.*

monocytogenes. As shown in Figure 3-3D, the *trans*-cinnamaldehyde concentration in the serum of 2% reduced-fat milk was higher than MBC (0.9 g/L, Table 3-3) at 0 h and 24 h when prepared by the EPM. In contrast, the concentration of *trans*-cinnamaldehyde in the serum of 2% reduced-fat milk (~ 1 g/L, Figure 3-3D) was lower than the MBC (1.4 g/L, Table 3-3) but higher than MIC (0.2 g/L) when prepared by the ETEM, which agrees with *ca.* 1 log CFU/mL reduction at both antimicrobial concentrations (Table 3-5).

Table 3-6 shows the anti-listerial activity of EOCs applied in full-fat milk. For 5 g/L thymol and carvacrol, growth of *L. monocytogenes* (negative log reductions) was observed in the ETEM treatments, while no growth (bacteriostasis) was seen with the EPM treatment. This again agrees with the EOC concentrations in the milk serum, i.e., much lower than 0.2 g/L (MIC) in the ETEM and near 0.2 g/L in the EPM (Figure 3-3A and 3-3B). Conversely, it was observed that 7 g/L eugenol inactivated *L. monocytogenes* by 0.4 log CFU/ml after 24 h when prepared with the EPM, compared to the > 1 log CFU/mL growth for the ETEM. The results corresponded to the concentration of eugenol in the milk serum (< 0.8 g/L, Figure 3-3C), being lower than the MBC (1.3 g/L) when prepared with the ETEM. Conversely, the concentration of eugenol in the serum of full fat milk via the EPM was around 0.9 g/L, which was close to the MBC of eugenol in TSB with 5% ethanol (1.0 g/L, Table 3-3). For *trans*-cinnamaldehyde, the concentration in the serum of full-fat milk via the ETEM was lower than its MBC (1.4 g/L, Table 3-3) but much higher than its MIC (0.2 g/L, Table 3-3), and corresponded to 0.8 and 1.3 log CFU/mL reduction in the ETEM and the EPM, respectively. The difference in log reductions between the ETEM and the EPM for *trans*-cinnamaldehyde was not significant. It was also observed that, while the concentration of *trans*-cinnamaldehyde dissolved via the EPM (Figure 3-3D) was higher than the MBC, complete inactivation of *L. monocytogenes* was not seen.

3.5 Discussion

The MICs and MBCs in the present study generally agree with the literatures. The MICs and MBCs of thymol and eugenol obtained at 32 ℃ in TSB were similar to those in our earlier study obtained using a 2-fold serial dilution method (Ma et al., 2013), with MIC and MBC of thymol and eugenol being 0.187 g/L and 0.375 g/L, and 0.75 g/L and 1.5 g/L, respectively. Similar MICs for thymol and carvacrol against *Listeria innocua* were observed by other researchers (Guarda et al., 2011). The MIC and MBC of *trans*-cinnamaldehyde against *L. innocua* at 35 ℃ were reported to be 0.5 g/L and 2 g/L, respectively (Hill et al., 2013), which are similar to the results in the present study (Table 3-3). The MIC of ethanol obtained in the present study agrees with a previous report showing strong inhibition of *L. monocytogenes* in TSB yeast extract by 5% v/v ethanol at 35 ℃ (Oh and Marshall, 1993).

The reduced activity of EOCs in complex food systems (milk in the present study) compared to that in microbial growth media and simple food systems also agrees with earlier studies. For example, 0.5 g/L thymol was needed in apple cider for inhibiting the growth of *Escherichia coli* O157:H7 ATCC 43889 and ATCC 43894, and *L. monocytogenes* strains Scott A and 101, while 9 times this concentration was needed for similar inhibition in 2% reduced fat milk (Shah et al., 2012a). In milk with various levels of milk fat, the MIC of eugenol against *E. coli* O157:H7 was 3.5, 4.5, and 5.5 g/L in skim, 2% reduced-fat, and full-fat milk, respectively, and the MBC was 1 g/L higher than MIC in the same medium (Shah et al., 2012a). For *L. monocytogenes* Scott A and 101, the MIC of eugenol were 3.5 g/L in skim milk, 4.5 g/L in 2% reduced-fat milk, and 6.5 g/L in full-fat milk, while the MBC were all 6.5 g/L (Shah et al., 2012a). Carvacrol reduced the growth of *Bacillus cereus* in brain heart infusion broth at 0.06 g/L but a 50-fold higher concentration was needed to achieve the same effect in various soups (Ultee

and Smid, 2001). A 100-fold increase in the concentration of a mixture of EO extracts from rosemary, sage and citrus in glycerol was needed to achieve similar inhibitory effects of *L. monocytogenes* in soft cheese when compared to treatments in TSB (MendozaYepes et al., 1997).

Proteins and lipids have been proposed as components interfering antimicrobial activities of EOCs in food matrices (Kühn et al., 2006). Milk is a good model food system to study such interference because it contains both proteins and lipids, and milk products with various fat levels and consistent protein content are readily available. Although the decreased antimicrobial activity of EOCs in milk with a higher fat content has been frequently observed (Gaysinsky et al., 2007; Shah et al., 2012a), the physicochemical mechanisms remain unclear. Bacteria have hydrophilic surfaces and are expected to be present in the aqueous phase (Gaysinsky et al., 2007; Weiss et al., 2005). Physically, the hydrophobic nature of EOCs enables their dissolution in phospholipids of the bacterial cell membrane, and a sufficient quantity of EOCs in phospholipids is needed to cause substantial changes in membrane structures and cell metabolism to inhibit and inactivate bacteria (Burt, 2004). Because the surface of bacterial cells has well-organized structures, it is likely that only the dissolved EOC molecules have small-enough dimensions to diffuse through the surface structure to access phospholipids. In microbial growth media, the MICs and MBCs (Table 3-3) of EOCs are typically below their solubility (Table 3-2), and all EOC molecules are available for interacting with bacteria. In milk, a much higher concentration of EOCs is needed to inhibit the growth of bacteria. When EOCs are added to milk above the solubility limit, a fraction of EOCs is expected to be dissolved and the remainder is present as dispersed particles. The dissolved EOCs can diffuse into porous casein micelles (McMahon and Oommen, 2013); bind with whey proteins such as β-lactoglobulin known for its hydrophobic

barrel available for loading hydrophobic compounds (Zhang et al., 2013b), or be attracted by fat globules. Casein micelles and whey proteins are present at about 2.6%w/w and 0.63%w/w in milk (Walstra et al., 2006) and cause about 10% reduction in the dissolved EOCs (Figure 3-3, skim milk samples versus Table 3-2). Fat globules appear to have strong ability to bind EOCs, causing a much lower concentration of EOCs in the milk serum than the solubility in simple solvents (Figure 3-3 and Table 3-2). The initially undissolved EOCs in the form of dispersed particles can continue to be dissolved in the continuous phase when there is a concentration gradient across the particle surface, and this portion of the dissolved EOCs also can bind with dairy proteins and fat globules. The dynamics of dissolving and binding processes determine the amount of EOCs dissolved in the milk serum that becomes available to interact with bacteria. This enables the correlation of EOC concentration in the milk serum and anti-listerial properties by comparison to the MIC and MBC data obtained in TSB, as presented above.

Ethanol is routinely used as a solvent to prepare stock solutions of EOCs before dilution to the required concentrations for microbiological experiments. Ethanol is considered bactericidal at high concentrations (60% to 75%) (Shelef and Seiter, 2010), and the impacts of low concentrations of ethanol on antimicrobial activities of EOCs are usually not addressed. The presence of low concentrations of ethanol lowers the polarity of the continuous phase and therefore increases the solubility (Table 3-2) and EOC concentration in the milk serum (Figure 3-3). This impacts the determined MICs and MBCs for EOCs with solubility comparable to the MBC (e.g., eugenol and *trans*-cinnamaldehyde, Tables 3-2 and 3-3). In milk, the increased EOC concentration in the milk serum prepared by the EPM (Figure 3-3) enables the enhanced reduction of bacteria, and the quicker mixing of EOCs in the EPM than the ETEM corresponds to greater log reductions in short times (Tables 3-4 to 3-6). The antimicrobial activity again can

be interpreted by comparing EOC concentration in the milk serum at 0 and 24 h (Figure 3-3) with the corresponding MIC/MBC (Table 3-3).

In terms of specific EOCs, thymol and carvacrol generally have better anti-listerial activity than eugenol and *trans*-cinnamaldehyde (Tables 3-3to 3-6). This trend is consistent with the structure characteristics observed for EOCs, showing antimicrobial activity following the order phenols > aldehydes > ketones > alcohols > esters > hydrocarbons (Koroch et al., 2007). *trans*-Cinnamaldehyde is more polar than other three EOCs in the present study (greatest water solubility, Table 3-2) and therefore may be less effective in affecting the structure of cytoplasmic membrane (Hyldgaard et al., 2012a). In milk, the aldehyde group of *trans*-cinnamaldehyde may allow stronger binding with proteins than the hydroxyl group of thymol, carvacrol and eugenol (Table 3-1). Binding between hydrophobic compounds and globulin proteins, like β-lactoglobulin, can be a long process (Kühn et al., 2006), and this may have caused the lowered concentration of *trans*-cinnamaldehyde in the milk serum after mixing with skim milk for 24 h (Figure 3-3D). The difference in binding properties may have caused the incomplete inhibition of the growth of *L. monocytogenes* in the full-fat milk by *trans*-cinnamaldehyde (Table 3-6), although its concentration in the milk serum is higher than the MBC (Figure 3-3 versus Table 3-3). Furthermore, the log-reduction at the 0 h time point was different when 2.5 g/L thymol and carvacrol were applied in skim milk using the ETEM (Table 3-4). Carvacrol is an isomer of thymol (Yanishlieva et al., 1999) and has similar MICs and MBCs as thymol (Table 3-3). Unlike the crystal form of thymol, carvacrol is present as a liquid at room temperature, which allows the quick mixing in the ETEM and likely causes the difference between the 2.5 g/L thymol and carvacrol treatments. The quicker mixing of 2.5 g/L carvacrol in skim milk is also consistent

with the same log reductions at 0 h using both the ETEM and the EPM, contrasted with the significant difference for 2.5 g/L thymol prepared by the two methods (Table 3-4).

Although the qualitative correlation between antimicrobial activity of EOCs and their concentration in the milk serum can be made by referencing to the MIC/MBC, the quantitative correlation of treatments prepared by the ETEM and EPM does not appear to be straightforward. Growth of *L. monocytogenes* in milk with low concentrations of ethanol after 24 h (Tables 3-4 to 3-6) makes it impossible to correct data from the EPM to eliminate the effect of ethanol. Because ethanol is generally not present in nonfermented foods, the EPM should be prevented if possible to assess realistic antimicrobial properties of EOCs expected in foods. From this perspective, mechanical methods of incorporating EOCs such as the ETEM evaluated in the present study, should be used to achieve solubility characteristics of EOCs and to evaluate delivery systems intended to improve the distribution and dissolution of EOCs. Conversely, the EPM offers convenience in sample preparation and can be used to enhance antimicrobial activity. When the EPM is used to prepare controls in studying delivery systems of antimicrobials, the increased antimicrobial activity by low concentrations of ethanol may result in inappropriate conclusions about the potential of delivery systems in improving the activity of EOCs in food matrices. Methods to address such scenario require future work.

3.6 Conclusions

Overall, the solubility and antimicrobial characteristics of EOCs were affected by the preparation methods. The solubility of EOCs was increased by low concentrations of ethanol and the increased solubility lowered the MICs and MBCs of EOCs with solubility comparable to MBC (eugenol and *trans*-cinnamaldehyde). In milk serum, the EOC concentration was reduced

to a greater extent by a higher fat level and was increased by low concentrations of ethanol resulting from the EPM. The EPM enabled faster distribution of EOCs to a higher level, which corresponded to the enhanced log reductions of *L. monocytogenes* after short-time (0 h) and long time (24 h) exposures. The EOC concentration quantified in the milk serum was correlated with log reductions of *L. monocytogenes* by comparing with the MIC and MBC determined in TSB. Generally, an EOC concentration in the milk serum higher than the MBC corresponded to complete inactivation of *L. monocytogenes*, while one between MIC and MBC agreed with partial reductions of the bacteria. Since the EPM increases antimicrobial activity of EOCs in milk, the ETEM is a more realistic representation of antimicrobial effectiveness expected in real food systems free of alcohol. The EPM offers convenience in sample preparation in laboratories, but the calibration of antimicrobial activity in foods due to low concentrations of alcohol remains a research question.

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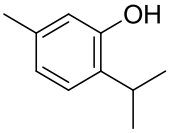
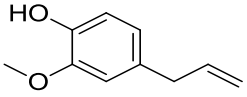
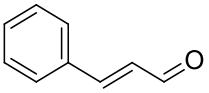
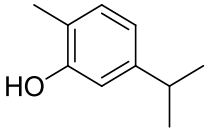
Appendix

Table 3-1. Water solubility of the studied essential oil components reported in references.

Compound	Structure	Solubility in water
Thymol		0.85 g/L at 25℃ (Griffin et al., 1999); 1.25 g/L at 20℃*;1.05 g/L at 20℃ (Helander et al., 1998)
Eugenol		0.64 g/L at 25℃ (Ben et al., 2006); 1.71 g/L at 25℃ (Miller and Hawthorne, 2000)
trans-Cinnamaldehyde		1.1 g/L at 20℃*; 1.76 g/L at 20℃ (Helander et al., 1998)
Carvacrol		1.25 g/L at 20℃*; 0.83 g/L at 25℃ (Griffin et al., 1999); 0.11 g/L at 25℃ (Ben et al., 2006); 1.0 g/L at 37℃ (Veldhuizen et al., 2006)

*http://www.chemicalbook.com/

Table 3-2. Characteristics of HPLC calibration curves and the measured solubility of essential oil components in water, TSB, 2% v/v ethanol, and 5% v/v ethanol at 21 °C. [*]

Compound	Peak area (*A*) – concentration (*C*) calibration curve				Solubility (g/L) in [†]:			
	Regression equation	R^2	Concn. range (mg/mL)	Recovery (%)	Water	TSB	2%v/v ethanol	5%v/v ethanol
Thymol	$A = 14558\ C$	0.9998	0-1	96.56 ± 1.43	0.48 ± 0.02 [b]	0.43 ± 0.01 [b]	0.52 ± 0.02 [ab]	0.61 ± 0.05 [a]
Carvacrol	$A = 13789\ C$	0.9999	0-1	99.64 ± 1.84	0.45 ± 0.02 [b]	0.41 ± 0.00 [b]	0.46 ± 0.00 [b]	0.57 ± 0.02 [a]
Eugenol	$A = 14190\ C$	0.9997	0-1	96.37 ± 2.74	1.35 ± 0.1 [b]	1.42 ± 0.01 [b]	1.49 ± 0.02 [b]	1.63 ± 0.12 [a]
Trans-Cinnamaldehyde	$A = 134163\ C$	0.9992	0-0.1	96.70 ± 3.83	1.41 ± 0.05 [bc]	1.51 ± 0.03 [c]	1.63 ± 0.04 [ab]	1.72 ± 0.13 [a]

[*] Numbers are mean ± standard daviation ($n = 3$).

[†] Different lowercase letters next to the numbers in the same row represent significant differences ($P < 0.05$).

Table 3-3. MIC and MBC of the four EOCs against *Listeria monocytogenes* Scott A in different concentrations of ethanol at 32 ℃ and 21 ℃ determined using the broth dilution method (n =4).

Compound	Ethanol concentration	MIC (g/L)†		MBC (g/L) †	
		32 ℃	21 ℃	32 ℃	21 ℃
Thymol	<1.3%*	0.2	0.2	0.3	0.3
	2.5%	0.2	0.2	0.3	0.3
	5%	0.2	<0.05‡	0.3	0.3
Carvacrol	<1.3%*	0.2	0.2	0.3	0.3
	2.5%	0.2	0.2	0.3	0.3
	5%	0.2	<0.0‡	0.3	0.3
Eugenol	<1.3% *	0.8	0.5	1.3	1.3
	2.5%	0.7	0.4	1.1	1.1
	5%	0.5	<0.2‡	1.0	1.0
***trans*-Cinnamaldehyde**	<1.3%*	0.2	0.2	1.4	1.4
	2.5%	0.2	0.1	1.2	1.2
	5%	0.2	<0.05‡	0.9	0.9
Ethanol		10%	5%	20%	20%

* The concentration derived from the stock solution with 95% aqueous ethanol to prepare an overall EOC concentration of 1.4 g/L. † g/L is the unit for MIC and MBC for all compounds except for ethanol. ‡ Below the lowest concentration used in the assay.

Table 3-4. Log reduction of *Listeria monocytogenes* Scott A treated with EOCs mixed via an end-to-end shaker or an ethanol stock solution in skim milk at 21 °C.

Compound	Overall concn	Log reduction*			
		End-to-end shaker		95% v/v ethanol	
		0 h	24 h	0 h	24 h
Thymol	5 g/L	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]
	2.5 g/L	0.3 ±0.5 [b]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]
Carvacrol	5 g/L	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]
	2.5 g/L	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]
trans-Cinnamaldehyde	9 g/L	0.2 ±0.3 [c]	1.3 ±0.5 [b]	0.4 ±0.5 [c]	>5.3 ±0.2 [a]
	4.5 g/L	0.1 ±0.2 [c]	0.8 ±0.2 [b]	0.1 ±0.4 [b]	5.0 ±0.7 [a]
Eugenol	7 g/L	>5.3 ±0.2 [a]	4.2 ±0.8 [b]	>5.3 ±0.2 [a]	>5.3 ±0.2 [a]
	3.5 g/L	0.3 ±0.3 [c]	2.8 ±0.4 [b]	4.7 ±1.3 [a]	>5.3 ±0.2 [a]
Controls					
Negative control (no antimicrobial)			-2.1 ±0.1 [C]		
Ethanol	4.75%v/v	0.1 ±0.2 [b]	-1.2 ± 0.1 [aA]		
	3.5%v/v	0.1 ±0.2 [b]	-1.6 ±0.2 [aB]		

* The log reduction is the difference between the population of *L. monocytogenes* in the negative control at time zero and that after mixing with EOCs using an end-to-end shaker for 30 min or a stock solution in 95% ethanol using brief vortexing, before (0 h) or after incubation at 21°C for 24 h. The population of *L. monocytogenes* in the negative control at 0 h was 6.2 ±0.5 log CFU/mL. Numbers are means ±standard deviations (n = 3). The detection limit was 1 log CFU/mL. Different lowercase letters next to the numbers in the same row and uppercase letters next to the controls in the same column represent significant differences ($P < 0.05$).

Table 3-5. Log reduction of *Listeria monocytogenes* Scott A treated with EOCs mixed via an end-to-end shaker or an ethanol stock solution in 2% reduced-fat milk at 21 °C.

Compound	Overall concn	Log reduction*			
		End-to-end shaker		95% v/v ethanol	
		0 h	24 h	0 h	24 h
Thymol	5 g/L	0.1 ± 0.2 [c]	1.3 ± 0.8 [b]	0.5 ± 0.4 [c]	>5.3 ± 0.1 [a]
	2.5 g/L	0.1 ± 0.2 [a]	-1.5 ± 0.5 [b]	0.1 ± 0.2 [a]	-0.9 ± 0.2 [b]
Carvacrol	5 g/L	0.3 ± 0.2 [b]	1.8 ± 1.6 [b]	0.6 ± 0.1 [b]	>5.3 ± 0.1 [a]
	2.5 g/L	0.0 ± 0.2 [a]	-1.2 ± 0.3 [b]	0.3 ± 0.2 [a]	-0.8 ± 0.2 [b]
trans-Cinnamaldehyde	9 g/L	0.3 ± 0.2 [c]	1.0 ± 0.4 [b]	0.3 ± 0.3 [c]	>5.3 ± 0.1 [a]
	4.5 g/L	0.2 ± 0.2 [a]	0.7 ± 0.3 [a]	0.2 ± 0.3 [a]	1.4 ± 1.4 [a]
Eugenol	7 g/L	0.2 ± 0.2 [b]	0.5 ± 0.4 [b]	0.3 ± 0.5 [b]	3.4 ± 1.4 [a]
	3.5 g/L	0.2 ± 0.2 [a]	-1.7 ± 0.1 [b]	0.2 ± 0.4 [a]	-0.7 ± 0.9 [ab]
Controls					
Negative control (no antimicrobial)			-2.2 ± 0.1 [C]		
Ethanol	4.75%v/v	0.0 ± 0.1 [b]	-1.1 ± 0.1 [aA]		
	3.5%v/v	0.0 ± 0.1 [b]	-1.7 ± 0.2 [aB]		

* The log reduction is the difference between the population of *L. monocytogenes* in the negative control at time zero and that after mixing with EOCs using an end-to-end shaker for 30 min or a stock solution in 95% ethanol using brief vortexing, before (0 h) or after incubation at 21°C for 24 h. The population of *L. monocytogenes* in the negative controls at 0 h was 6.3 ± 0.1 log CFU/mL. Numbers are means ± standard deviations (n = 3). The detection limit was 1 log CFU/mL. Different lowercase letters next to the numbers in the same row and uppercase letters next to the controls in the same column represent significant differences ($P < 0.05$).

Table 3-6. Log reductions of *Listeria monocytogenes* Scott A treated with EOC mixed via an end-to-end shaker and ethanol stock solution in full fat milk at 21 °C.

Compound	Overall concn	Log reduction*			
		End-to-end shaker		95% v/v ethanol	
		0 h	24 h	0 h	24 h
Thymol	5 g/L	-0.1 ±0.2 ^{ab}	-0.8 ±0.1 ^{b}	0.0 ±0.0 ^{a}	0.3 ±0.5 ^{a}
	2.5 g/L	-0.1 ±0.1 ^{a}	-1.9 ±0.1 ^{b}	0.0 ±0.2 ^{a}	-1.2 ±0.1 ^{b}
Carvacrol	5 g/L	-0.1 ±0.1 ^{b}	-0.2 ±0.3 ^{b}	0.4 ±0.1 ^{a}	0.5 ±0.6 ^{a}
	2.5 g/L	-0.1 ±0.0 ^{a}	-2.0 ±0.0 ^{c}	0.0 ±0.1 ^{a}	-1.0 ±0.1 ^{b}
trans-Cinnamaldehyde	9 g/L	0.1 ±0.1 ^{b}	0.8 ±0.3 ^{a}	0.1 ±0.2 ^{b}	1.3 ±0.9 ^{a}
	4.5 g/L	0.0 ±0.1 ^{a}	0.3 ±0.3 ^{a}	0.1 ±0.3 ^{a}	0.3 ±0.2 ^{a}
Eugenol	7 g/L	0.0 ±0.1 ^{a}	-1.2 ±0.2 ^{b}	0.1 ±0.2 ^{a}	0.4 ±0.2 ^{a}
	3.5 g/L	0.0 ±0.0 ^{a}	-1.8 ±0.2 ^{c}	0.1 ±0.2 ^{a}	-1.0 ±0.3 ^{b}
Controls					
Negative control (no antimicrobial)			-2.2 ±0.1 ^{C}		
Ethanol	4.75%v/v	0.1 ±0.2 ^{b}	-1.0 ±0.2 ^{aA}		
	3.5%v/v	0.1 ±0.1 ^{b}	-1.6 ±0.2 ^{aB}		

* The log reduction is the difference between the population of *L. monocytogenes* in the negative control at time zero and that after mixing with EOCs using an end-to-end shaker for 30 min or a stock solution in 95% ethanol using brief vortexing, before (0 h) or after incubation at 21°C for 24 h. The population of *L. monocytogenes* at time point 0 h was 6.4 ±0.1 log CFU/mL. Different lowercase letters next to the numbers in the same row and uppercase letters next to the controls in the same column represent significant differences ($P < 0.05$). Numbers are means ±standard deviations ($n = 3$).

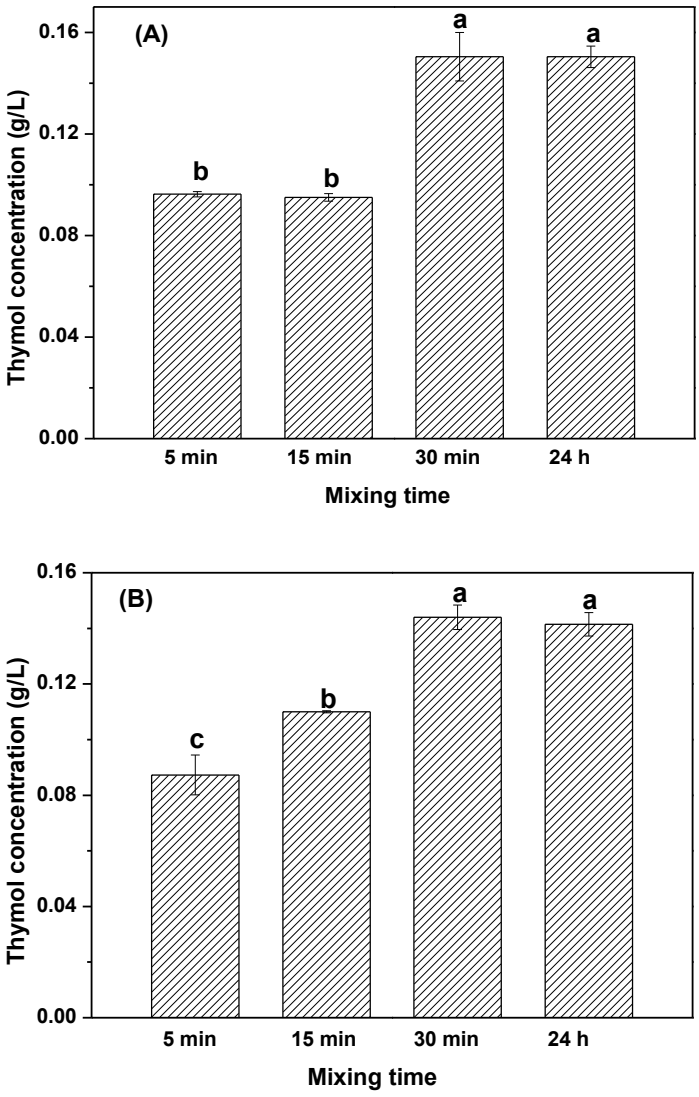


Figure 3-1. The thymol concentration detected in the serum of full-fat milk after mixing with an end-to-end shaker (A) or vortexing (B) for different durations. Error bars are standard deviations (n = 3). Different letters above bars indicate differences between mean values ($P < 0.05$).

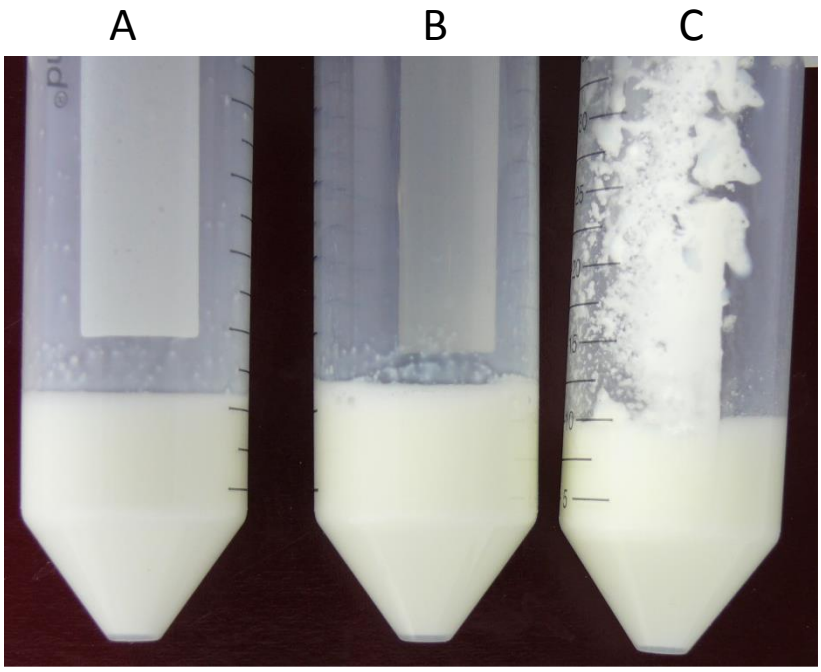


Figure 3-2. Appearance of full fat milk mixed with 5 g/L thymol crystals using an end-to-end shaker for 24 h (A), 500 μL of a stock solution with 100 g/L thymol in 95% aqueous ethanol (B), and 5 g/L thymol crystals by low-speed vortexing for 24 h (C).

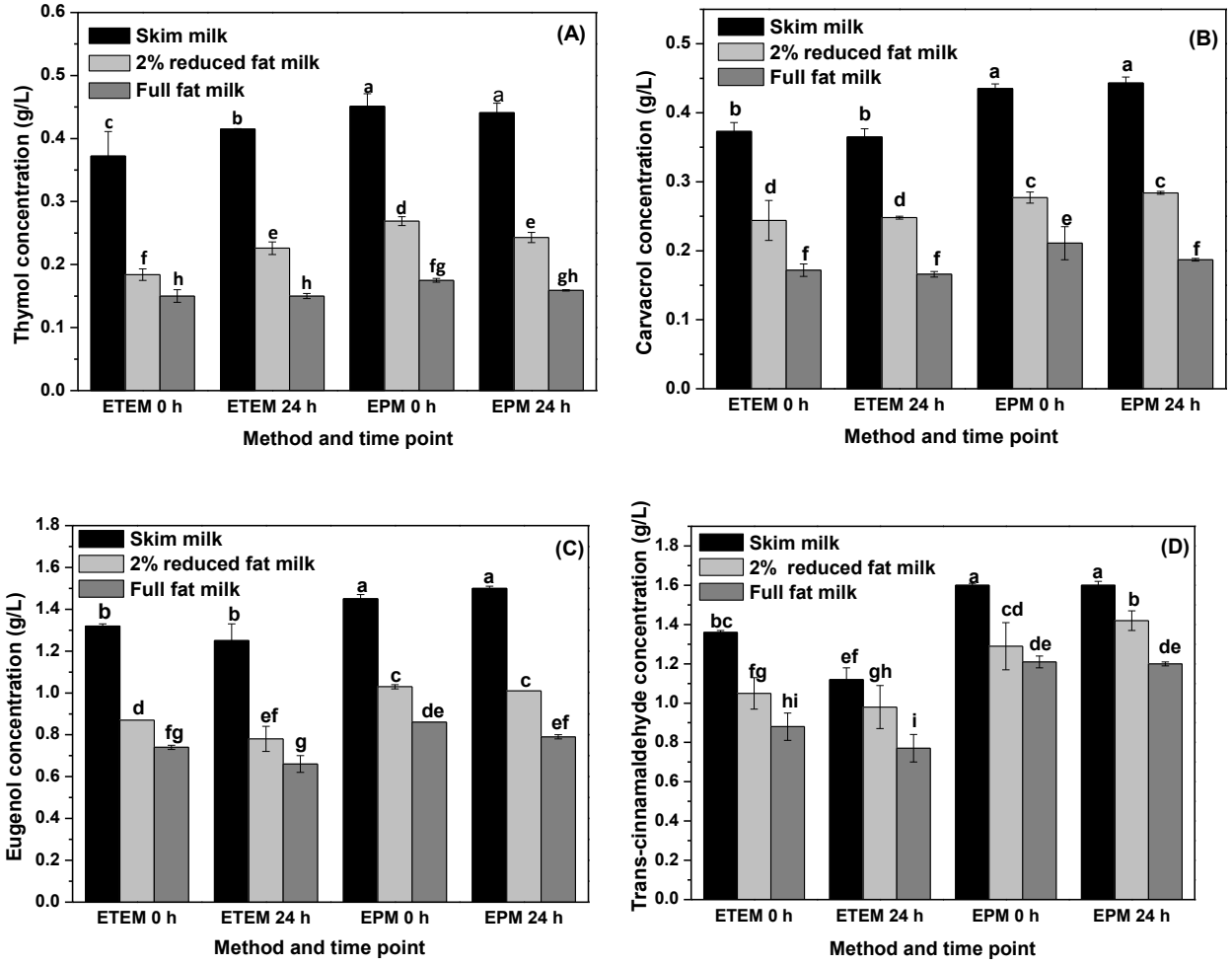


Figure 3-3. Concentration of EOCs in the serum of milk with different fat levels after mixing with 5 g/L thymol (A), 5 g/L carvacrol (B), 7 g/L eugenol (C), and 9 g/L *trans*-cinnamaldehyde (D) using an end-to-end shaker for 30 min (ETEM) or with a stock solution in 95% ethanol (EPM), before (0 h) and after incubation at 21 ℃ for 24 h. Error bars are standard deviations (n = 3). Different letters above bars indicate differences between mean values ($P < 0.05$).

Chapter 4. Physical and antimicrobial properties of spray-dried zein-casein nanocapsules with co-encapsulated eugenol and thymol

A version of this chapter was submitted to *Journal of Food Engineering* by Huaiqiong Chen, Yue Zhang and Qixin Zhong. My primary contributions to this manuscript include sample preparation, data collection and analysis, results interpretation and writing. Yue Zhang attributed to the antimicrobial data collection.

4.1 Abstract

Encapsulation of essential oil components in zein/casein complex nanoparticles using an anti-solvent precipitation method is well-established, but the properties of nanocapsules after spray-drying have not been studied. In the present work, eugenol and thymol were co-encapsulated in zein/casein nanoparticles at pH 6.0-8.0, and the properties of resulting spray-dried powders were characterized. The spray-dried zein/casein complexes were hydrated easily and the resulting dispersions with particles smaller than 200 nm were stable. The encapsulated EOCs showed the controlled release in 24 h, with the encapsulated eugenol showing a higher release rate than thymol. The encapsulated eugenol and thymol were present in milk whey at a concentration much lower than their overall concentrations (2.5 mg/mL each) and solubility but bactericidal and bacteriostatic effects were observed for *Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A, respectively. Therefore, spray-dried capsules may have the potential to be used as antimicrobial preservatives in food products.

Key words: zein, sodium caseinate, eugenol, thymol, co-encapsulation, spray-drying

4.2 Introduction

Outbreaks of foodborne illnesses due to consumption of ready-to-eat meals contaminated with pathogens cause negative social and economic impacts. Consequently, the use of efficient antimicrobial preservatives in combination with other intervention strategies is increasingly being recognized, especially the naturally derived antimicrobials that provide label friendliness. Essential oils (EOs) or their components (EOCs) extracted from various parts of edible, medicinal, and herbal plants have been frequently studied due to their excellent activities as natural antimicrobial preservatives against bacteria, viruses, fungi, parasites, and insects (Burt, 2004). Many EOs and EOCs are classified by the U.S. Food and Drug Administration as generally recognized as safe (GRAS) (Burt, 2004; Shaaban et al., 2012). Thymol, carvacrol, and eugenol, major components in EOs derived from thyme, oregano, and clove bud, are amongst the most-studied EOCs (Castillo et al., 2014; Chinou et al., 2009; Johny et al., 2010; Pan et al., 2014). The hydrophobic and volatile nature of EOCs requires technologies such as delivery systems to achieve uniform distribution and controlled release in aqueous food systems.

It is also well known that there is synergistic antimicrobial activity when some EOCs are used in combination. For example, the minimum bactericidal concentration (MBC) of carvacrol, thymol, and eugenol against *Listeria innocua* was 150, 250, and 450 mg ŧkg^{-1}, respectively (Garc ́a-Garc ́a et al., 2011). When their binary mixtures were studied, the combinations of 62.5 mg ŧkg^{-1} thymol and 75 mg ŧkg^{-1} carvacrol, or 56.25 mg ŧkg^{-1} thymol and 125 mg ŧkg^{-1} eugenol completely inhibited the growth of *L. innocua*. The ternary mixture of carvacrol–thymol–eugenol at respective concentrations of 75, 31.25, and 56.25 mg ŧkg^{-1} was also effective in completely inhibiting *L. innocua*. Therefore, these combinations could reduce their doses as antimicrobial

preservatives to lower the cost and potential impacts on sensory quality. However, studies on co-encapsulating EOCs in one delivery system are scarce.

Nano-/microencapsulation of EOCs in food biopolymers is a group of technologies that can possibly solve challenges facing their applications. This has been studied recently for thymol encapsulated in sodium caseinate (NaCas) (Pan et al., 2014) or whey protein-maltodextrin conjugates (Shah et al., 2012a). The encapsulated thymol was more effective than unencapsulated thymol in inhibiting foodborne pathogens in milk, resulting from the enhanced distribution and solubility. Much work however is needed before delivery systems of EOCs can be used in the food industry. For example, these delivery systems are preferably produced as spray-dried powder for convenience of storage, transportation, and utilization.

In the present work, zein, a group of prolamins (alcohol-soluble proteins) from corn, was studied as a GRAS biopolymer to produce spray-dried powder with co-nanoencapsulated thymol and eugenol. Zein has more than 50% hydrophobic amino acid residues on the surface and is not water soluble (Chen et al., 2013, 2014b). Zein is only soluble in 55-90% aqueous alcohol and easily precipitates as nanoparticles after mixing with water to an overall alcohol concentration incapable of dissolving zein (Zhong and Jin, 2009a). This anti-solvent precipitation property has been used to encapsulate fish oil (Zhong et al., 2009), α-tocopherol (Luo et al., 2011), vitamin D3 (Luo et al., 2012), vitamin E (Zou and Gu, 2013) and curcumin (Gomez-Estaca et al., 2012; Patel et al., 2010b) in zein nanoparticles. A challenge of utilizing hydrophobic zein nanoparticles is their poor dispersibility in aqueous systems with acidity close to the isoelectric point (*pI*) of zein, *ca.* pH 6.2 (Patel et al., 2010b). It is particularly more problematic for freeze-dried or spray-dried zein nanoparticles due to the bad redispersibility of zein. To solve this problem, water soluble and amphiphilic biopolymers can be used to form complexes with zein nanoparticles to

create repulsive steric and electrostatic forces. In our previous study (Chen and Zhong, 2014), zein and NaCas were co-dissolved in hot 50% aqueous ethanol adjusted to pH 8.0 before anti-solvent precipitation to form nanoparticles. We observed that nanoparticles contained κ-casein and zein and the spray-dried powder was easily re-dispersed in water with good stability during storage. In the present study, we are interested in applying the complex nanoparticles to encapsulate volatile EOCs to prepare spray-dried powder.

The first objective of the present work was to study the properties of zein/casein complexes in co-encapsulating thymol and eugenol. The second objective was to study encapsulation properties after preparing spray-dried powder from nanocomplexes. The third objective was to characterize release properties of thymol and eugenol from spray-dried powder and their solubility in milk. The fourth objective was to evaluate the antimicrobial activity of spray-dried capsules against bacteria in milk.

4.3 Materials and methods

4.3.1 Materials

Purified α-zein was purchased from Acros Organics (Morris Plains, NJ). NaCas was a product from American Casein Company (Burlington, NI). Anhydrous ethanol was purchased from Decon Laboratories Inc. (King of Prussia, PA). HPLC grade methanol and water were purchased from Fisher Scientific (Pittsburgh, PA). Tryptic soy broth (TSB, Remel$^{®TM}$, Fisher Scientific) medium was prepared by dissolving 30 g powder in 1000 mL water. Tryptic soy agar (TSA) was prepared by adding 12 g agar (Fisher Scientific) into the TSB medium. Ultra-high-temperature (UHT) processed 2% reduced-fat milk (Simple Truth Organic^{TM}, Kroger Co., San Diego, CA) was purchased from a local grocery store.

4.3.2 Sample preparation

The previous method of preparing zein/casein nanoparticles was adopted to co-encapsulate eugenol and thymol (Chen and Zhong, 2014), with modification. Firstly, 50 mL of 50%v/v aqueous ethanol with 5 mM sodium phosphate was adjusted to pH 6.0, 7.0, or 8.0, followed by mixing with 2 g zein, 2 g NaCas, 1 g thymol, and 1 g eugenol, and the pH was readjusted if needed. After heating the mixture at 90 ℃ for 30 min, the hot solution was sheared into 150 mL of room temperature (21 °C) buffer with 5 mM sodium phosphate adjusted to the same pH as the mixture solution by homogenization at 10,000 rpm for 2 min (IKA® 25 digital ULTRA TURRAX®, IKA® Works, Inc., Wilmington, NC). The dispersion was then spray-dried (mini spray dryer B-290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) using the following parameters: inlet temperature of 105 ℃, outlet temperature of 60 ℃, a feed rate of 15%, and an aspirator setting of 100%. Spray-dried powder was collected and stored at -20 ℃ before use. Another set of samples with only one EOC was prepared by dissolving 2 g thymol or 2 g eugenol in the aqueous ethanol with same amounts of zein (2 g) and NaCas (2 g) adjusted to pH 8.0. Two replicates of spray-dried samples were prepared for each treatment.

4.3.3 Particle size and morphology

Dimensions of particles in fresh dispersions or those reconstituted with spray-dried powder were measured using a dynamic light scattering (DLS) instrument (Delsa Nano C particle size/zeta potential analyzer, Beckman Coulter, Fullerton, CA). The volume fraction-length ($d_{4,3}$) mean diameters were calculated based on number (n_i) of particles with diameter d_i using Eq. (1).

$$d_{4,3} = \frac{\sum_{i=1} n_i {d_i}^4}{\sum_{i=1} n_i {d_i}^3} \quad (1)$$

Atomic force microscopy (AFM, model Multimode 8, Bruker Corp., Santa Barbara, CA) was used to characterize the morphology of particles. Spray-dried powder was hydrated in deionized water at 1 mg/mL and diluted to 40 ppm solids in deionized water. Four μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks and dried overnight. The samples were scanned using a rectangular cantilever probe (FESPA, Bruker Corp.) with aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m. Images were generated with a preset scan area of 5.0 × 5.0 μm at a scanning speed of 1 Hz. By using the instrument software, two-dimensional images were used to estimate average particle size. Three-dimensional topographic images were used to generate the mean heights of detected particles.

Scanning electron microscopy (SEM) was used to observe the morphology of spray-dried powder. The powdered sample was glued directly onto an adhesive tape mounted on the specimen stub and sputter-coated with a gold layer of *ca.* 5 nm thickness before imaging using a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany).

4.3.4 Encapsulation performance

To determine the total thymol/eugenol content in spray-dried powder, 10 mL of 60% aqueous ethanol was used to dissolve 10 mg powder by mixing for 3 h on an end-to-end shaker. After centrifugation at 4545*g* for 10 min (Sorvall RC-5B plus; Sorvall, Newtown, CT) at 21 ℃, the supernatant was filtered through a 0.45μm polyvinylidene fluoride (PVDF) syringe filter (Fisher Scientific, Co., Pittsburgh, PA) to collect the permeate for HPLC analysis. To determine the free oil content in spray-dried powder, 10 mg powder was mixed with 2 mL distilled water and vortexed for 30 s, and then the dispersion was centrifuged at 13150*g* for 5 min (model

MiniSpin® plus, Eppendorf, Westbury, NY) and the supernatant was filtered as above to obtain the sample for HPLC analysis.

To quantify eugenol and thymol, a reversed phase HPLC system (1200 series, Agilent Technologies, Waldbronn, Germany) was used. The system consisted of a quaternary pump module, a degasser, an auto-sampler, a temperature-controlled column chamber, and an Agilent diode array detector. Chromatograms were recorded and integrated by the 1200 LC Chromatography Data System. Eugenol and thymol were simultaneously determined by the HPLC method established previously (Chen et al., 2014a). All experiments were performed on an Agilent ZORBAX Eclipse Plus C18 HPLC column (5 μm, 150 mm × 4.6 mm, Agilent, Palo Alto, CA), protected by a ZORBAX Eclipse Plus C-18 guard column (4.6 ×12.5 mm, 5 μm). A binary mixture of methanol and water was used as the mobile phase applied at a linear gradient from 20% to 80% methanol in the first 20 min, an isocratic step of 80% methanol for 5 min, and another linear gradient from 80% to 20% methanol in 5 min. The flow rate was 0.5 mg/mL throughout, and the temperature was set at 25 ℃. The chromatogram was acquired at 275 nm. The standard curve for each compound was established using solutions with 0.1-1 mg/mL eugenol or thymol as an external standard. Experiments were performed in duplicate for two spray-dried samples (n = 4) and encapsulation efficiency, loading percentage, and EOC content change% were calculated using Eq. (2), (3), and (4), respectively. The EOC content change% is used to evaluate the loss of an EOC with respect to other compounds during spray-drying (Xiao et al., 2011b; Xiao et al., 2011c) because a small amount of mass is used in lab-scale spray drying experiments and sample collection errors can cause imprecise evaluation of encapsulation performance as defined in Eq. 2.

$$\text{Encapsulation efficiency\%} = \frac{\text{Total EOC in powder (g)} - \text{Free EOC in powder (g)}}{\text{Total EOC before spray drying (g)}} \times 100\% \qquad (2)$$

$$\text{Loading\%} = \frac{\text{Total EOC in powder (g)} - \text{Free EOC in powder (g)}}{\text{Powder mass (g)}} \times 100\% \quad (3)$$

$$\text{EOC content change\%} = (1 - \frac{\text{EOC\% in spray-dried powder}}{\text{EOC\% in non-solvent mass before spray drying}}) \times 100\% \quad (4)$$

4.3.5 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to study changes of chemical structures in spray-dried nanoparticles. Ten mg of powder was mixed with 100 mg of potassium bromide (KBr) powder, and the mixture was pressed into a disk for spectrum recording. The spectra were acquired at 400-4000 cm^{-1} in 64 scans with the resolution of 4 cm^{-1} utilizing a Nicolet Nexus 670 IR spectrometer (Thermo Nicolet Corp., Madison, MI) which was equipped with a Germanium attenuated total reflection (ATR) accessory.

4.3.6 Release kinetics of thymol/eugenol from nanocapsules

The spray-dried samples were used to characterize *in vitro* release kinetics of thymol and eugenol. The powder was suspended at 10 mg/mL in 5 mL of phosphate buffered-saline (PBS, pH 7.0), which was then transferred into a dialysis tubing with a molecular-weight-cut-off of 3500 Da (catalog No.21-152-10, Thermo Fisher Scientific, Pittsburgh, PA). The dialysis tubing was sealed and placed in a 125 mL Erlenmeyer flask with 100 mL PBS (pH 7.0). During stirring at 300 rpm on a magnetic stirrer at room temperature (21 °C), 20 mL solution was taken out at designated time intervals and an equivalent volume of fresh PBS (7.0) was supplemented. The withdrawn solution was filtered through a 0.45 μm PVDF syringe membrane filter for the HPLC analysis as above. The percentage of cumulatively-released eugenol and thymol was calculated according to Eq. (5). Controls of free eugenol and thymol were prepared at 1 mg/mL in PBS (pH 7.0) with 0.5% w/v Tween 20, which was used to increase the solubility of eugenol and thymol. Five mL of the control solution was contained in the dialysis tubing and assayed as above. Two

powder/control replicates were tested, and two measurement replicates were conducted for each sample.

$$C_{\mathrm{ti}}(\%) = \frac{20\sum_{n=1}^{i-1} a_n + 100a_i}{m_0} \times 100\% \qquad (5)$$

where C_{ti} (%) is the cumulatively released eugenol/thymol at time t_{i}; a_{i} is the eugenol and thymol concentration (mg/mL) in the withdrawn solution at the sampling time t_{i}; and m_{o} is the total eugenol or thymol content (mg) in the dialysis tubing.

4.3.7 Quantification of eugenol and thymol dissolved in the milk whey

Previously, we found that EOC concentration in the continuous phase of milk (whey) is critical to antimicrobial activity (Chen et al., 2014a). To quantify the amount of eugenol/thymol in milk whey, the spray-dried powder containing 50 mg EOCs (eugenol and thymol combined) was weighed in a 50 mL centrifugation tube, followed by adding 10 mL milk and vortexing to disperse the powder. After incubation at room temperature (21 ℃) for 0 and 24 h, samples were adjusted to pH 4.6 using 1 M HCl to precipitate caseins, followed by centrifugation at 4545*g* for 10 min (Sorvall RC-5B plus; Sorvall, Newtown, CT) and filtration of the supernatant through a 0.45 μm PVDF syringe membrane filter for the HPLC analysis. Four samples, two from each of two spray-dried replicates, were measured. For free eugenol and thymol control, 25 mg eugenol and 25 mg thymol were weighed directly in the centrifuge tube and mixed with 10 mL milk on an end-to-end shaker for 30 min at 21 °C, followed by the same procedures as above.

4.3.8 Growth kinetics of bacteria

Spray-dried powder prepared at pH 7.0 was hydrated in 2% reduced-fat milk to test the antimicrobial activity against gram-negative *Escherichia coli* O157:H7 strain ATCC 43895 and gram-positive *Listeria monocytogenes* Scott A. The bacteria strains were obtained from the culture collection of the Department of Food Science and Technology at the University of

Tennessee (Knoxville, TN). The stock culture, stored in glycerol at -20 ℃, was transferred in TSB at 37 ℃ for *E. coli* O157:H7 and 32 ℃ for *L. monocytogenes* for 2 consecutive days and was diluted to 8 log CFU/mL as the working culture. Fresh culture was prepared for each replicate. Similar to the quantification of eugenol and thymol in milk whey, an appropriate amount of spray-dried powder was hydrated in 10 mL milk to obtain a total EOC concentration (eugenol+thymol) of 5 mg/mL, and 100 μL working culture was added to the milk to get an overall bacterial population of 6 log CFU/mL. For free EOC treatments, 50 mg thymol or eugenol or a combination of 25 mg thymol and 25 mg eugenol was weighed directly to pre-autoclaved glass tubes, and 10 mL 2% reduced-fat milk was added. The tubes were then attached to an end-to-end shaker and mixed for 30 min (Chen et al., 2014a), followed by adding the working culture as above. After incubation at 21 ℃ for 0, 4, 8, 24, and 48 h, viable cells were enumerated using the TSA spread plating method with a detection limit of 1 log CFU/mL. Spray-dried samples had four measurements, with two from each of two independent replicates, and free EOC treatments and control were tested in duplicate.

4.3.9 Statistical analysis

Statistical analyses were performed using the SAS software (version 9.3, SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between pairs of means were compared using a Tukey's test. The significance level was set at 0.05.

4.4 Results and discussion

4.4.1 Particle dimension of fresh dispersions

DLS was used to determine the particle size of fresh dispersions prepared by precipitation at pH 6.0-8.0. The $d_{4,3}$ (Table 4-1) was significantly smaller at a higher pH, decreasing from 174

nm at pH 6.0 to 107 nm at pH 8.0. The polydispersity index was below 0.21, which ensured the reliability of data from DLS (Wu et al., 2012a) and indicated the uniform size distribution (Luo et al., 2012). The bigger $d_{4,3}$ at a lower pH between 6.0 and 8.0 may be caused by particle flocculation because of stronger hydrophobic interactions at acidity closer to the *pI* of zein (pH 6.2) (Patel et al., 2010a) and reduced number of negative charges of caseins (*pI* == ~pH 4.6) (Patel et al., 2010a).

4.4.2 Morphology of spray-dried powder

The surface morphology of spray-dried powder was observed using SEM, demonstrated in Figure 4-1. A wide range of particles with a dimension from 0.5 to 20 μm was observed for all powdered samples. These dimensions are bigger than those of individual nanoparticles (Table 4-1), which suggests that multiple nanoparticles are present in one atomized droplet during spray drying (Hogan et al., 2001; Zhang and Zhong, 2013). While a few big mostly-spherical particles were observed for each sample, most particles appeared to be collapsed, and no apparent difference in the morphology was noticed for samples prepared at different pHs. Compared to rough and porous surfaces of capsules directly spray-dried from zein solutions (Xiao et al., 2011b), the surface of capsules in Figure 4-1 was smooth (Figure 4-1A-D). It was also previously observed that the addition of Tween® 20 in aqueous ethanol solution of zein improved the surface smoothness of spray-dried powder, because of changes in protein interactions and surface compositions of atomized droplets during drying (Xiao et al., 2011c). In the present study, zein nanoparticles formed complexes with caseins after anti-solvent precipitation, as previously characterized (Chen and Zhong, 2014), and were well-dispersed before spray-drying, which was different from spray drying zein solutions with a much higher ethanol (12.5% vs. 70% v/v) concentration. Free caseins in dispersions may have additionally contributed to surface

smoothness of spray-dried particles in the present study, because smooth particles were observed after spray-drying NaCas directly, partially due to its excellent interfacial activity (Hogan et al., 2001; Zhang and Zhong, 2013).

4.4.3 Encapsulation performance

The encapsulation parameters after spray-drying dispersions are listed in Table 4-2. For the thymol and eugenol alone treatments, even though thymol had a higher free oil amount, the total oil amount and loading% of thymol were significantly higher than eugenol ($P < 0.05$), corresponding to the lower oil content change% and higher encapsulation efficiency. Thymol has a higher vapor pressure than eugenol (1333 versus 667 Pa) at the inlet temperature (105 ℃) of spray drying (Stull, 1947), indicating a potentially higher loss of thymol than eugenol during spray drying. However, the opposite phenomenon was observed in this study. Structurally, thymol and eugenol have a respective water-solubility of 0.48 and 1.35 g/L at 21 °C, and therefore thymol is more hydrophobic than eugenol (Chen et al., 2014a). The concentration of thymol in the aqueous phase is expected to be lower than eugenol because no free thymol or eugenol was observed after the liquid-to-liquid dispersion. The greater percentage of thymol encapsulated in zein nanoparticles and the stronger hydrophobic attraction by zein may have resulted in less evaporation of thymol than eugenol during spray drying. When the total values of thymol and eugenol co-encapsulated at pH 8.0 were compared to treatments with one EOC (Table 4-2), all encapsulation parameters of the co-encapsulation treatment were between those with thymol or eugenol only.

When co-encapsulation treatments were compared (Table 4-2), both total oil content and encapsulation efficiency in spray-dried powder decreased when the preparation pH decreased from 8.0 to 6.0. This may have been caused by bigger particle sizes at a lower pH (Table 4-1).

During spray-drying, smaller particles have higher mass and heat transfer rates that result in a faster formation of a semi-permeable membrane around atomized droplets to reduce the loss of volatile compounds (Jafari et al., 2007). As a result, the encapsulation efficiency is lower for dispersions prepared at a lower pH with bigger particles.

Like treatments with only one EOC, thymol in co-encapsulation treatments had a higher total oil amount, loading%, encapsulation efficiency, and lower oil content change than eugenol (Table 4-2). However, the free oil amount of thymol in co-encapsulation samples was lower than eugenol, which is in opposite to treatments with one EOC. This may be because the more hydrophilic eugenol is preferentially excluded from zein nanoparticles and can be extracted more easily from spray-dried particles by water than thymol during the determination of free EOC.

The encapsulation efficiency and oil content change in this study are respectively far higher and lower than previous studies when aqueous ethanol solutions of zein with co-dissolved thymol and nisin were spray-dried directly (Xiao et al., 2011c). This indicates that pre-encapsulation of eugenol and thymol in zein nanoparticle/casein complexes by the liquid-to-liquid dispersion increases the diffusion resistance for the pre-encapsulated EOCs and therefore reduces their evaporation during spray drying.

4.4.4 FTIR analysis of spray-dried powder

FTIR was used to study whether the encapsulation changed the secondary structures of zein/casein. As presented in Figure 4-2, compared to bare zein/casein complexes reported previously (Chen and Zhong, 2014), encapsulation of thymol/eugenol did not change the peaks belonging to Amide B, Amide I, and Amide II for all samples. This indicates that eugenol and thymol mixed together with zein/casein physically (Wu et al., 2012a) and no chemical reaction occurred during encapsulation and spray drying. Because zein, eugenol, and thymol are

hydrophobic molecules, hydrophobic interactions may be the main force involved in encapsulation (Luo et al., 2012).

4.4.5 Particle dimension and structure of reconstituted dispersions

After hydrating spray-dried powder in distilled water, $d_{4,3}$ of all treatments increased by 10-30 nm when compared to fresh dispersions, with the treatment at pH 8.0 showing significant increase (Table 4-1). The increase can be caused by incomplete hydration of spray-dried nanocomplexes or structural changes of nanoparticles within atomized droplets during spray drying. The latter can be caused by redistribution of thymol/eugenol that became vaporized and condensed during spray drying and subsequent cooling. Regardless, all dispersions had $d_{4,3}$ smaller than 200 nm and polydispersity below 0.21. Therefore, the spray-dried particles can be hydrated easily as nanodispersions. The stability of the reconstituted dispersions was tested at 4 ℃ after adding 0.05%w/v sodium azide to prevent the microbial growth (Chen and Zhong, 2014). After 15-d storage, particle size did not differ ($P > 0.05$) from that at day 0 (Table 4-1) and no precipitation was observed for all treatments. This indicates excellent stability of these dispersions.

The morphology of particles in reconstituted dispersions was characterized using AFM. Discrete particles were observed for all samples (Figure 4-3). The particle dimension and height estimated from AFM are summarized in Table 4-3. The particle size from AFM was in good proximity with $d_{4,3}$ and also increased as pH decreased from 8.0 to 6.0 (Tables 4-1 and 4-3).

4.4.6 Release profiles

Figure 4-4 shows the release profiles of eugenol and thymol from nanoparticles in PBS at pH 7.0. Both eugenol and thymol demonstrated similar trends showing sustained release in 24 h. Generally, eugenol was released faster than thymol. In the first hour, the released eugenol was

twice amount of the released thymol, which agrees with a higher amount of free eugenol (Table 4-2). Furthermore, this may be due to weaker hydrophobic attraction between eugenol and zein than that of thymol and zein, as discussed previously. There was no significant ($P > 0.05$) difference in the release profile of samples spray-dried from dispersions prepared at different pHs, with a tendency of powders prepared at pH 8.0 released faster than powders prepared at pH 7.0 and 6.0. This may have resulted from a higher amount of free oil in the powder prepared at pH 8.0 (Table 4-2) and smaller particles enabling a higher mass transfer rate (Table 4-1).When free eugenol and thymol were compared, the overall release rate and percentage of release after 24 h were higher than the zein/casein capsules, which can be due to the lack of hydrophobic attraction by zein and the facilitated dissolution by 0.5% Tween® 20.

In our previous study, thymol encapsulated in zein by spray-drying directly showed around 40% release even at pH 2.0 when zein capsules are expected to be more porous than at pH 7.0 because of the increased solubility of zein (Xiao et al., 2011c). When an appropriate amount of Tween® 20 was incorporated in capsules, the release rate was significantly improved because of the reduced hydrophobic interaction between thymol and zein by Tween® 20 (Xiao et al., 2011c). We showed previously that nanoparticles as prepared in this study had both κ-casein and zein (Chen and Zhong, 2014). It is possible that the inclusion of κ-casein in nanoparticles could increase the overall particle porosity and weaken the hydrophobic interaction between eugenol/thymol and zein. In another study using zein/casein to encapsulate thymol, a higher mass ratio of thymol:zein resulted in faster release of thymol, reaching 40% release at pH 7.0 in 13 h for the treatment with a thymol:zein ratio of 1:2.5 (Li et al., 2013a). The faster release rate of thymol in the present study may again have been caused by the presence of κ-casein in zein nanoparticles (Chen and Zhong, 2014).

4.4.7 Concentration of thymol and eugenol in milk whey

The concentrations of thymol and eugenol after precipitation of caseins in 2% reduced fat milk hydrated with spray-dried powder are compared in Figure 4-5. No difference ($P > 0.05$) was observed for samples prepared from different pH conditions. The results were in agreement with the release data in Figure 4-4. After 24 h incubation at room temperature, the concentration did not change, which was similar to our previous studies (Chen et al., 2014a; Xue et al., 2013). The free eugenol control showed a higher concentration than the encapsulated ones, while no difference was observed for thymol treatments (Figure 4-5). These concentrations were much lower than the water solubility of eugenol (1.35 mg/mL) and thymol (0.48 mg/mL) at 21 ℃ (Chen et al., 2014a), and the higher concentration of eugenol in the milk whey may be due to its higher solubility than thymol.

4.4.8 Antibacterial activity in 2% reduced-fat milk

Since the concentration of eugenol and thymol in the milk whey were not different ($P > 0.05$) for samples prepared from different pHs (Figure 4-4) and the pH of milk is around 7, spray-dried powder co-encapsulating eugenol and thymol prepared at pH 7.0 was used to study the antimicrobial activity in 2% reduced fat milk. As presented in Figure 4-6, 5 mg/mL free thymol reduced *E. coli* O157:H7 by 1.1 log CFU/ml at the time point of 0 h and completely inhibited the bacterium after 8 h with no recovery in 48 h. In contrast, 5 mg/mL free eugenol showed limited inhibition on the growth rate of *E. coli* O157:H7, and 7 log CFU/mL *E. coli* O157:H7 was detected after 48 h, which was only 2 log CFU/mL lower than the control without EOC. The combination of 2.5 mg/mL each of free thymol and eugenol also showed bactericidal effect after 24 h incubation, but there was a recovery after 48 h. In comparison, at an overall

concentration of 5 mg/mL encapsulated EOCs with about equal mass of eugenol and thymol, the growth of *E. coli* O157:H7 was completely inhibited in 8 h and no recovery was observed in 48 h.

For *L. monocytogenes*, treatments except 5 mg/mL free eugenol showed a reduction of 0.8-1.2 log CFU/mL at the 0 h time point, followed by insignificant changes. For free eugenol at 5 mg/mL, *L. monocytogenes* recovered to the initial population after 48 h which was 2.5 log CFU/mL lower than the control at 48 h.

As established previously, bactericidal effects in milk were observed when the EOC concentration in milk whey was higher than the minimum bactericidal concentration (MBC) tested in TSB, while an EOC concentration in milk whey below MBC and above minimum inhibition concentration (MIC) corresponded to partial inhibition or bacteriostatic behavior (Chen et al., 2014a; Pan et al., 2013). The MIC of eugenol and thymol against *L. monocytogenes* Scott A in TSB was previously determined to be 0.8 and 0.2 mg/mL, respectively (Chen et al., 2014a), and the MIC of eugenol and thymol against *E. coli* O157:H7 ATCC 43895 in TSB was 0.75 and 0.0938 mg/mL, respectively (Ma et al., 2013). In the present study, the concentration of eugenol and thymol in milk whey was 0.30~0.37 and 0.051~0.061 mg/mL, respectively, that were below the corresponding MIC for both bacteria. However, bactericidal effect of *E. coli* O157:H7 and bacteriostatic effect of *L. monocytogenes* Scott A were observed after 24 and 48 h (Figure 4-6). There appeared to have synergism in antimicrobial activity of both free and encapsulated thymol and eugenol in milk, which is in agreement with other studies based on free EOCs (Garc á-Garc á et al., 2011). Detailed mechanisms using microbial growth media however were not attempted. Nevertheless, results in Figure 4-6 showed that co-encapsulated thymol and eugenol remained their activity and improved long-term effectiveness against *E. coli* O157:H7

after 48 h when compared to the combination with same amounts of free EOCs. There appeared to have synergism in antimicrobial activity of both free and encapsulated thymol and eugenol in milk, which is in accordance to others study for free EOCs (Garc á-Garc á et al., 2011). Because high concentration of EOCs are required in food matrices to inhibit the growth of bactericia (Ma et al., 2013; Pan et al., 2013; Shah et al., 2012a; Shah et al., 2013b; Xue et al., 2013) and each EOC has different flavor characteristics, the combination of EOCs may be used to adjust flavor profiles (Leffingwell and Leffingwell, 1991), while achieving antimicrobial activity.

4.5 Conclusions

In summary, eugenol and thymol were co-encapsulated in zein/casein complexes with a dimension smaller than 200 nm, and the dimension was bigger at a lower pH between 6.0 and 8.0. Pre-encapsulation in nanoparticles resulted in better retention of volatile EOCs in spray-dried powder than previous studies spray-drying the solution directly. The encapsulation performance was better for samples prepared at 8.0 than that at pH 7.0 and 6.0. The spray-dried samples were rehydrated easily, and the reconstituted dispersions were stable during refrigerated storage for 15 d. The encapsulated EOCs showed the controlled release in 24 h, with the encapsulated eugenol showing a higher release rate than thymol. The encapsulated eugenol and thymol were present in milk whey at a concentration much lower than their overall concentrations and solubility but was effective in inhibiting the growth of *E. coli* O157:H7 and *L. monocytogenes*. Therefore, the spray-dried encapsulated eugenol and thymol capsules may be applicable as antimicrobial preservatives in food products.

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Appendix

Table 4-1. The volume fraction-length mean diameters ($d_{4,3}$) and polydispersity (PDI) of fresh dispersions prepared at pH 6.0-8.0 and those reconstituted from spray-dried samples before and after 15-d storage at 4 ℃.*

Sample	**Fresh dispersions**		**Reconstituted dispersions (day 0)**		**Reconstituted dispersions (day 15)**	
pH	$d_{4,3}$ (nm)	PDI	$d_{4,3}$ (nm)	PDI	$d_{4,3}$ (nm)	PDI
8.0	107 ± 1^{d}	0.16 ± 0.01^{ab}	132 ± 5^{cd}	0.21 ± 0.02^{ab}	142 ± 2^{bc}	0.17 ± 0.02^{ab}
7.0	131 ± 7^{cd}	0.20 ± 0.01^{ab}	157 ± 4^{abc}	0.13 ± 0.00^{b}	164 ± 6^{ab}	0.20 ± 0.01^{ab}
6.0	174 ± 9^{a}	0.20 ± 0.01^{ab}	182 ± 7^{a}	0.17 ± 0.05^{ab}	175 ± 10^{a}	0.22 ± 0.04^{a}

*Values are means ± standard deviations from two independent replicates, each tested twice (n = 4). Different superscript letters for each parameter indicate statistical difference ($P < 0.05$).

Table 4-2. Performance of encapsulating eugenol and thymol individually or simultaneously in zein/casein nanocomplexes precipitated at pH 6.0-8.0 followed by spray-drying.*

EOC	pH	Total oil amount (mg/100 mg powder)		Free oil amount (mg/100 mg powder)		Loading (%)		EOC content change (%)†		Encapsulation efficiency (%) †	
		Eugenol	Thymol	Eugenol	Thymol	Eugenol	Thymol	Eugenol	Thymol	Eugenol	Thymol
Eugenol and thymol	8.0	9.81 ± 0.81^{ab}	$10.17 \pm 0.66^{a, B}$	7.12 ± 0.44^{a}	$5.86 \pm 0.52^{bc, A}$	2.69 ± 0.44^{b}	$4.30 \pm 0.52^{a, B}$	38.07 ± 5.85^{b}	$38.67 \pm 3.13^{ab, B}$	16.64 ± 2.71^{b}	$26.03 \pm 3.20^{a, B}$
	7.0	8.90 ± 0.26^{cd}	$9.62 \pm 0.24^{abc, BC}$	6.08 ± 0.31^{bc}	$5.46 \pm 0.24^{bc, B}$	2.64 ± 0.29^{b}	$4.16 \pm 0.24^{a, B}$	46.17 ± 1.56^{ab}	$40.86 \pm 1.53^{ab, AB}$	16.23 ± 1.79^{b}	$25.54 \pm 1.50^{a, B}$
	6.0	8.66 ± 0.29^{d}	$9.29 \pm 0.32^{bcd, CD}$	6.16 ± 0.12^{b}	$5.38 \pm 0.07^{c,B}$	2.50 ± 0.12^{b}	$3.91 \pm 0.07^{a,}_{BC}$	47.18 ± 0.90^{a}	$42.42 \pm 2.74^{ab, AB}$	15.35 ± 0.72^{b}	$24.03 \pm 0.46^{a, B}$
Thymol	8.0	-	22.5 ± 0.48^{A}	-	13.87 ± 0.58^{A}	-	8.53 ± 0.58^{A}	-	31.05 ± 1.02^{C}	-	25.74 ± 1.79^{A}
Eugenol	8.0	16.6 ± 1.15^{D}	-	11.38 ± 0.53^{B}	-	5.51 ± 0.53^{C}	-	49.05 ± 3.53^{A}	-	16.00 ± 1.63^{C}	-

*Values are means ± standard deviations from four measurements, two each from two spray-dried samples. Different lower-case superscript letters indicate differences among co-encapsulated samples, while different uppercase letters represent differences when the combination of thymol and eugenol in co-encapsulated samples is compared with treatments with only one EOC ($P < 0.05$).

†The theoretical loading is 16.28 mg/100 mg powder for each of co-encapsulated eugenol and thymol and 32.56 mg/100 mg powder when only thymol or eugenol was encapsulated. In these two parameters, the average values of co-encapsulated EOCs were compared for treatments with only one EOC.

Table 4-3. Mean particle size and height estimated from AFM for eugenol and thymol co-encapsulated in zein/casein complex at pH 6.0-8.0.*

Preparation pH	Mean particle size (nm)	Mean particle height (nm)
8.0	99.1 ± 10.1 [b]	46.1 ± 3.7 [a]
7.0	165.3 ± 24.5 [a]	52.7 ± 8.3 [a]
6.0	195.2 ± 13.6 [a]	65.6 ± 19.7 [a]

* Values are means ± standard deviations of means from particles in three different AFM images. Different superscript letters in each column indicate significant difference ($P < 0.05$).

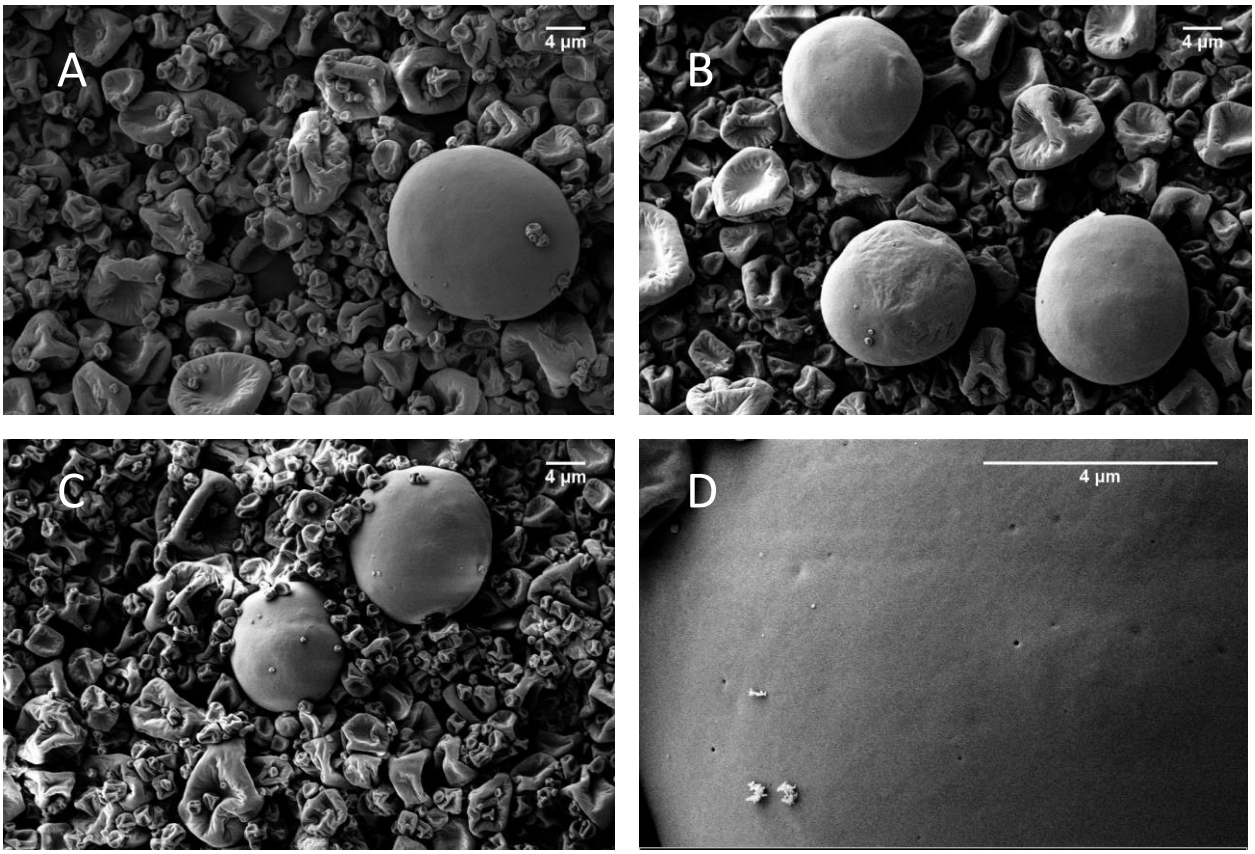

Figure 4-1. SEM images of spray-dried powder produced from dispersions with eugenol and thymol co-encapsulated in zein/casein complexes precipitated at pH 8.0 (A), pH 7.0 (B), and pH 6.0 (C). Figure D is a magnified view of the biggest particle in C.

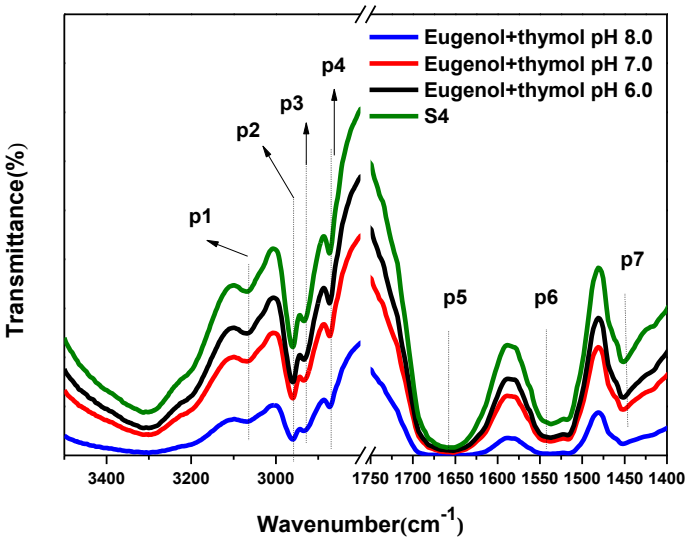

Figure 4-2. FTIR spectra of samples at wavenumbers of 1400-1700^{-1} and 2800-3200 cm^{-1}. Capsules with co-encapsulated eugenol and thymol were prepared at pH 6.0-8.0. Sample S4 is zein/casein nanocomplexes produced at pH 8.0 without EOC, reported in our earlier study (Chen and Zhong, 2014). p1~p4 are peaks belonging to Amide B; p5 is the peak belonging to Amide I; p6 and p7 are peaks belonging to Amide II.

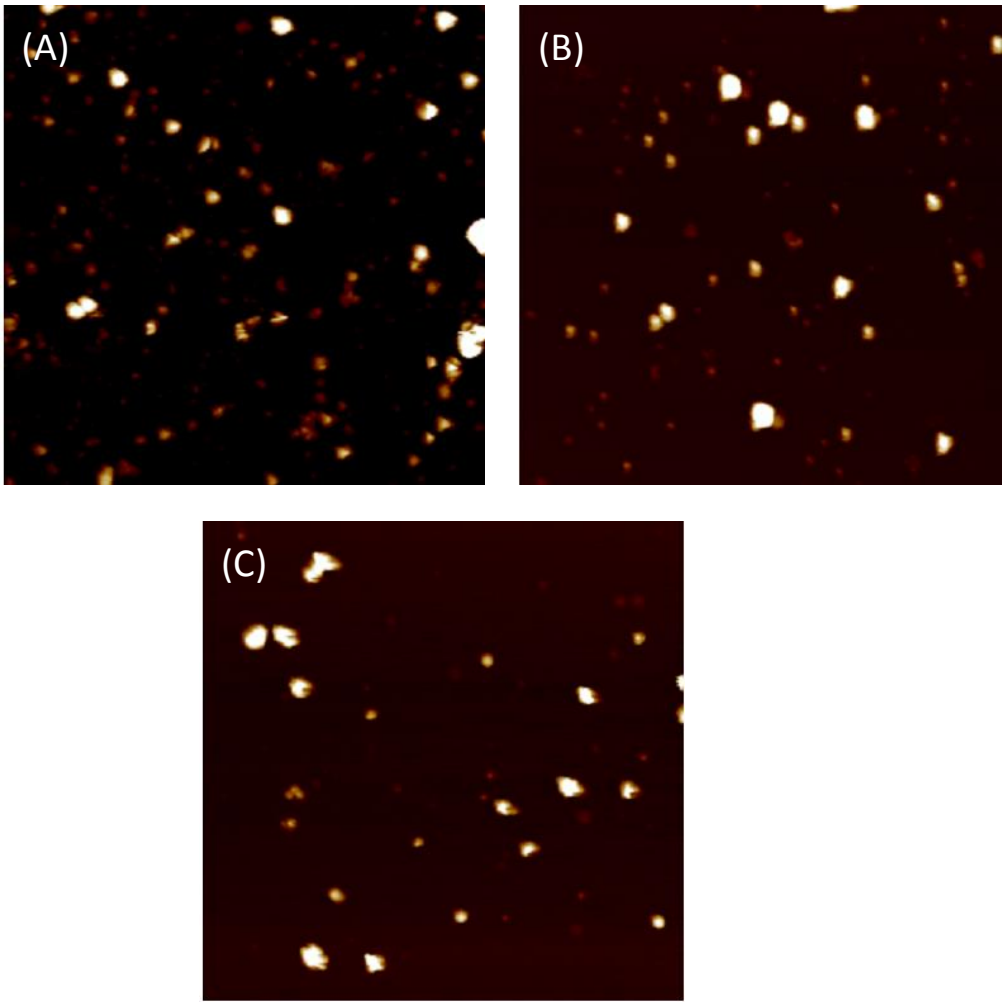

Figure 4-3. AFM images (5 μm × 5 μm) of zein/casein complexes co-precipitated with eugenol and thymol at pH 8.0 (A), 7.0 (B) and 6.0 (C). Dispersions were prepared from spray-dried powder before dilution for imaging.

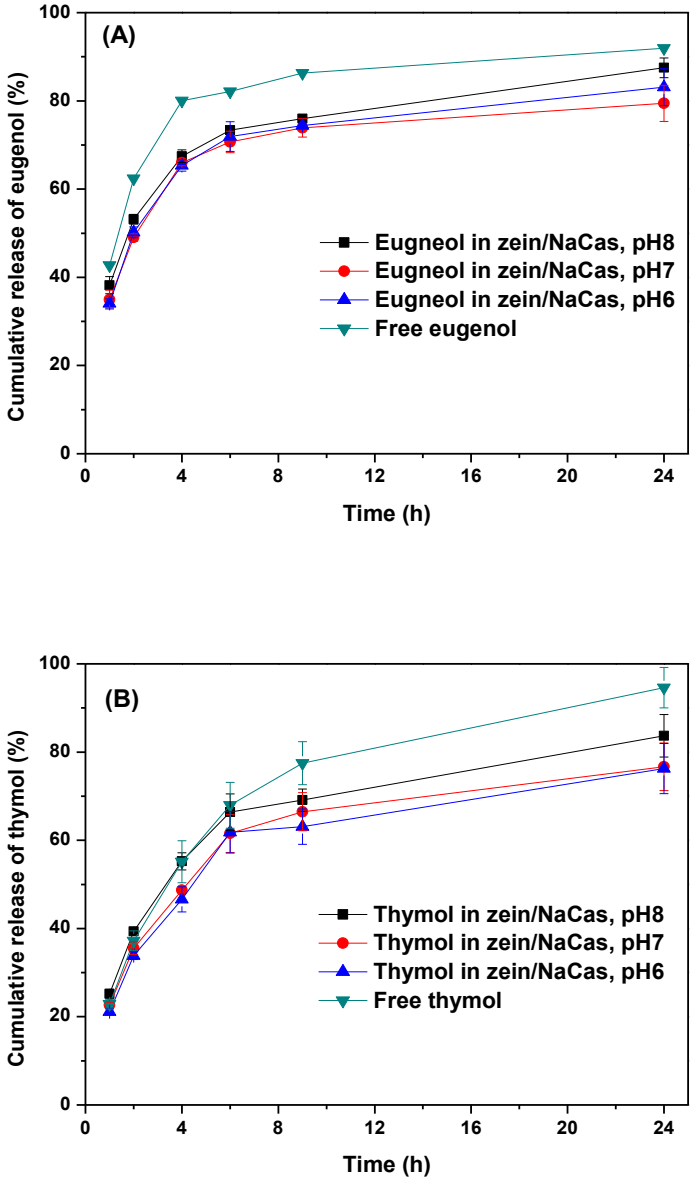

Figure 4-4. Release kinetics of eugenol (A) and thymol (B) in reconstituted dispersions (at pH 7.0 and 21 °C) with zein/casein complexes prepared by precipitation at pH 6.0-8.0.

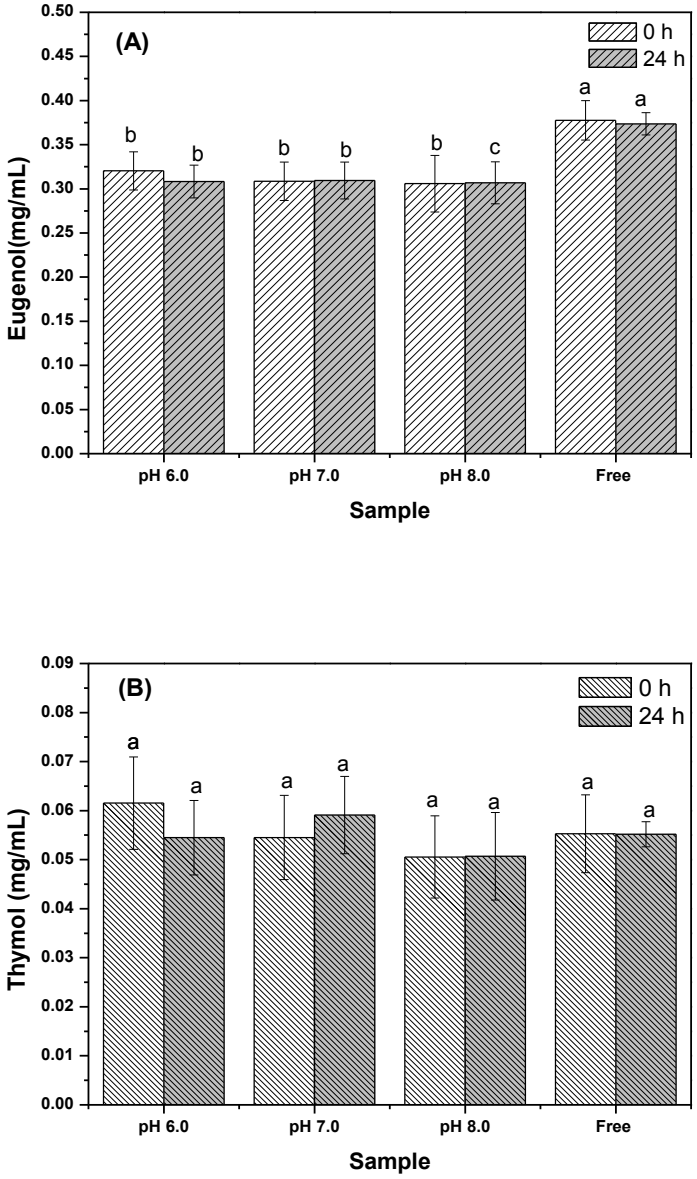

Figure 4-5. The concentrations of eugenol (A) and thymol (B) in milk whey after mixing spray-dried powder with about 2.5 mg/mL each of eugenol and thymol, before (0 h) and after incubation at 21 ℃ for 24 h. Spray-dried capsules with co-encapsulated eugenol and thymol were prepared at pH 6.0-8.0. Free eugenol and thymol were tested at same concentrations. Error bars are standard deviations (n = 4). Different letters above bars indicate differences in the mean of the same EOC ($P < 0.05$).

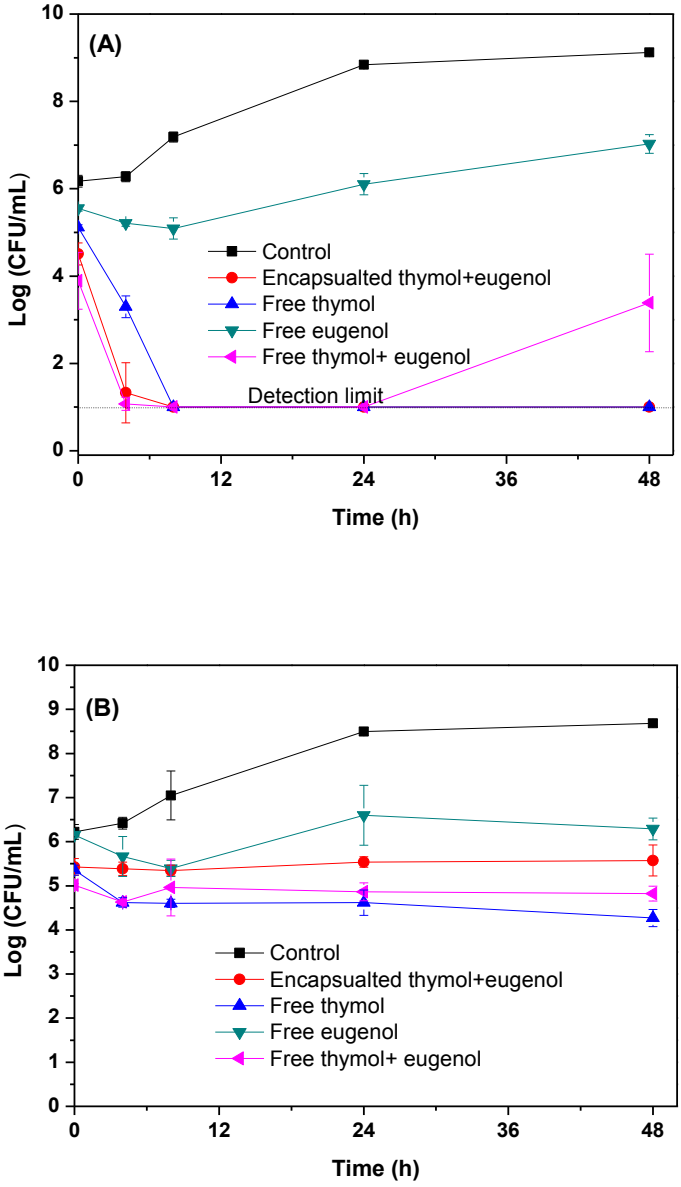

Figure 4-6. Inhibition of *Escherichia coli* O157:H7 ATCC 43895 (A) and *Listeria monocytogenes* Scott A (B) at 21 ℃ in 2% reduced-fat milk by free or encapsulated EOCs. Free EOCs were tested for 5 mg/mL thymol or eugenol, or 2.5 mg/mL each. The encapsulated EOCs, prepared at pH 7.0 and tested at an overall concentration of 5 mg/mL, contained around 2.5 mg/mL each of thymol and eugenol. Error bars are standard deviations ($n \geq 2$).

Chapter 5. A novel method of preparing stable zein nanoparticle dispersions for encapsulation of peppermint oil

A version of this chapter was accepted by Food Hydrocolloids:

Huaiqiong Chen, and Qixin Zhong. 201x. A novel method of preparing stable zein nanoparticle dispersions for encapsulation of peppermint oil. *Food Hydrocolloids*. Accepted.

My primary contributions to this manuscript include sample preparation, data collection and analysis, results interpretation and writing.

5.1 Abstract

Zein is a group of alcohol-soluble maize proteins and is typically dissolved in 70-80% aqueous ethanol before dispersion into water to precipitate zein as nanoparticles, which can simultaneously encapsulate various lipophilic compounds co-dissolved in aqueous ethanol. Strategies are needed to prevent the precipitation of hydrophobic zein nanoparticles at a wide pH range and prevent the use of flammable ethanol in industrial production. The objective of this work was to replace ethanol with nonflammable propylene glycol to prepare zein nanoparticles and stabilize nanoparticles using gum arabic (GA). Nanoparticles smaller than 200 nm were produced using a stir plate, and GA prevented their precipitation at pH 3.0-8.0. Both electrostatic and hydrophobic interactions contributed to the adsorption of GA on zein nanoparticles, with the former being more significant at a lower pH corresponding to a higher surface load. Encapsulation of peppermint oil in zein nanoparticle-GA complexes did not significantly change particle dimension and dispersion stability. The gradual release of peppermint oil from freeze-dried samples was observed at pH 2.0-8.0, reaching complete release in a shorter time at a lower pH. The established method may enable the preparation of stable zein nanoparticles for various applications in the food industry.

Keywords: zein; gum arabic; propylene glycol; peppermint oil; release kinetics

5.2 Introduction

Zein is a group of alcohol-soluble proteins extracted from corn gluten meal. Being prolamins, the surface of zein molecules includes more than 50% hydrophobic amino acid residues (Chen et al., 2013, 2014b; Luo and Wang, 2014) making them water insoluble. To incorporate zein in aqueous systems, zein can be prepared as dispersible nanoparticles by dispersing a stock aqueous ethanol solution of zein into water (Patel et al., 2010a; Zhong and Jin, 2009b), where zein precipitates to form nanoparticles because the mixture with a lowered content of ethanol becomes a non-solvent of zein. The process can be used to encapsulate various lipophilic compounds that are co-dissolved in the aqueous ethanol stock solution and co-precipitated with zein during dispersion in water (Patel et al., 2010b; Wu et al., 2012a). Despite being generally-recognized-as-safe (GRAS), ethanol is not permitted in non-alcohol foods and is not friendly in the industrial production due to its flammability. Propylene glycol (PG) is also a GRAS compound that can be used as an additive in non-alcohol foods, is less flammable than ethanol, and is another solvent of zein at room temperature (Lawton, 2002). However, few studies have used PG as a solvent of zein.

The poor dispersibility of hydrophobic zein nanoparticles in aqueous food products is a well-known limitation, which is partially due to the isoelectric point (*pI*) of zein being at neutral pH (Patel et al., 2010a; Zhang et al., 2014). Sodium caseinate has been studied as a biopolymer that adsorbs on zein nanoparticles with and without encapsulated compounds to provide repulsive electrostatic and steric interactions to prevent particle aggregation (Chen and Zhong, 2014; Li et al., 2012a; Patel et al., 2010a; Patel et al., 2010b; Wang et al., 2013; Zhang et al., 2014). A drawback of this approach is the instability of complex particles at acidic pH due to the precipitation of caseins. Compared to proteins, many polysaccharides have ionizable side groups

such as carboxylate groups with an acidic pKa and therefore provide negative charges of protein-polysaccharide complexes at acidic pH. For example, pectin, with carboxylate groups having a pKa of 2.5-4.5 (Sriamornsak, 2003), has been studied to form stable complexes with proteins such as β-lactoglobulin (Sperber et al., 2009), whey protein isolate (Krzeminski et al., 2014; Salminen and Weiss, 2014), and wheat gluten protein (Correa et al., 2014). Pectin has also been studied to form hydrogels with zein for pH-stimulated delivery of drugs (Bobokalonov et al., 2012; Mukhidinov et al., 2011). Gum arabic (GA) is another frequently studied polysaccharide. GA is a naturally occurring anionic polysaccharide−protein conjugate comprised of three fractions - arabinogalactan–protein, arabinogalactan and glycoprotein that make up about 10, 90–99, and 1% mass of GA products, respectively (Wang et al., 2011; Weinbreck et al., 2004). These three fractions differ in molecular size and protein content and are depicted as a "wattle blossom" type structure (Dickinson, 2003). GA is highly water soluble and its concentrated solutions have a viscosity much lower than other polysaccharides (McNamee et al., 1998; Weinbreck et al., 2004). GA has been studied to stabilize sodium caseinate and whey protein in a wide pH range (Weinbreck et al., 2004; Ye et al., 2012; Ye et al., 2006), but its ability to stabilize zein is still unknown.

In this study, a new approach was studied to fabricate zein nanoparticles by advancing our previous study (Chen and Zhong, 2014). PG was used as a solvent of zein to replace ethanol, and GA replaced sodium caseinate to stabilize zein nanoparticles after anti-solvent precipitation. Because GA is an excellent emulsifying agent and adsorbs on hydrophobic solid surface (Bouyer et al., 2013; Wang et al., 2011), we hypothesize that GA could adsorb on zein nanoparticles to provide steric and electrostatic repulsions to stabilize nanoparticles in a wide pH range.

The objectives of this study were to (1) characterize zein nanoparticle formation and the stability as impacted by GA, (2) understand interaction forces between zein nanoparticles and GA, and (3) study the ability of zein-GA complexes to encapsulate and control the release of peppermint oil. Peppermint oil was chosen as a model essential oil because it has not been encapsulated in zein nanoparticles by the liquid-to-liquid dispersion process.

5.3 Materials and methods

5.3.1 Materials

Purified α-zein was purchased from Acros Organics (Morris Plains, NJ). GA and PG were obtained from Fisher Scientific (Pittsburgh, PA). GA had a protein content around 2% w/w based on our assessment. 8-Anilino-1-naphthalene sulfonate (ANS) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Peppermint oil was a product from NOW Food Company (Bloomingdale, IL). Other chemicals such as hexane were obtained from Fisher Scientific (Pittsburgh, PA).

5.3.2 Sample preparation

Stock solution of zein was prepared by dissolving at 5% w/w in a binary mixture of PG and water with a mass ratio of 8:2. GA stock solution was prepared at 20% w/w in distilled water under gentle stirring for overnight at room temperature (21 °C) for complete hydration, followed by centrifugation at 4564 *g* for 10 min (Sorvall RC-5B plus, Sorvall, Newtown, CT) to remove insoluble impurities. Both stock solutions were stored at 4 ℃ before use. Eight hundred μL of zein stock solution was added drop-by-drop into 39 mL deionized water while being mixed on a magnetic stir plate at 350 rpm. Subsequently, 300 μL of GA stock solution was added into the zein dispersion to obtain a zein: GA mass ratio of 2:3. This ratio was selected based on

preliminary trials using ratios of 2:1, 1:1, 2:3, and 1:2, and the dispersion prepared at a ratio of 2:3 was the most stable and the turbidity was the lowest. The freshly prepared dispersions were characterized for particle size, turbidity, zeta-potential, morphology, and other physical properties as below.

5.3.3 Particle size and zeta-potential measurements

The particle size and zeta-potential of freshly prepared dispersions were measured using a Delsa Nano C particle size/zeta potential analyzer (Beckman Coulter, Fullerton, CA). Dispersions were diluted to an appropriate concentration in deionized water before adjusting pH using 1 M HCl or NaOH for particle size and zeta-potential measurements. Each treatment was measured using triple independent dispersion replicates, each measured for two times. The volume fraction-length ($d_{4,3}$) and volume-area ($d_{3,2}$) mean diameters were calculated using Eq. (1) and (2), respectively.

$$d_{4,3} = \frac{\sum_{i=1} n_i di^4}{\sum_{i=1} n_i di^3} \quad (1)$$

$$d_{3,2} = \frac{\sum_{i=1} n_i di^3}{\sum_{i=1} n_i di^2} \quad (2)$$

where n_i is the number of particles with a diameter of d_i.

5.3.4 Turbidity measurement

The turbidity of dispersions was measured for absorbance at 600 nm (Abs_{600}) with a UV–vis spectrophotometer (model Evolution 201, Thermo Scientific Co., Waltham, MA). The measurements were conducted for at least two independent dispersion replicates.

5.3.5 Protein solubility

The solubility of zein was measured as a function of pH. The freshly prepared dispersions with and without GA were adjusted to pH 3.0–8.0 with 1 M HCl or NaOH. After incubation for 30 min at room temperature, samples were centrifuged at 14,100*g* for 10 min (model MiniSpin®

plus, Eppendorf, Westbury, NY). The protein content in the supernatant was measured by the bicinchoninic acid (BCA) method, as detailed previously (Chen and Zhong, 2014). The solubility was defined as the percentage of the measured protein concentration to that of the corresponding solution at pH 11 that completely dissolved zein (Kasran et al., 2013; Luo et al., 2014a).

5.3.6 Estimation of mass ratio of zein and GA in complexes

The mass ratio of zein and GA in complexes was estimated using our previous method (Chen and Zhong, 2014). The freshly prepared dispersion was centrifuged at 6700*g* for 20 min to collect nanoparticles as the precipitate. The collected precipitate was washed three times using deionized water to remove free biopolymers, with centrifugation at 6700*g* for 20 min in each repetition. The washed precipitate was mixed with 90% aqueous ethanol to extract zein. After centrifugation at 6700*g* for 5 min, the supernatant with zein was used to quantify protein content using the BCA method with bovine serum albumin as a reference protein. The precipitate was re-dissolved in deionized water to quantify GA using the phenol–sulfuric acid method that is the easiest and most reliable colorimetric method for carbohydrate determination (Masuko et al., 2005). The colorimetric assay conditions were adopted from the literature (Masuko et al., 2005; Ye et al., 2006). Briefly, 5% w/v phenol was prepared in deionized water as a stock solution. Two hundred μL of the GA solution was mixed with 200 μL of the 5% phenol solution and 1 mL of 9 M sulfuric acid. After brief vortexing, the mixture was heated at 90 ℃ for 5 min. After cooling in a room temperature (~21 °C) water bath for 5 min, the absorbance at 485 nm was measured using the above spectrophotometer. GA was used to establish a calibration curve in the colorimetric assay (Ye et al., 2006).

5.3.7 Estimation of surface coverage of GA on zein nanoparticles

Surface load (Γ), mg surfactant on unit area of droplet surface, is commonly estimated to evaluate emulsifying activity of surfactants (Nakauma et al., 2008). In the present work, the amount of GA adsorbed on zein nanoparticles was similarly estimated according to Eq. (3) using zein and GA concentrations determined as above (after removing free GA by centrifugation)

$$\Gamma\ \left(\frac{\text{mg}}{\text{m}^2}\right) = \frac{C_{\text{GA}}\ d_{3,2}}{6\phi} \times 0.001 \tag{3}$$

where C_{GA} is the GA concentration (mg/mL) determined in the complexes, $d_{3,2}$ is the mean diameter (nm) calculated using Eq. (2), and ϕ is the volume fraction of zein particles determined by Eq. (4) using the concentration of zein in complexes (C_{zein}, mg/mL) and zein density (ρ_{zein}) of 1.22 g/mL (Gong et al., 2006).

$$\phi = \frac{C_{\text{zein}}}{\rho_{\text{zein}}} \times 0.001 \tag{4}$$

5.3.8 Surface hydrophobicity of nanoparticles

Binding between fluorescent probe ANS and nanoparticles was studied using a previous method with modification (Chen and Zhong, 2014; Ye et al., 2012). ANS was dissolved at 8 mM in phosphate buffered saline (PBS) adjusted to pH 3-8. The fresh 0.1% w/v zein dispersion with and without GA was diluted 20-fold in PBS and adjusted to pH 3-8. The 0.5% w/v GA solution was also studied. Four mL zein dispersion or GA solution was mixed with 20 μL ANS solution at the same pH. The fluorescence spectrum was acquired using a RF-1501 Fluorospectrophotometer (Shimadzu Corp., Tokyo, Japan). An excitation wavelength of 370 nm was used to collect the emission spectrum from 400 to 600 nm using an emission slit of 20 nm.

5.3.9 Atomic force microscopy (AFM)

The freshly prepared dispersion was adjusted to pH 5.0 and incubated at room temperature for 30 min followed by dilution in deionized water (pre-adjusted to pH 5.0) to an

overall solute concentration of 10 ppm. Four μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA). A rectangular cantilever with an aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m (FESPA, Bruker Corp.) and a Multimode VIII microscope (Bruker AXS, Billerica, MA) were used to scan the sample. Images were generated at the tapping mode with a preset area of 5.0 × 5.0 μm at a scanning speed of 1 Hz. By using the instrument software, two-dimensional images were used to estimate average particle size. Three-dimensional topographic images were used to generate the mean particle heights.

5.3.10 Encapsulation of peppermint oil in zein/GA complexes

5.3.10.1 Encapsulation method

Peppermint oil and zein were dissolved at a mass ratio of 1:4, 1:2, or 1:1 in 80% aqueous PG, with zein concentration set at 5% w/w. The mixture was dispersed into water at an overall zein concentration of 0.2% w/w. Besides the freshly prepared dispersions, another set of samples was freeze-dried and stored at −20 °C before characterization of release kinetics of peppermint oil.

5.3.10.2 Efficiency of encapsulating peppermint oil

To measure encapsulation efficiency, 10 mg freeze-dried sample was mixed with 2 mL distilled water, vortexed for 30 s, and centrifuged at 14,100*g* for 5 min to obtain the supernatant to determine free peppermint oil. Another 10 mg freeze-dried sample was mixed in 60% aqueous ethanol for 1 h and centrifuged at 14,100*g* for 5 min to get the supernatant to measure the total peppermint oil. The absorbance at 240 nm was determined for the supernatants using the above UV-vis spectrophotometer. After subtraction by the absorbance of the supernatant from the zein/GA control dispersion prepared as above without peppermint oil, the amounts of total and

free peppermint oil were determined based on a standard curve established with standard solutions with peppermint oil dissolved at 0.03-0.5 mg/mL in 60% aqueous ethanol. The encapsulation efficiency was then calculated based on Eq. 5 (Wu et al., 2012a). Triplicate samples were measured.Encapsulation efficiency (%) =

$$\frac{\text{Total peppermint oil in freeze-dried sample} - \text{free oil in freeze-dired sample}}{\text{Total peppermint oil before freeze-drying}} \times 100\% \quad (5)$$

5.3.10.3 Release kinetics of encapsulated peppermint oil

The kinetics of peppermint oil released from zein nanoparticles was characterized using our earlier method, with modification (Xiao et al., 2011b; Xiao et al., 2011c). Ten mg of the freeze-dried powder was suspended in 1.0 mL of 10 mM PBS, pre-adjusted to pH 2.0, 6.0 and 8.0, in a 1.5 mL micro-centrifuge tube. For the "0 h" time point, the sample was vortexed for 1 min and then centrifuged at 14,100*g* for 5 min. The 700 μL supernatant was taken to determine the amount of released peppermint oil, followed by supplementing 700 μL of the corresponding fresh PBS to the remaining suspension. For later time points, an end-to-end shaker was used to continuously mix the suspensions at room temperature (21 ℃). At a chosen time point, the same procedures described above were repeated to determine the amount of released peppermint oil. Four replicates were tested for each sample.

Three mL hexane was mixed with the 700 μL supernatant obtained above to extract peppermint oil by vortexing for 2 min, and the upper hexane phase after clear separation into two phases was measured for absorbance at 240 nm to determine peppermint oil concentration as above. The percentage of cumulatively released peppermint oil was calculated according to Eq. (6).

$$R_{\text{ti}}(\%) = \frac{3\sum_{n=1}^{i-1} p_n + \frac{30}{7} p_i}{P_0} \times 100\% \quad (6)$$

where R_{ti} (%) is the cumulatively released peppermint oil after time t_i, the i^{th} time of sampling; p_i is the peppermint oil concentration in the 700 μL supernatant determined at the sampling time t_i; and P_0 is the total peppermint oil content in the 10 mg powder. To determine P_0, 4 mg powder was continuously mixed in 4 mL hexane for 1 h on an end-to-end shaker, and the absorbance of the separated hexane phase was measured for absorbance at 240 nm to determine peppermint oil mass as described previously.

5.3.11 Statistical analysis

Statistical analyses were performed using the SAS software (version 9.3, SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between pairs of means were compared using a Tukey's test. The significance level was set at 0.05.

5.4 Results and discussion

5.4.1 Stabilization of zein nanoparticles by GA

As shown in Figure 5-1A, zein nanoparticles were tentatively stable at pH 3.0-5.0 but precipitated at pH 6.0-8.0, near its neutral *pI* (Patel et al., 2010a). The addition of GA effectively prevented the precipitation of zein nanoparticles at the entire pH range tested, with the sample at pH 3.0 being slightly more turbid than other pH conditions (Figure 5-1A). The $d_{4,3}$ of zein/GA dispersion ranged from 100 to 165 nm and was generally bigger at a lower pH (Figure 5-1B). The Abs_{600} (Figure 5-1C) was in agreement with the appearance (Figure 5-1A) and $d_{4,3}$ (Figure 5-1B), with that at pH 3.0 being significantly higher than other pHs ($P < 0.05$). After heating at 80 ℃ for 30 min, the zein/GA dispersion at pH 3.0 and pH 4.0 became more turbid, with significant increases in $d_{4,3}$ and Abs_{600} (Figure 5-1B&C, $P < 0.05$). This may have been caused by the reduced solubility of GA as pH became close to the pKa of carboxylate groups (Chanamai and

McClements, 2002; Moschakis et al., 2010). At pH 5.0-8.0, there were no noticeable differences in visual appearance, $d_{4,3}$, and Abs_{600} before and after heating the zein/GA dispersions. In comparison, zein nanoparticles without GA precipitated at all tested pH conditions after heating, which is likely due to the exposure of a greater amount of hydrophobic amino acids after thermal denaturation (Chi et al., 2003). These results indicated that zein nanoparticles were stabilized by GA. GA is a well-known naturally occurring protein-polysaccharide conjugates with good emulsifying properties. In the "wattle blossom" model, the predominantly hydrophobic and protein-rich backbone of GA adsorbs onto oil surface and several conjugated polyelectrolytes extend to the continuous aqueous phase to prevent the aggregation of oil droplets by repulsive electrostatic and steric interactions (Dickinson, 2003). It is likely that GA stabilized zein nanoparticles by adsorbing on the hydrophobic protein particle surface, as hypothesized previously.

The stability of zein dispersions was further evaluated for protein solubility using percentages of protein remaining in the supernatant after centrifugation (Figure 5-2). For zein alone, its solubility was 8% at pH 4.0-8.0 but was 66% at pH 3.0. This agreed with the poor stability of zein dispersions at pH 6.0-8.0 (Figure 5-1A) and temporary stability at pH 4.0 and 5.0 that showed precipitation after 30 min (data not shown). After supplementing GA, the solubility of zein was significantly improved at pH 4.0-8.0 but reduced at pH 3.0. At pH 3.0, zein behaves like polyelectrolytes and could be unfolded or swollen, providing a larger solvent-accessible area and eventually an improved solubility (Li et al., 2012c). The respective $d_{4,3}$ of zein dispersion without and with GA at pH 3.0 was 82 and 165 nm, which resulted in better retention of zein in the supernatant after centrifuging the dispersion without GA. The data further suggested the adsorption of GA on zein nanoparticles at pH 3.0, and the reduced solubility of GA at acidity

around pKa of carboxylate groups [~pH 2.2, (Chanamai and McClements, 2002; Moschakis et al., 2010)] caused the flocculation of zein particles.

5.4.2 Surface properties of zein nanoparticles

5.4.2.1 Zeta-potential

To verify the successful coating of GA on zein nanoparticle surfaces and understand colloidal forces stabilizing zein nanoparticles, the zeta potentials of zein nanoparticles, GA, and their mixture were measured at pH 3.0-8.0 (Figure 5-3). For zein nanoparticles, the zeta potential changed from positive to negative with increasing pH, and the pH corresponding to zero zeta potential (*pI*) was observed around 6.2, which is in a good agreement with the literature (Patel et al., 2010a; Zhang et al., 2014). The zeta potential of GA was negative at pH 3.0-8.0, with a decreasing magnitude at a lower pH. This is expected because carboxylate groups of GA contribute to its negative charges (Chanamai and McClements, 2002) and the pKa of carboxylate groups is *ca*. 2.2 (Chanamai and McClements, 2002; Moschakis et al., 2010). The zein dispersion with GA showed a similar zeta-potential profile as GA. After removing free GA by centrifugation, the measured zeta-potential became less negative and was between those of zein nanoparticles and GA, which indicated the adsorption of GA on zein nanoparticles. The lowered zeta-potential magnitude at a lower pH indicates the weakened electrostatic repulsion induced the aggregation of GA-coated zein nanoparticles, corresponding to bigger $d_{4,3}$ in Figure 5-1B.

5.4.2.2 Surface load of GA on zein nanoparticles

To understand the composition of zein nanoparticles after interacting with GA, free GA was removed by centrifugation, and the quantities of GA and zein in nanoparticle complexes were determined. As presented in Figure 5-4, zein:GA mass ratio was bigger and Γ was smaller

at a higher pH (decreasing from ~ 9 mg/m^2 at pH 3 to ~3 mg/m^2 at pH 6-8), indicating that a greater amount of GA was associated with zein at a lower pH below the pI of zein. This is because zein is more soluble (Figure 5-2) and swells to a higher extent at a lower pH, corresponding to a bigger area for adsorption of GA. In addition, zein becomes overall more positively-charged at a lower pH that favors the binding with negatively-charged GA (Figure 5-3). When 10.0% GA was used to emulsify 15% w/w medium chain glycerides, Γ was determined to be similar (about 7.8 mg/m^2) at pH 3.0-6.0 (Nakauma et al., 2008). The pH-dependence of Γ in the present study is caused by the significance of electrostatic attraction for the adsorption of GA on zein, which is different from hydrophobic interactions when GA is used to emulsify lipids.

5.4.2.3 Surface hydrophobicity

To further characterize particle surface properties, ANS was used as a probe to study surface hydrophobicity of nanoparticles. The fluorescence intensity after interacting ANS with zein nanoparticles, GA, and their mixture at pH 3.0-8.0 is shown in Figure 5-5. GA solution had little fluorescence at the entire pH range. This is expected because GA is an overall very water-soluble conjugate with only about 2% protein (Dickinson, 2003), as verified in the present study using the BCA method. The fluorescence intensity of zein nanoparticles was higher at a lower pH. For zein particles mixed with GA, the fluorescence intensity was also higher at a lower pH but was lower than that of zein, with the intensity being closer to that of GA as pH increased to 8.0.

ANS preferentially interacts with solvent-accessible hydrophobic pockets or patches of proteins, producing a marked increase in the emission intensity (Ye et al., 2012). Zein is more soluble at a lower acidic pH, which reduces the particle size or increases the porosity of zein

nanoparticles (Wongsasulak et al., 2014). This overall increases the accessible total surface area available for binding by ANS and therefore the higher fluorescence intensity at a lower pH (Figure 5-5). When GA adsorbs on zein nanoparticles, the fluorescence data in Figure 5-5 provides insights of surface structures of zein nanoparticle-GA complexes. At a lower pH, a greater amount of GA adsorbs on zein nanoparticles (Figure 5-4), but the fluorescence intensity of the mixture is higher. Conversely, zein is more positively charged (Figure 5-3) and more soluble at a lower pH (Figure 5-2). It is possible that GA binds with zein nanoparticles via positively charged amino acid residues at low pHs, leaving hydrophobic patches still available for binding with ANS. As pH increases, zein has fewer positive surface charges and becomes overall negatively-charged at pH 7.0 and 8.0 (Figure 5-2), but the negatively charged GA binds with zein nanoparticles at pH 3.0-8.0 (Figure 5-3 and 5-4). This suggests that the adsorption of GA on zein nanoparticles changes from the domination by electrostatic forces to the significant contribution by hydrophobic forces when pH increases from 3.0 to 8.0. The blocking of hydrophobic patches on zein particle surface reduces the ANS binding capacity and lowers fluorescence intensity, decreasing to a magnitude close to GA at pH 8.0 (Figure 5-5).

5.4.3 Significance of electrostatic interactions on GA adsorption on zein nanoparticles

Even though zein has a high proportion (>50%) of hydrophobic amino acid residues (Chen et al., 2013), hydrophilic surface amino acid residues, mostly glutamines (Matsushima et al., 1997a), provide surface charges of zein nanoparticles. GA is overall a negatively-charged polysaccharide with a small fraction of covalently-bonded hydrophobic protein (Wang et al., 2011; Weinbreck et al., 2004). The significance of electrostatic interactions on zein-GA binding was studied by increasing the ionic strength from 0 to 50 mM (using NaCl) at pH 3.0-8.0 (Weinbreck et al., 2004; Zhang et al., 2013a). As shown in Figure 5-6, the successive addition of

NaCl to the zein/GA dispersion resulted in the gradual increase in turbidity and eventually precipitation. At 50 mM NaCl, the quantified Γ decreased at all pH conditions when compared to treatments with 0 mM NaCl (Figure 5-4), indicating the detachment of GA at each pH regardless of the overall surface charge of zein nanoparticles (Figure 5-3). The results indicated the significance of electrostatic attraction on GA adsorption on zein nanoparticles. The adsorption of GA on overall negatively-charged zein nanoparticles at pH 7.0 and 8.0 likely is enabled by binding on basic amino acid residues (Dickinson, 2003).

5.4.4 Structures studied by AFM

Nanoscale structures of GA, zein and zein/GA complex at pH 5.0 were studied using AFM, shown in Figure 5-7 for images and Table 5-1 for the estimated mean particle size and height. GA itself showed mostly spherical structures (Figure 5-7A) with an average dimension of 109 nm (ranging from 44 to 257 nm), and an average particle height of 23.3 nm. The results were in a good agreement with a previous study (Ikeda et al., 2005). Zein particles had a wide size range, from 110 to 1159 nm, with an average dimension of 250 nm and average particle height of 51.3 nm. The bigger structures appeared to be aggregated nanoparticles, as evidenced by visible precipitates in Figure 5-1A. When GA was mixed with zein nanoparticles, most particles were spherical and discrete, indicating zein nanoparticles coated by GA were quite stable against aggregation. The GA/zein complexes had average particle size of 143 nm, with a range from 44 to 349 nm, and the average particle height (43.8 nm) was smaller than that of zein nanoparticles without GA (51.3 nm). The AFM results confirmed the coating of GA on zein nanoparticles.

5.4.5 Properties of peppermint oil encapsulated in zein nanoparticles stabilized by GA

5.4.5.1 Encapsulation efficiency

The efficiency of encapsulating peppermint oil in zein/GA dispersions at different mass ratios of peppermint oil: zein is listed in Table 5-2. When the peppermint oil: zein mass ratio increased, the encapsulation efficiency decreased. The dispersion prepared from peppermint oil and zein dissolved at a mass ratio of 1:2 in 80% aqueous PG was used for further particle size and zeta-potential analyses. The dispersion after freeze-drying was used to characterize release properties.

5.4.5.2 Particle size and zeta-potential

The dispersion prepared with peppermint oil and zein at a mass ratio of 1:2 was adjusted to pH 3.0-8.0, and the appearance, $d_{4,3}$ and zeta potential are shown in Figure 5-8. Like dispersions without peppermint oil (Figure 5-1B), a bigger $d_{4,3}$ was observed at a lower pH, increasing from 108 nm at pH 8.0 to 130 nm at pH 3.0 (Figure 5-8B). Nanoparticles with and without peppermint oil had similar $d_{4,3}$ at pH 5.0-8.0 (Figure 5-1B and 5-8B), but those with peppermint oil were significantly smaller than bare particles at pH 3.0 and 4.0 ($P < 0.05$). The AFM results studied at pH 5.0 for peppermint oil-loaded nanoparticles also showed the smaller particle size (129 nm) and height (24 nm) than bare nanoparticles at the same pH (143 nm in particle size and 43 nm in height; Table 5-1 and Figure 5-7). When peppermint oil was loaded in zein particles without GA, the $d_{4,3}$ at pH 3.0 (78 nm) and 4.0 (82 nm) was also smaller than bare zein nanoparticles (82 nm at pH 3.0, 90 nm at pH 4.0). As presented previously, zein is more soluble and particles are more porous at a lower pH. Encapsulation of peppermint oil in zein may enhance its intra-particle attraction and therefore the packing of nanoparticles, especially at pH 3.0.

When turbidity was compared (Figure 5-1A vs. 5-8A), the encapsulation of peppermint oil (Figure 5-8A) increased the turbidity of dispersions. The trend seems to disagree with the smaller or similar $d_{4,3}$ of the dispersions with oil, as presented above. In DLS experiments, the intensity of samples with peppermint oil was 2-3 times higher than those without peppermint oil at the same dilution ratio (data not shown), which may indicate a larger number of particles in the former when oil was encapsulated (Luo et al., 2013; Teng et al., 2013). The greater number of particles (with more mass deriving from peppermint oil) can increase turbidity.

The zeta-potential of zein/GA after loading peppermint oil showed a similar trend as the zein/GA dispersion without centrifugation, and the dispersions with and without peppermint oil did not show significant difference ($P > 0.05$) at pH 4.0-8.0 (Figure 5-8B vs. Figure 5-3). The smaller magnitude of negative zeta-potential at pH 3.0 again is due to the acidity close to the pKa of carboxylate groups. Encapsulation of peppermint oil in zein nanoparticles increased the magnitude of negative zeta-potential at pH 3.0, from -7.1 mV (Figure 5-3) to -26.6 mV (Figure 5-8B). As discussed previously, the entrapment of peppermint oil may have increased the compactness of zein nanoparticles, which reduces the amount of positively charged zein molecules neutralizing negative charges of GA. Similar phenomena (decreased particle size and increased magnitude of negative zeta-potential) were observed after thymol was encapsulated in sodium caseinate (Pan et al., 2014).

5.4.5.3 Release properties

In vitro release kinetics of peppermint oil from zein/GA complexes after hydration of freeze-dried powder in PBS at pH 2.0, 6.0, and 8.0 is presented in Figure 5-9. The gradual release of peppermint oil from the complexes was observed at all three pHs. Similar to our early studies of encapsulating thymol in zein by spray drying (Xiao et al., 2011b; Xiao et al., 2011c),

the faster release of peppermint oil was observed at a lower pH, reaching 100% release in 72 h at pH 2.0 and 120 h at pH 6.0 and 8.0. As discussed above, zein has a higher solubility at a more acidic pH and the increased porosity of zein nanoparticles may have caused the faster release of peppermint oil at a lower pH.

5.5 Conclusions

In summary, our work demonstrated a novel method to prepare stable zein nanoparticles with $d_{4,3}$ smaller than 170 nm using less flammable PG and low intensity mixing (using a stir plate). The supplementation of GA stabilized zein nanoparticles at pH 3-8 and improved protein solubility at pH 4.0-8.0. Both electrostatic and hydrophobic interactions contributed to the adsorption of GA on zein nanoparticles, with the former being more significant at a lower pH. Conditions of forming zein nanoparticles were successfully used to encapsulate peppermint oil. The dispersions after reconstituting freeze-dried powder in PBS at pH 2.0, 6.0, and 8.0 showed the gradual release of peppermint oil, reaching complete release in a shorter time at a lower pH. Therefore, the studied method can be used to incorporate lipophilic compounds such as flavor oils in aqueous dispersions with various acidities.

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Appendix

Table 5-1. Mean particle size and height estimated from AFM * for gum arabic (GA), zein, zein/GA dispersion and peppermint oil loaded zein/GA dispersion at pH 5.0.

Sample	Mean particle size (nm)	Particle size range (nm)	Mean particle height (nm)
Gum arabic	109.3 ±33.53 [b]	44-257	23.3 ±5.5 [b]
Zein	250.3 ±37.9 [a]	110-1159	51.3 ±7.2 [a]
Zein/gum arabic	143.2 ±7.6 [b]	47-349	43.8 ±9.8 [a]
Zein/gum arabic loaded with peppermint oil	128.7 ±37.4 [b]	67-196	24.1 ±3.4 [b]

* From particles in three different AFM images. Different superscript letters represent significant differences in the mean ($P < 0.05$).

Table 5-2. Efficiency of encapsulating peppermint oil in zein nanoparticles.

Zein : peppermint oil mass ratio	Encapsulation efficiency (%)*
4:1	89.2 ±4.9 [a]
2:1	83.9 ±7.4 [a]
1:1	53.6 ±6.9 [b]

* Numbers are mean ± standard deviation from three replicates. Different superscript letters represent significant differences in the mean ($P < 0.05$).

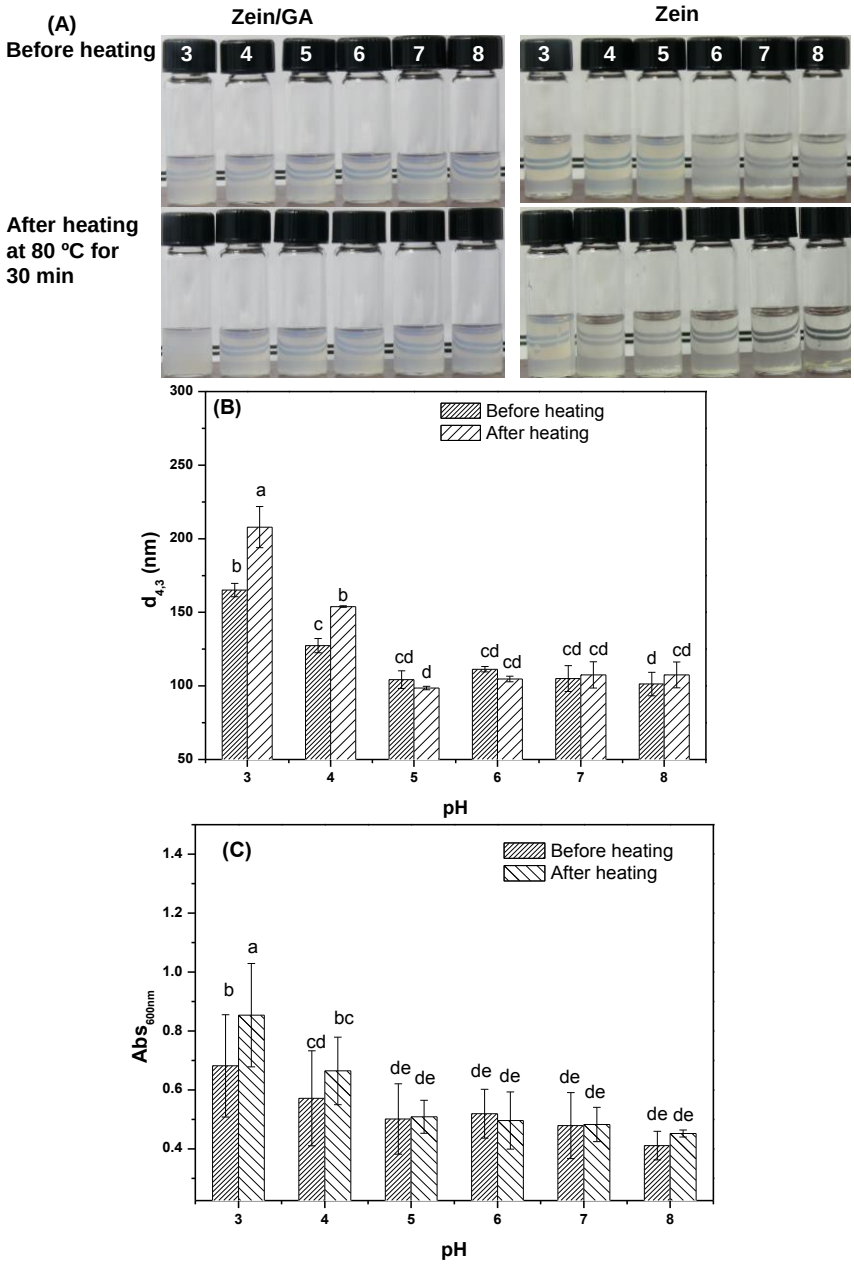

Figure 5-1. Appearance of 0.1% w/v zein dispersion with and without 0.15 % w/v gum arabic (GA), before and after heating at 80 °C for 30 min (A). Absorbance at 600 nm (Abs_{600nm}, B) and volume fraction-length mean diameter ($d_{4,3}$, C) are shown for dispersions with GA only. Nanodispersions were prepared around neutral pH, with or without adding GA, and adjusted to pH 3.0-8.0 before characterization. Different letters above bars represent significant differences in particle dimension or sample absorbance ($P < 0.05$).

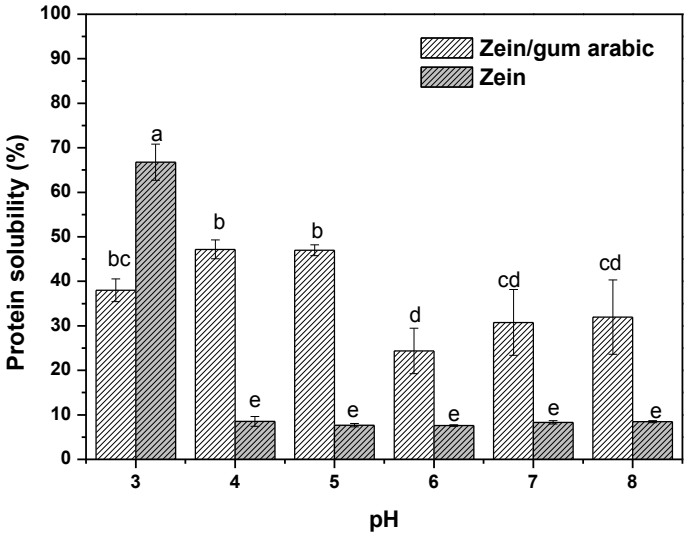

Figure 5-2. Zein solubility in dispersions with and without gum arabic at pH 3.0-8.0 and 21 °C. The percentages of soluble proteins were based on the residual protein content in the supernatant after centrifugation at 14,100*g* for 10 min with respect to the control at pH 11.0 that completely dissolved zein. Different lower-case letters above the bar represent significant differences in the mean ($P < 0.05$).

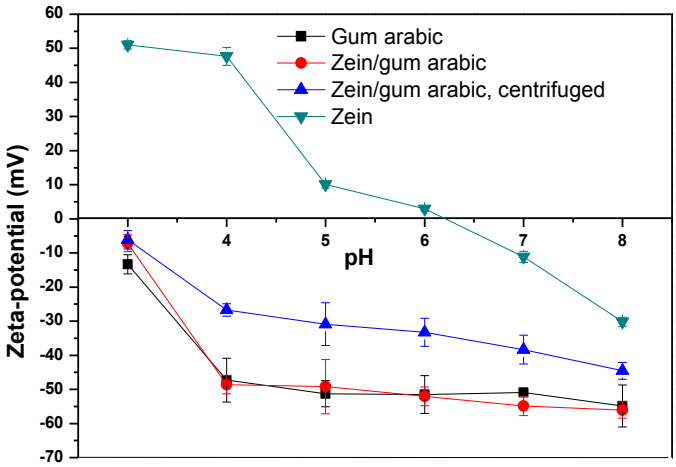

Figure 5-3. Zeta-potential of gum arabic, zein nanoparticles, and their mixtures at pH 3.0-8.0. The ein/gum arabic dispersions after centrifugation at 6700*g* for 20 min to remove free gum arabic were also measured for comparison.

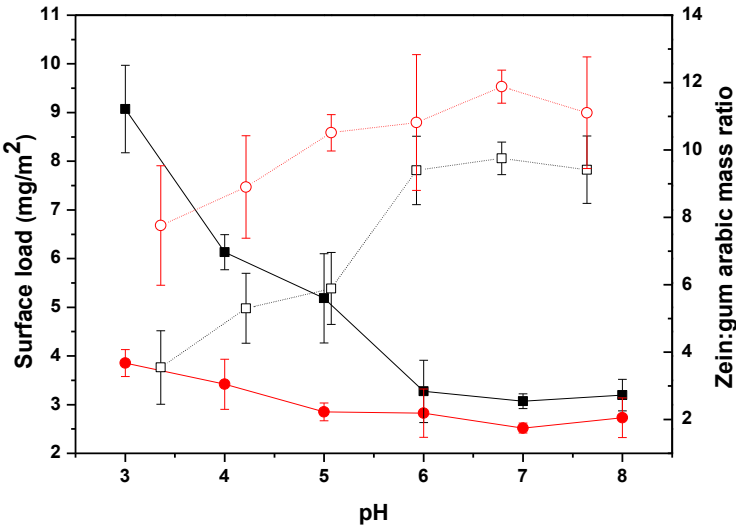

Figure 5-4. Surface load (solid symbols) of gum arabic on zein nanoparticles and mass ratio (open symbols) of zein:gum arabic in complexes at pH 3.0-8.0 and 0 (squares) or 50 (circles) mM NaCl.

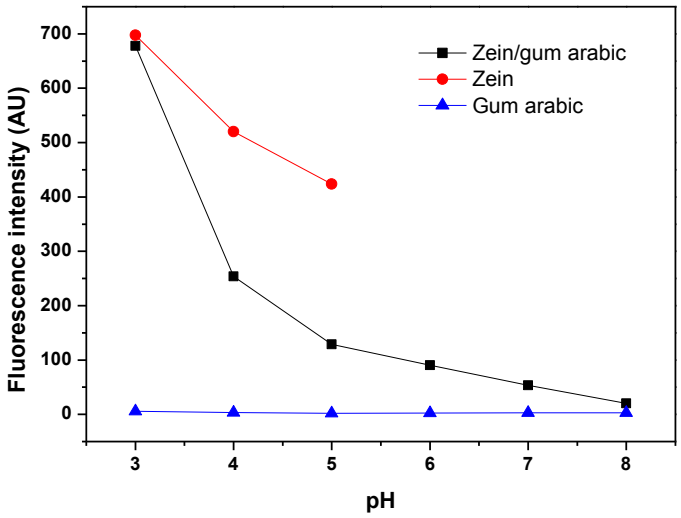

Figure 5-5. Maximum ANS fluorescence intensity of 0.5% w/v gum arabic solution and 0.1% w/v zein nanoparticle dispersions with and without 0.15% w/v gum arabic at pH 3.0-8.0. The zein-only samples at pH 6.0-8.0 were not measured because of the insolubility of zein and poor repeatability of replicates.

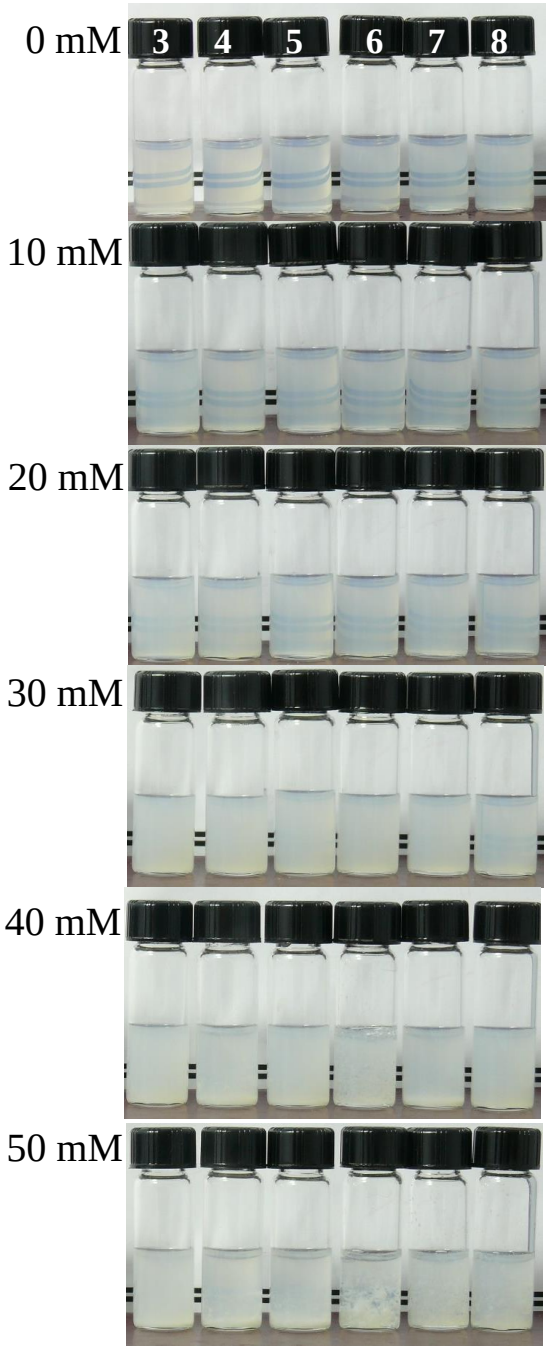

Figure 5-6. Effects of 0-50 mM NaCl on the stability of zein/gum arabic dispersions at pH 3.0-8.0 (labeled on the top panel).

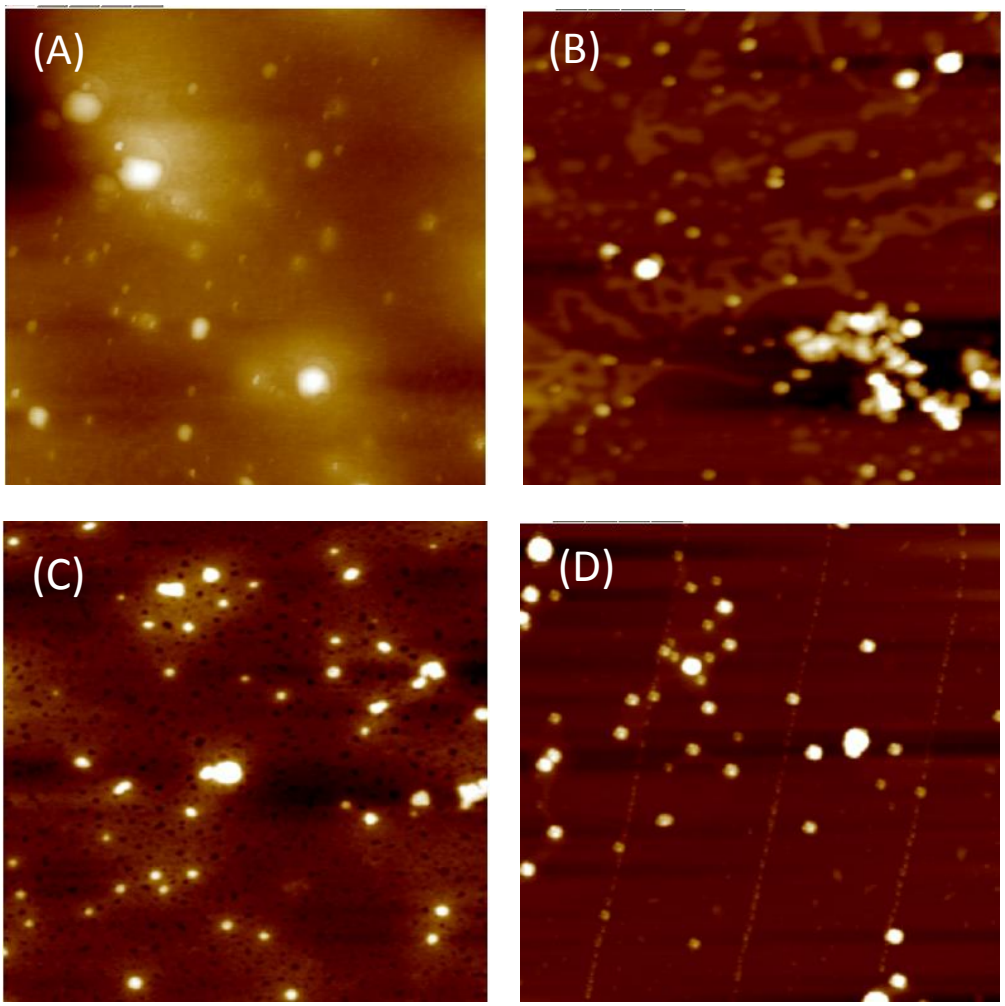

Figure 5-7. Representative AFM images (5 μm×5 μm) of gum arabic (A), zein (B), zein/gum arabic complex (C), and peppermint oil-loaded zein/gum arabic complex (D) at pH 5.0.

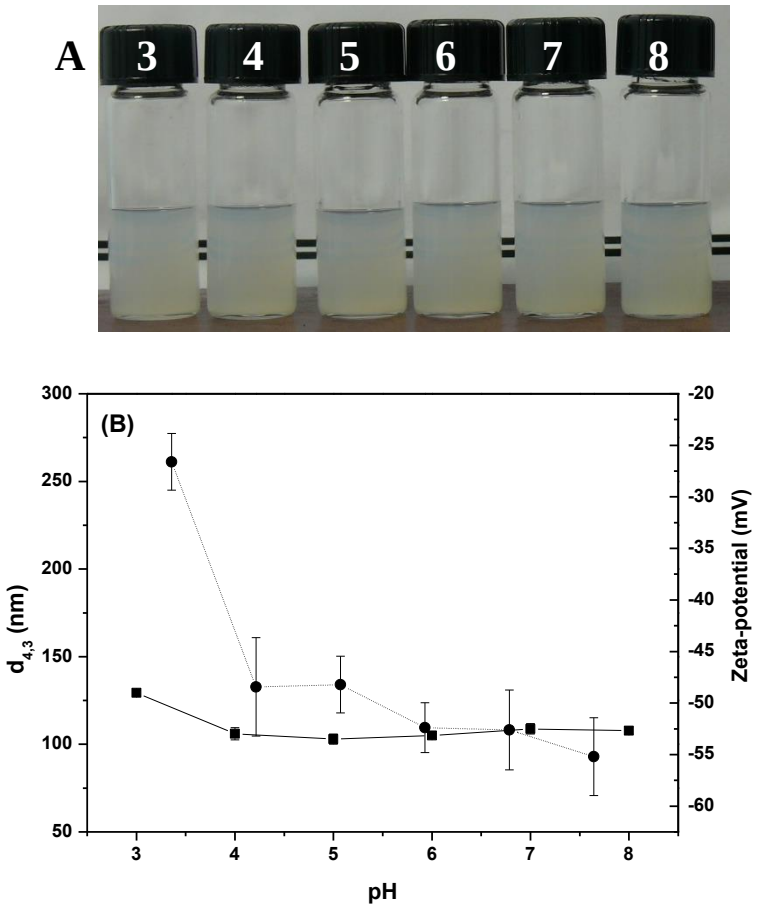

Figure 5-8. Appearance (A), particle size (squares in B), and zeta-potential (circles in B) of 0.1% w/v zein nanodispersions encapsulated with 0.05% w/v peppermint oil and stabilized by 0.15% w/v gum arabic at pH 3.0-8.0.

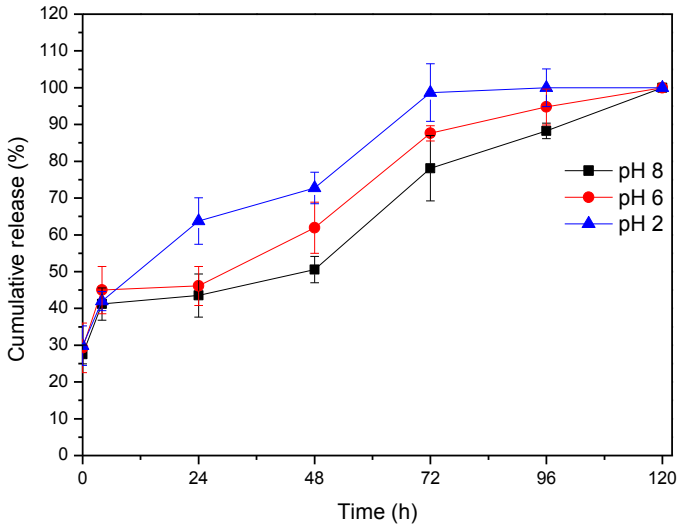

Figure 5-9. The cumulative release of peppermint oil from zein/GA complex at pH 2.0, 6.0, and 8.0 at 21 °C.

Chapter 6. Concluding remarks and future work

6.1 Conclusions

This dissertation demonstrated that the stability of zein nanoparticles in aqueous dispersions can be improved by forming complexes with surface active polysaccharides or proteins and the established complexes can be used to deliver essential oils.

When NaCas was co-dissolved with zein in hot 50% aqueous ethanol adjusted to pH 8.0 followed by anti-solvent precipitation to form nanoparticles, κ-casein, instead of α- and β-caseins, associated more with zein nanoparticles and produced smaller particles than other treatments. The spray-dried powder was easily re-dispersed in water with good stability at increased ionic strengths during storage. This approach also reduced the use of ethanol in producing zein nanoparticles.

When the above approach was used to co-encapsualte eugenol and thymol in zein/NaCas nanocomplexes, the spray-dried capsules also had good re-dispersibility in water and the dispersions were stable and had particles smaller than 200 nm. The encapsulation efficiency of EOCs with prior anti-solvent precipitation followed by spray-drying was better than the previous studies spray-drying the ethanol solution co-dissolving zein and thymol directly. The encapsulated EOCs showed controlled release in PBS (pH 7.0) in 24 h, and encapsulated eugenol showed a higher release rate than thymol. The co-encapsulated eugenol and thymol were present in milk whey at a concentration much lower than their overall concentrations and solubility but were effective in inhibiting the growth of *E. coli* O157:H7 ATCC 43895 and *L. monocytogenes* Scott A. The findings indicate the potential of applying the spray-dried capsules as preservatives in food.

When gum arabic was used to stabilize zein nanoparticles, dispersions were stable in a wide pH range, from 3.0-8.0. Zein interacted with gum arabic by both electrostatic and

hydrophobic interactions, with the former being more significant at a lower pH. Conditions of forming zein/gum arabic nanocomplexes were successfully used to encapsulate peppermint oil, with insignificant changes in particle size. Peppermint oil showed a gradual release from the freeze-dried powder tested in PBS at pH 2.0, 6.0, and 8.0, and the lower pH promoted the faster release. Additionally, less flammable and GRAS propylene glycol was used to substitute ethanol to dissolve zein, which is an alternative solvent for the industry to reduce the use of flammable ethanol.

Besides improving the stability of zein nanoparticles in aqueous dispersions, a comparison of using ethanol as a solvent for pre-dissolving EOCs with simple mixing EOCs with test media was conducted. There was a strong relationship between solubility of EOCs in aqueous phase with their antimicrobial activity. Ethanol affected the solubility of EOCs in aqueous phase and thereof overestimated the antimicrobial activity of free EOCs in TSB and food matrix. Direct mixing EOCs with food samples such as end-to-end shaking for 30 min may be more rational for preparing free EOCs to compare with the results of encapsulated EOCs. The findings are important to design delivery systems to deliver high concentration of EOCs in foods.

6.2 Future work

The results in the present dissertation showed that appropriate proteins and polysaccharides can be effective in stabilizing zein nanoparticles produced by the liquid-to-liquid dispersion method. However, some interesting points could be further investigated. Firstly, although zein-casein complexes had good re-dispersibility and stability, the encapsulated EOCs were released in 24 h that is relatively short for food applications. Methods are needed to lower the release rate of EOCs without impacting the re-dispersibility of the spray-dried complexes. One possible method is to add another layer between zein nanoparticles and sodium caseinate. For example, zein nanoparticles can be first stabilized by one polysaccharide that can be further attached with sodium caseinate. Secondly, co-encapsulation of EOCs has good potential to improve the antimicrobial activity so as to reduce sensory impacts of EOCs. One possible method is to use some highly acceptable flavor oils, e.g. citrus oil, to combine with these antimicrobial oils. Some flavor oils also have antimicrobial activities. If these flavor oils have synergistic effects with antimicrobial EOs, their combinations can be applied in foods to both reduce the amount of antimicrobial EOs and minimize their odor and taste.

VITA

Huaiqiong Chen was born on December 26, 1986 in Fujian, China. She entered Beijing Normal University in 2004 after she graduated from Xianyou No.1 Middle School. She majored in Biological Science, and was trained to be a high school teacher in biology. At the same time, she took classes and obtained her double major in International Economy and Trade. However, because of the curiosity in scientific research, she continued to work on a M.S. degree in medicine of Peking Union Medical College. In the three years, she focused on learning chemical analysis knowledge and techniques and finished her thesis on the quality evaluation of Chinese Traditional Medicine, especially agarwood. During the job hunting before graduation, she became interested in food science research. She decided to pursue a doctoral degree in Food Science. Fortunately, she became a graduate student in the Department of Food Science and Technology at the University of Tennessee, Knoxville, working under the guidance of Dr. Qixin Zhong. Her dissertation research was focused on stabilizing zein nanoparticles dispersions by forming zein-biopolymer complexes for delivery of essential oils in food matrices.