Microencapsulation of Oregano, Rosemary and Sage Essential Oils and Preliminary Study on Incorporating Essential Oils into Edible Films

by

Xiaoyu Luo

Department of Food Science and Agricultural Chemistry
Macdonald Campus, McGill University
Montreal, Quebec
Canada

A Thesis Submitted to the Graduate and Post-Doctoral Studies Office
in partial fulfillment of the requirements of the degree of
Master of Science

August 2017

Suggested Short Title:

Microencapsulation of Essential Oils

ABSTRACT

The increasing demand for functional food products has been a driving force in the food industry in recent decades. One of the examples is the application of essential oils, due to their antioxidant, antifungal and antibacterial activities. As most essential oil species are susceptible to degrade or evaporate readily, the development of certain techniques to overcome this drawback is the major focus of this study. The objectives of this study involved the evaluation of a process for microencapsulation of oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils in β-lactoglobulin (β-lg) by freeze-drying, a comparative study of the potential of inulin as an encapsulating material for oregano essential oil and a preliminary study on incorporating essential oils in edible films. The encapsulation efficiency obtained with β-lg (28.35 – 42.13%) was slightly higher than that obtained with inulin (11.22 – 33.86%) due to the emulsifying capacity of the protein molecules. All the β-lg capsules containing essential oils exhibited significant antioxidant activities in a β-carotene assay, with the percentage of inhibition ranging between 41.05% and 51.02%. The dried capsules containing oregano essential oil tended to be more stable with respect to water sorption, compared to those containing the other essential oils. However, in phosphate buffer, rosemary and sage essential oils exhibited a higher release rate from the protein microcapsules. The incorporation of essential oil in β-lg-based edible films apparently increased the level of brittleness, resulting in films that readily cracked. On the other hand, the edible films containing oregano essential oil tended to possess better moisture barrier properties than those prepared without addition of essential oil due to the presence of more hydrophobic compounds. Fourier transform infrared (FTIR) analysis on microcapsules, emulsions and edible films demonstrated that the presence of essential oils would significantly influence the structure of β-lg, evidenced by the band shifts or intensity changes of infrared spectra and the alterations of relative proportions of protein secondary structures, particularly α-helix and β-sheet.

RÉSUMÉ

La sensibilisation croissante aux produits alimentaires fonctionnels a été un défi pour l'industrie alimentaire au cours des dernières décennies. L'un des exemples est l'application d'huiles essentielles, en raison de leurs activités antioxydantes, antifongiques et antibactériennes visibles. Comme la plupart des espèces d'huile essentielle sont susceptibles de se dégrader ou de s'évaporer facilement, le développement de certaines techniques pour surmonter cet inconvénient est l'objet principal de cette étude. Les objectifs de cette étude ont porté sur l'évaluation du processus de microencapsulation des huiles essentielles d'origan (*Origanum vulgare* L.), de romarin (*Rosmarinus officinalis* L.) et de sauge (*Salvia officinalis* L.) par lyophilisation dans la β-lactoglobuline (β-lg), l'étude comparative du potentiel d'inuline pour l'encapsulation de l'huile essentielle d'origan et l'étude préliminaire sur l'incorporation d'huiles essentielles dans les films comestibles. L'efficacité d'encapsulation pour β-lg (28.35 – 42.13 %) était légèrement supérieure à celle de l'inuline (11.22 – 33.86 %) en raison de la capacité d'émulsification de la β-lactoglobuline. Pour les poudres contenant toutes les huiles essentielles, l'activité antioxydante a été jugée efficace dans un dosage avec du β-carotène, avec le pourcentage d'inhibition entre 41.05 % et 51.02 %. Les capsules séchées contenant de l'huile essentielle d'origan ont tendance à être plus stables en ce qui concerne la sorption de l'eau, par rapport aux traitements avec les autres huiles essentielles. Cependant, dans le tampon phosphate, les huiles essentielles de romarin et de sauge présentaient un taux de libération plus élevé à partir des microcapsules protéiques. L'incorporation d'huiles essentielles dans des films comestibles à base de β-lg a apparemment augmenté le degré de fragilité, ce qui a donné lieu à des films facilement fissurés. D'autre part, les films comestibles contenant de l'huile essentielle d'origan ont tendance à posséder une meilleure propriété de barrière à l'humidité en raison de la présence de composés plus hydrophobes. L'analyse par infrarouge à transformée de Fourier (FTIR) sur les microcapsules, les emulsions et les films comestibles a démontré que la présence d'huiles essentielles influencerait de manière significative la structure de β-lg, démontrée par les changements de bande ou les changements d'intensité des spectres infrarouges et les altérations des proportions relatives de structures secondaires des protéines, en particulier α-hélice et β-feuille.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my thesis supervisor, Dr. Ashraf A. Ismail, for offering me the opportunity to have this wonderful journey at McGill University. This study cannot be accomplished without his patience, support, guidance, and encouragement. Moreover, Dr. Ismail is a knowledgeable scholar, whose devotion towards science will continuously inspire me for pursuing my future studies in food science.

I would also like to say thank you to Dr. Jacqueline Sedman, for her professional editing skills and valuable thoughts for preparing my seminar presentations.

I am also grateful the Department of Food Science and Agricultural Chemistry, for the graduate excellence award I held during my first year of the study.

Special thanks to Jagpreet Kaur Gill and Jin Li, for their kindly training and help in conducting the freeze-dryer.

I wish to thank all my colleagues from McGill IR group, for their friendship and help. I am also thankful to other friends I made in Montreal. My experience was uniquely amazing because of everyone of you.

Last but not the least, I would like to take this opportunity to thank my beloved parents, for their financial support throughout both my undergraduate and graduate studies, and more importantly for their endless love and encouragements. I am also grateful to my grandparents and I know you always love me, no matter where you are. I would like to extend my thanks to others who have ever helped and inspired me to pursue my dreams: my relatives, my best friends overseas and those friends in Vancouver. I will not have such an unexceptionable experience without anyone of you. I love you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATR	Attenuated total reflectance
β-lg	β-Lactoglobulin
EO	Essential oil
FSD	Fourier self-deconvolution
FTIR	Fourier transform infrared
IR	Infrared
PBS	Phosphate buffer solution
SSD	Sum of squared deviations
UV	Ultraviolet

CHAPTER 1

GENERAL INTRODUCTION

In recent years, with remarkably increasing consumer demands for functional foods, essential oil production becomes significant in the food and pharmaceutical industries, particularly because of the excellent antioxidant and antimicrobial activities of essential oils. However, due to their high volatility and susceptibility to environmental factors, essential oils must be protected from degradation by means of encapsulation technology. Microencapsulation is the process which helps preserve bioactive compounds from the environment by entrapping the small molecules within certain matrices made from biopolymers. Moreover, the process of microencapsulation helps carry and deliver the active components for further usage. In general, emulsions are prepared and encapsulation is performed by different techniques such as spray-drying, freeze-drying, coacervation, and extrusion. Thus, developing efficient encapsulation techniques which can yield stable products and high level of essential oil recovery as well as retain significant functionality is one of the present challenges in the food processing industry. Also, the properties of the microcapsules can be influenced if there are significant interactions between the molecules making up the protective matrix and the bioactive compounds.

In this study, microencapsulation of three types of essential oils (oregano, rosemary and sage essential oils) by freeze-drying was investigated. The wall material was selected to be β-lactoglobulin due to its excellent emulsifying capacity. The encapsulation potential of inulin was also studied in comparison. The physical and chemical characteristics of the microcapsules produced from different essential oils were evaluated and compared as well as the interactions between the core and coating materials. The essential oils were also added during protein-based edible film production in order to evaluate their impact on film properties. By conducting this study, the compatibility of the wall material and the core material, the efficiency of the drying technique and the difference between different essential oils were assessed. Therefore, processes for microencapsulation of essential oils can be further improved in the future.

In general, the main objectives of this study are to evaluate the physical and chemical properties of particles containing microencapsulated oregano, rosemary and sage essential oils produced by freeze-drying; to investigate the interactions between β-lactoglobulin and essential oils; and to incorporate essential oils into protein-based edible films. Chapter 2 is a literature review on the main concepts of this study on microencapsulation in food applications. In Chapter 3, the microencapsulation of essential oils in β-lactoglobulin by freeze-drying and the impact of essential oils on protein conformation are investigated. Chapter 4 is a short chapter that addresses the effect of using inulin as the coating material for encapsulation of oregano essential oil, as compared to protein in the previous chapter. Chapter 5 describes a preliminary study on incorporating essential oils into protein-based edible films. In the final chapter, the conclusions drawn based on the results obtained in Chapters 3, 4 and 5 are summarized and recommendations for future work are also elaborated.

CHAPTER 2

LITERATURE REVIEW

2.1 Essential Oils

2.1.1 General Introduction

Essential oils are defined by previous researchers as the concentrated hydrophobic liquids extracted from aromatic herbs and they contain a collection of volatile compounds which give the aroma profile (Valgimigli, 2012). Essential oils were initially used by Egyptians before 3000 BC in ritual ceremonies. They were also used as cleaning agents due to lack of sanitizers. Greeks were among the earliest individuals using essential oils in food preparation. Specifically, essential oils were used by Greeks in the production of wines, aromatic vinegars and breath-refreshing gums (Valgimigli, 2012). In addition, the essential oils, involving rosemary, lemon, or thyme oils, were used by the French for perfume preparation.

In the contemporary world, the species of essential oils have been extensively discovered and their functionalities have been largely enhanced. Currently there are 3000 known essential oils and 300 of them are being used commercially as food additives, sanitizers and natural remedies, in the fields of agriculture and medicine (Valgimigli, 2012). Based on the statistical data on the consumption of essential oils in 2007, the flavor industry is the largest consumer in the world (Baser & Buchbauer, 2010).

The functionality of essential oils largely depends on their variations in chemical composition. Normally there are 20-60 chemicals present in each type of essential oil and two or three of them are considered as predominant compounds due to large abundance (Valgimigli, 2012). The most common compounds are phenolic molecules and terpenoids. Due to the presence of these active compounds, essential oils are considered to possess biological functions on plants and human systems. Since terpenoids and phenolic compounds are highly hazardous for most fungi and insects, their presence in herbs is effective in protecting the plants from pathogens and predators (Valgimigli, 2012). The biological impacts in the human body are

diverse, since essential oils have been reported to possess antimicrobial, antioxidant, anti-inflammatory, anticancer, analgesic and sedative activities (Valgimigli, 2012).

Essential oils can be derived or extracted from raw materials by various techniques. The most conventional extraction methods are steam or water distillation (Baser & Buchbauer, 2010). Other traditional methods involve solvent extraction methods and cold-pressing techniques (Valgimigli, 2012). The modern extraction techniques can be generally divided into three categories: modified distillation methods, modified solvent extraction methods and headspace methods (Valgimigli, 2012).

2.1.2 Oregano (*Origanum vulgare* L.) Essential Oil

Oregano has been used as flavoring and seasoning agents in fish, meat and sauces since ancient times in Italian, Greek and Mexican dishes (Sahin *et al.*, 2004). Among the large number of species that have been designated as oregano, *Origanum vulgare* has been found to be one of the most common species. The essential oil extracted from oregano has been extensively studied and found to be among the most effective antibacterial and antioxidant agents.

2.1.2.1 Chemical Composition

It is important to notice the functionality of oregano essential oil is highly dependent on its composition, which varies based on different factors such as temperature, moisture content, soil quality and daylight length (Ceylan *et al.*, 2003; Farooqi *et al*., 1999; Russo *et al.*, 1998; Viuda-Martos *et al.*, 2010). Based on previous studies on oregano (*Origanum vulgare* L.) essential oil composition, the major components are carvacrol, thymol, *p*-cymene and α-pinene (Govaris *et al.*, 2010; Hussain *et al*., 2011; Pozzatti *et al*., 2010; Rosato *et al*., 2009). In particular, the two phenolic compounds carvacrol and thymol usually constitute 70-80% of oregano essential oil and show highly specific activities compared to other components, such as antimicrobial, antioxidant and antiviral activities as well as aroma and flavor properties (Dorman & Deans, 2000; Lee *et al.*, 2008; Sokmen *et al*., 2004). The chemical structures of carvacrol and thymol are shown in Figure 2.1.

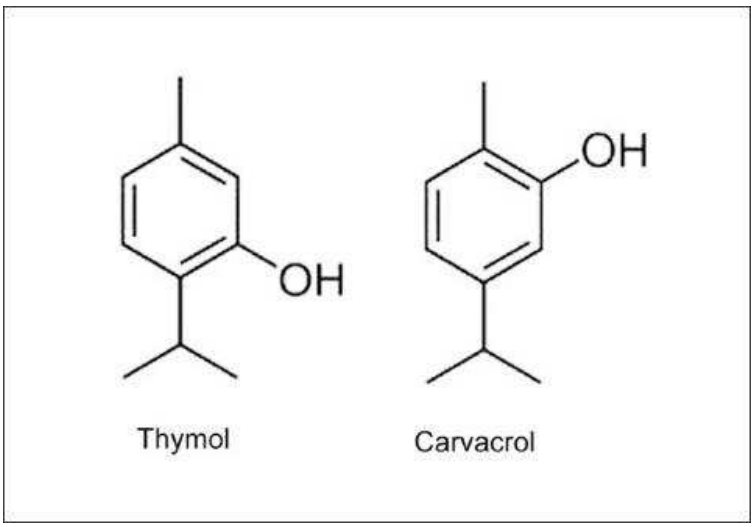

Figure 2.1. Chemical structures of carvacrol and thymol.

2.1.2.2 Functions and Applications in Food Science

Among the several functions exhibited by oregano essential oil, the most significant ones in terms of food industry applications are considered to be antimicrobial, antioxidant and sensorial effects.

The antioxidant activities of oregano essential oil and its major components have been confirmed to be effective against lipid oxidation in fatty foods (Lagouri *et al.*, 2010). A few studies have shown that the direct addition of oregano (*Origanum vulgare* L.) essential oil in food commodities such as extra virgin olive oil, peanuts, tuna salad and sea bream can significantly increase their oxidative stability (Asensio *et al*., 2012; Goulas & Kontominas, 2007; Olmedo *et al.*, 2009; Sorensen *et al.*, 2010). Another study applying milk protein-based edible films containing oregano essential oil (1% w/v) on beef muscle slices showed significant inhibition effect on lipid oxidation and bacterial growth (Oussalah *et al.,* 2004). Research by Kulisic *et al.* (2007) revealed significant protective effects of oregano essential oil on copper-induced human low-density lipoprotein oxidation.

The application of using oregano essential oil as an antimicrobial food additive is being improved to tackle food safety issues. The most common technique is to incorporate oregano oil into biodegradable edible films to extend the shelf life of food systems. A study by Du *et al.* (2009) showed that tomato-based films containing oregano essential oil were essentially

effective against the growth of *Salmonella enterica* and *Listeria monocytogenes*. Other recent studies indicated that the addition of oregano essential oil into whey-protein based edible films was effective at inhibiting the bacterial growth of *Listeria innocua*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Pseudomonas fragi* (Fernandez-Pan *et al.*, 2012; Royo *et al.*, 2010). Moreover, Oussalah *et al.* (2004) applied oregano films onto meat surfaces containing 10^3 CFU/cm^2 *E. coli* O157:H7 and 1.12 log reduction of the bacterial population was observed.

Another application is using oregano essential oil and its main components as antimicrobials to treat fresh fruits and vegetables. This application on apples showed significant reduction in microbial growth of *Botrytis cinerea* and *Penicillium expansum* (Lopez-Reyes *et al.*, 2010). Treatments of lemons with carvacrol and thymol (10-500 μL/L of carvacrol, thymol or 1:1 mixture) by Perez-Alfonso *et al.* (2012) resulted in a reduction in fungal degradation induced by *Penicillium digitatum* and *P. italisum*. Furthermore, De Azeredo *et al.* (2011) found there were significant inhibition effects on bacterial microflora, by using a combination of oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis* L.) essential oils (0.003-80 μL/mL) on vegetable produce.

2.1.3 Rosemary (*Rosmarinus officinalis* L.) Essential Oil

Originally grown in Mediterranean countries, rosemary (*Rosmarinus officinalis* L.) is a long-lasting evergreen aromatic herb with needle-shaped dark green leaves (Turasan *et al.*, 2015). Nowadays this plant is grown all over the world and its leaves and flowers yield highly aromatic essential oils. In history, rosemary essential oil was initially employed for food preservation and medical antiseptic purposes before the invention of refrigeration. In current society, rosemary oil is extensively utilized to treat nervous system and blood circulation diseases. Furthermore, it is used as disinfectant due to its antipathogenic properties. In addition, due to its pleasant flavor profile, it can be used in the cosmetic industry as well as for daily culinary purposes (Turasan *et al.*, 2015).

2.1.3.1 Chemical Composition

The chemical composition of rosemary essential oil is diverse. Several previous studies have shown that the most abundant compounds in rosemary essential oil are 1,8-cineol, α-pinene, camphor, camphene, borneol, β-pinene, linalool, verbenone and myrcene (Bousbia *et al.*, 2009;

Jalali-Heravi *et al.*, 2011; Orhan *et al*., 2008). The chemical structures of these major components are shown in Figure 2.2. The percentage of each chemical present in rosemary oil varies with the origin of the plants, due to both intrinsic factors such as genetic inheritance (Zaouali *et al.,* 2012) and extrinsic factors such as soil quality and climate conditions (Brewer, 2011).

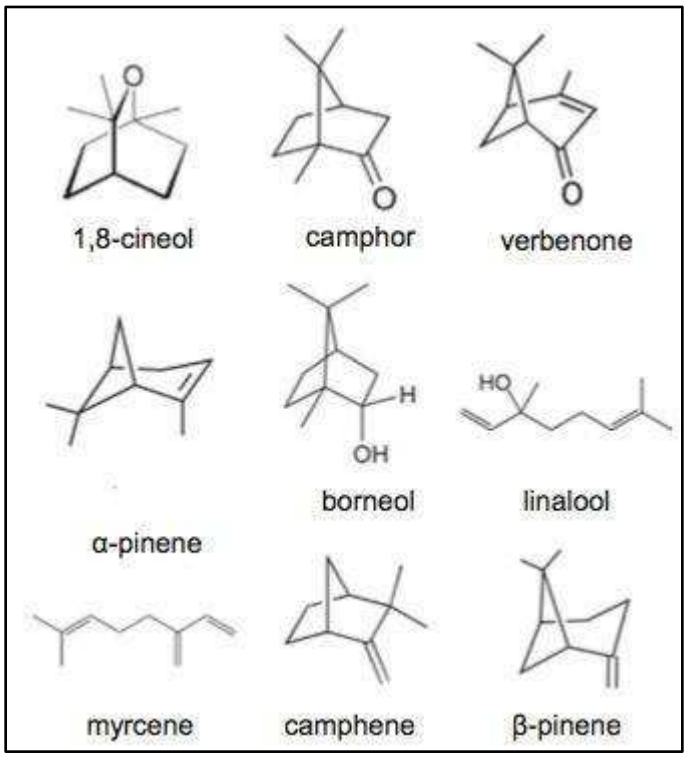

Figure 2.2. Chemical structures of the major components identified in rosemary essential oil.

2.1.3.2 Functions and Applications in Food Science

Particularly from the perspective of food science, the major functions of rosemary essential oil are as natural food preservatives and livestock growth promoters.

The potential of rosemary essential oil for use as a food preservative is attributed to its excellent antioxidant, antimicrobial and antifungal activities, which have been extensively studied by previous researchers. Wang *et al.* (2008) compared the antioxidant activity of rosemary essential oil with that of its major components (1,8-cineol, α-pinene and β-pinene) and found it to be a stronger antioxidant than any of the pure components. Some other researchers have reported very good inhibitory effects of rosemary essential oil against foodborne pathogens

such as *E. coli*, *Bacillus cereus*, and *Staphylococcus aureus* (Burt, 2004). Generally Gram-positive bacteria are more sensitive to essential oils since the Gram-negative bacteria have a more hydrophilic cell wall which prevents the penetration of hydrophobic compounds through the cell membrane (Burt, 2004). By comparing the antibacterial effect of rosemary essential oil with that of the pure main components (1,8-cineol and α-pinene), it has been shown that a synergistic effect may be present among the volatile compounds (Jiang *et al.*, 2011). Furthermore, the antifungal activity of rosemary essential oil from Greece against five phytopathogenic fungal species (*Sclerotinia sclerotiorum, Phytophthora nicotianae, Sclerotium cepivorum, Fusarium oxysporum* f. sp. *dianthi* and *Fusarium proliferatum*) has been evaluated and *Ph. nicotianae* has been found to be the most sensitive (Pitarokili *et al.,* 2008).

The use of antibiotics in livestock production has been a public concern due to the potential risks from antibiotic-resistant bacteria. Because of the excellent antimicrobial activity, essential oils are considered as alternatives to antibiotics so as to control microbial growth in the rumen (Benchaar *et al.*, 2008). Previous research demonstrated that rosemary essential oil can modify rumen fermentation by interacting with bacterial cell membranes and suppressing bacterial growth (Calsamiglia *et al.*, 2007; Castillejos *et al.*, 2008). Yesilbag *et al.* (2011) tested the effects of dietary supplementation of rosemary essential oil on meat quality and found the broiler meat quality improved with addition of rosemary oil. Another study showed a supplementation of rosemary essential oil in the diet of quail resulted in higher live weight and weight gain, which suggested the potential of rosemary oil as a natural growth promoter for quail (Yesilbag *et al*., 2012).

2.1.4 Sage (*Salvia officinalis* L.) Essential Oil

Similar to oregano and rosemary, the origin of sage (*Salvia officinalis* L.) is also in the Mediterranean area. This plant is a bushy medicinal perennial herb with dark green oval leaves. Sage has been used for medicinal purposes by Greeks, Egyptians and Romans from ancient times and now is mainly being used in pharmacology and the food industry. Interest in the use of sage essential oil in medicinal applications is due to its potential as an antiperspirant, antibiotic, estrogenic, hypoglycemic, diuretic, carminative and tonic agent (Cenic-Milosevic *et al.*, 2013). To be specific, Badiee *et al*. (2012) reported significant medicinal effects of sage (*S. officinalis*) essential oil on respiratory and digestive syndromes, heart and blood circulation, and endocrine

diseases. Another study demonstrated the contribution of sage to treat Alzheimer's disease due to its estrogenic and sedative effects (Mohammad, 2011). In addition, sage was reported to hasten wound healing (Anitha *et al.*, 2013).

2.1.4.1 Chemical Composition

Although the relative abundance of specific compounds in essential oils varies with different factors, the most abundant ones found in all sage essential oils are α-thujone, camphor, β-thujone and 1,8-cineol (Edris *et al.*, 2007; Langa *et al.*, 2009; Taarit *et al.*, 2009). The chemical structures are shown in Figure 2.3. In general, the types of chemicals present in sage essential oil can be divided into three categories: monoterpenoids, triterpenoids and flavonoids. All the chemicals in Figure 2.3 belong to the monoterpenoid category, which usually accounts for over 60% of sage essential oil. The presence of these components gives sage essential oil strong anti-inflammatory, antimicrobial, antioxidant, antitumor and antihypertensive effects.

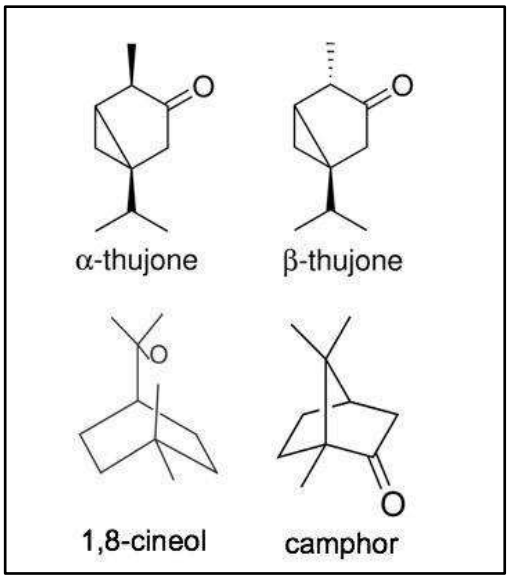

Figure 2.3. Chemical structures of the major components identified in sage essential oil.

2.1.4.2 Functions and Applications in Food Science

Sage has been used as a spice since ancient times in European cuisine. Sage and its essential oil not only contribute a certain flavor and odor profile in cooked meats but also help extend the shelf life of meat products. In modern society, the trend of applying sage and its essential oil becomes increasingly important in the food industry.

Similar to that of oregano and rosemary essential oil, sage oil also possesses significant antioxidant activity which aids in controlling autoxidation rates of food commodities. It is known that lipid oxidation is a key factor determining the shelf life of high-fat foods, especially meat products, due to the production of off-flavors and undesirable odors (Kenawi, 2012). Several research studies showed significant antioxidant effects of sage oils in food applications. Roman *et al.* (2009) demonstrated that the phenolic compounds from sage extracts possess radical-scavenging activity. Another study indicated that the minor components in sage oil such as carnosic acid and carnosol contribute to the antioxidant activity (Pizzale *et al.*, 2002). Studies by Salem and Ibrahim (2010) and Lopez-Bote *et al.* (1998) showed the potential of using sage oil as a natural antioxidant in sausages and broilers to prolong storage time. The oil oxidation rate in mayonnaise can also be attenuated by sage essential oil (Rasmy *et al.*, 2012).

The antimicrobial activity of sage and sage essential oil is also significant. The research by Basuny *et al.* (2011) revealed that addition of 200-1000 ppm sage essential oil to bakery products was effective in inhibiting microbial growth when tested against six species of microorganisms: *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis* and *Staphylococcus aureus*. Another study on the application of sage extract (0.3-0.5%) in beef minced with soy protein showed significant shelf life increase and attenuated bacterial growth (Ahmed & Ismail, 2010). Due to the strong antimicrobial effects, sage essential oil can be used as a feed supplement (Kahraman, 2009).

Additionally, sage possesses potential in controlling insects and pests in agricultural products. In a particular research, the addition of sage leaves for long-term storage of legume seeds significantly retained the physical and biological quality of seeds (Dal *et al.*, 2001). Another study recommended applying sage essential oil as an alternative means to preserve seeds during long-term storage (Larocque *et al*., 1999).

2.2 Microencapsulation

2.2.1 General Introduction

Approximately 60 years ago, encapsulation was initially introduced into biotechnology in order to more efficiently separate the production cells and the metabolites (Nedovic *et al*., 2011). In recent decades, the concept of microencapsulation has become popular in the pharmaceutical

and food industries, due to the potential of delivering optimal amount of specific bioactive compounds in newly designed products (Gouin, 2004). In most cases, the bioactive components are susceptible to environmental, industrial and physiological conditions and require microencapsulation for effective protection. In the food processing industry, the term "microencapsulation" can be defined as the process of incorporating sensitive small molecules into an inert shell material which protects the core components from environmental interferences (Ghosh, 2006). The core materials usually refer to the entrapped components that may exist in solid, liquid or gas state, with various functional properties. The common encapsulated components in food industry include flavor compounds, antimicrobials, antioxidants, minerals, vitamins and probiotics (McClements, 2012). The application of various encapsulation techniques can therefore raise the efficacy of the bioactive agents by increasing their solubility and stability and by controlling their release (Chen *et al*., 2006). Moreover, microencapsulation is a means to help mask undesirable aroma or taste from the bioactive ingredients (Abd El-Salam & El-Shibiny, 2012). In addition, encapsulation modifies the physical properties of the original materials for easier handling, processing and distribution (Desai & Park, 2005).

In general, there are four categories of microcapsule morphology (Figure 2.4). The simplest one consists of only a single type of core material and a single type of coating material, forming a single core-shell structure. The second shape is a multi-walled structure which denotes that a single core component is covered by multiple layers of coating materials. The form in which various core materials are encapsulated in a shell is considered to be a polynuclear structure. In addition, matrix encapsulation suggests the core material is homogeneously present in the coating material (Ghosh, 2006).

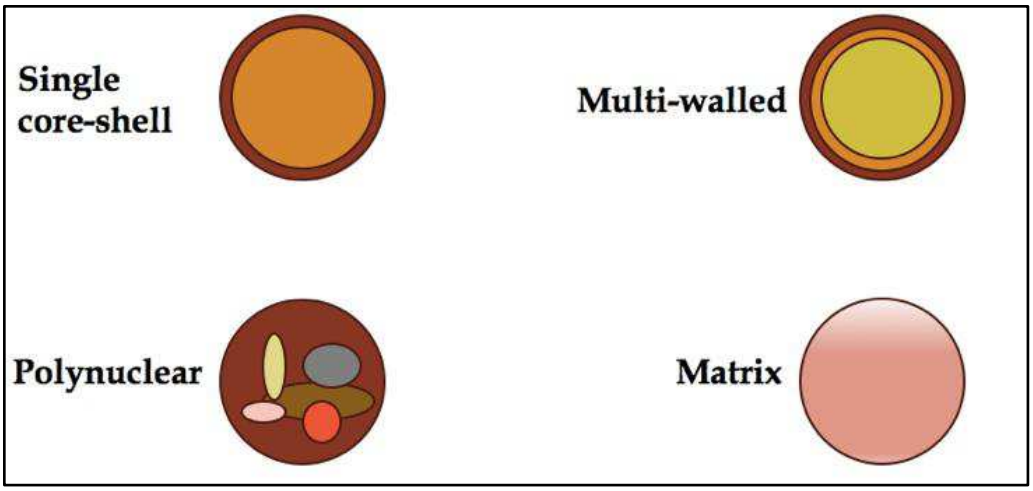

Figure 2.4. Categories of microencapsulation morphology.

2.2.2 Coating Materials

The most common coating materials used in the food industry are biodegradable polymers such as carbohydrates, proteins, gums, lipids and so on (Turasan *et al.*, 2015). Different coating materials possess different emulsifying and film-forming properties, and thus the choice of coating materials is an essential step for the success of the microencapsulation process. In particular, coating materials must protect the core components from physical loss and chemical degradation during processing and storage (Baranauskiene *et al.*, 2006). For the coating materials exhibiting higher encapsulation efficiencies, they have to show good emulsifying capacity, be chemically inert with the core materials, exhibit low viscosity in high concentrations and have good solubility for the release of the core materials (Ghosh, 2006). Table 2.1 shows the commonly employed coating materials for volatile food components and the corresponding methods being used.

Table 2.1. Commonly used coating materials for encapsulation of volatile food components.

Category	Coating Materials	Commonly Used Methods
Carbohydrate	Starch, maltodextrins, chitosan, corn syrup solids, dextran, modified starch, cyclodextrins	Spray drying, freeze drying, extrusion, coacervation, inclusion, complexation,
Cellulose	Carboxymethylcellulose, methyl cellulose, ethyl cellulose	Coacervation, spray drying, edible films
Gum	Gum acacia, agar, sodium alginate, carrageenan	Spray drying, syringe methods
Lipids	Wax, paraffin, beeswax, diacylglycerols, oils, fats	Emulsions, liposomes, film formation
Proteins	Gluten, casein, whey protein, gelatin, albumins, peptides	Emulsion, spray drying, edible films

2.2.3 Encapsulation Techniques

There are numerous techniques used for microencapsulation and no single one can be applied to all materials. The selection of a specific encapsulation method is largely dependent on the nature of the core and wall materials, as well as the designated capsule properties. Moreover, the encapsulation efficiency and the cost are other important considerations (Augustin & Sanguansri, 2008). It is also crucial to select the proper encapsulation technique that can protect the bioactives throughout the industrial process and during storage (Gibbs *et al*., 1999). The common techniques used for bioactive components include emulsification, spray-drying, freeze-drying, coacervation, extrusion, supercritical fluid extraction, fluidized bed and development of biodegradable edible films (Augustin & Sanguansri, 2008; Gouin, 2004; Shahidi & Han, 1993).

Among the various encapsulation techniques, emulsification is widely applied in the food and pharmaceutical industries. Generally, emulsification can be applied for encapsulation of lipophilic bioactive agents in aqueous solutions, which can be used directly in liquid form or in powder form by a drying process (spray- or freeze-drying). An emulsion is composed of two or

more immiscible liquids, usually water and oil. The type of the emulsion depends on the relative proportions of water and oil present in the system. The four common water and oil emulsion systems are shown in Figure 2.5, where O and W represent oil and water. If the system consists of oil droplets dispersed in water, it is called an oil in water (O/W) emulsion, and vice versa. In both cases, there is usually involved the presence of an emulsifier, namely, the surfactant that assists in stabilizing the emulsion system (Holmberg *et al.*, 2002). There are also double emulsions (W/O/W and O/W/O) which are usually employed for controlled delivery and release of drugs (Holmberg *et al.*, 2002). In food systems, the diameter of the emulsion droplets varies from 0.1 to 100 micrometers (Fang & Bhandari, 2010). Normally, the emulsions are prepared by using a mechanical device known as a homogenizer, which is discussed in Section 2.2.3.1. The prepared emulsions can be subsequently dried for further usage.

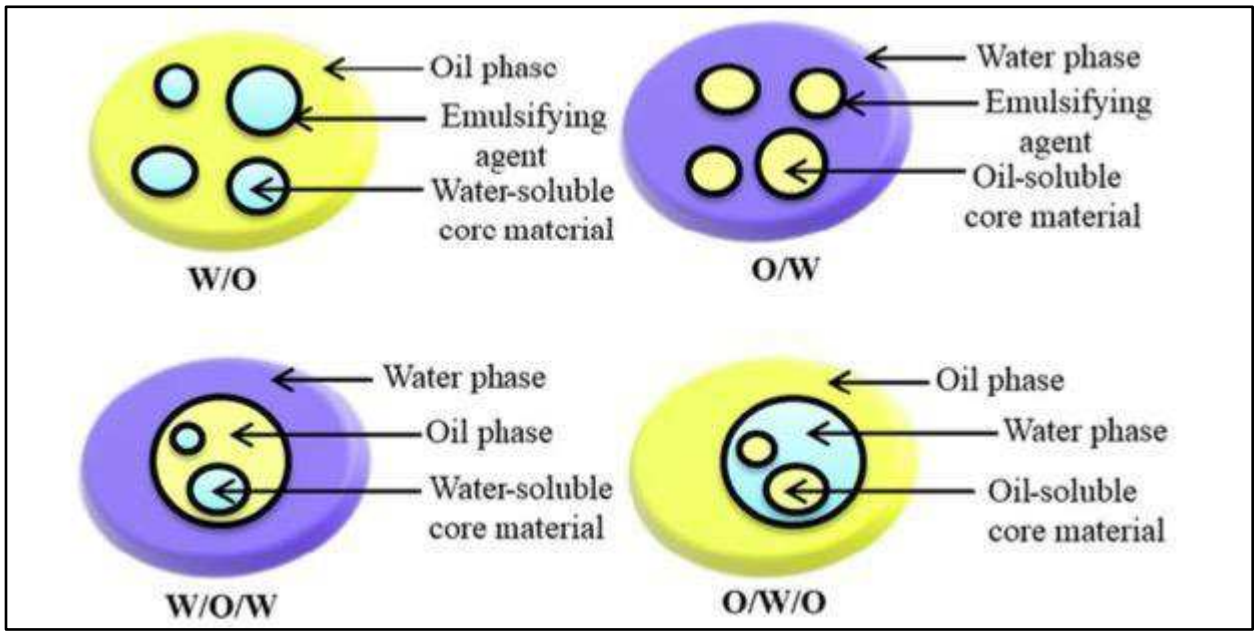

Figure 2.5. Illustration of the four emulsion systems (Bakry *et al.*, 2016).

2.2.3.1 High-Shear Homogenization

As mentioned above, the mechanical process that prepares the emulsion system is referred to as homogenization. This is an important step because it significantly influences the overall encapsulation efficiency, structural properties, product stability and rheology

(McClements, 2012). Modern homogenization techniques used in the food industry mainly involve high-shear homogenization, ultrasonic homogenization and microfluidization (McClements. 2005). These techniques are commonly simple to operate and cost effective, which leads to extensive usage in food sectors (McClements *et al.*, 2009).

Among the homogenization techniques, high-shear homogenization is the most common and conventional one employed in the food industry. This technique applies high-speed mixers directly to the oil and water emulsions and produces small-size droplets in a short period of time (McClements, 2005). It is considered to be the most effective homogenization method because it consumes less energy and is more effective in reducing emulsion droplet sizes (Bi *et al.*, 2014; Salager *et al.*, 2004). Figure 2.6 shows the mechanism of a typical high-shear homogenizer. During the process, the emulsion mixture is added into the container followed by turning on the high-speed mixer. The mixer usually generates a speed as high as 3600 revolutions per minute and results in longitudinal, radial and rotational gradients. As a result, the oil/water interfaces are destroyed and larger droplets are broken into smaller ones (Turasan *et al.*, 2015). After homogenization, the emulsion droplet size can be as small as 2 μm (McClements, 2005).

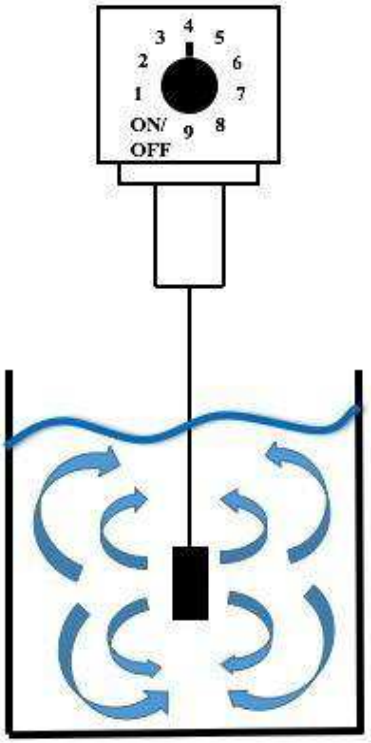

Figure 2.6. Simplified mechanism of a typical high-shear homogenizer.

2.2.3.2 Drying Techniques

As mentioned above, drying techniques are important methods for microencapsulation processes since they produce stable powder form of capsules which are easy for further use. Based on current status, the most commonly employed drying methods for essential oil encapsulation are spray-drying and freeze-drying (Akhtar & Dickinson, 2007; Fernandes *et al.*, 2013; Huynh *et al*., 2008; Jafari *et al.*, 2008).

2.2.3.2.1 Spray-Drying

Spray-drying is considered to be one of the oldest techniques for encapsulation (Fang & Bhandari, 2012). The earliest application of spray-drying was traced back to the 1800s in the production of dairy products (Filkova *et al.*, 2007) and the first spray-drying patent was established in 1872 (Bhandari *et al.*, 2008). In recent decades, many studies have been conducted to successfully encapsulate oils by spray-drying in the food (Domian *et al.*, 2014; Huang *et al.*, 2014; Liu *et al*., 2014), pharmaceutical (Liu & Yang, 2011) and pesticide industries (Lopez *et al*., 2014). Specific for essential oils, previous studies have successfully accomplished the encapsulation of basil, lime and oregano essential oils by spray-drying (Botrel *et al*., 2012; Bringas-Lantigua *et al.*, 2012; Garcia *et al.*, 2012).

In general, spray-drying involves the atomization of the emulsion in a drying chamber at high temperature, followed by fast water evaporation. The microencapsulation by spray-drying is usually composed of four steps: (i) emulsion preparation, (ii) homogenization, (iii) atomization, and (iv) dehydration. The first two stages have been discussed in previous paragraphs. Atomization is regarded as the most important step because it determines the drying time, the final droplet quality, and the drying efficiency (Filkova *et al.*, 2007). The viscosity of the emulsion is crucial since it can significantly interfere with the atomization process, thereby influencing the drying rate (Rosenberg *et al.*, 1990). The temperature conditions, involving the feed temperature, air inlet temperature and air outlet temperature, are essential in spray-drying in order to obtain high encapsulation efficiency (Liu *et al.*, 2004). Thus, such temperatures should be optimally controlled during the drying process.

Among the various microencapsulation techniques, spray-drying is an inexpensive method with the advantage of producing reproducible simple microcapsules that are easy to be

scaled up, indicating the good compatibility of usage in the industrial sector (Pu *et al.*, 2011; Schafroth *et al.*, 2012). Due to its economical effect, it is a good substitute for conventional drying processes. The cost is usually 30 to 50 times lower than freeze-drying. However, there are studies demonstrating the energy waste in the form of heat during the process (Gharsallaoui *et al.*, 2007). Another limitation is that spray-drying is not suitable for thermally sensitive compounds due to the high temperature conditions of atomization (Fang & Bhandari, 2012). Furthermore, spray-drying cannot be applied to high-sugar core materials since their high viscosity causes stickiness and reduces the drying efficiency (Bhandari *et al.*, 1997). Also, the selection of wall materials for spray-drying is relatively limited since aqueous solutions are usually prepared (Gouin, 2004).

2.2.3.2.2 Freeze-Drying

Freeze-drying, also referred as lyophilization or cryodesiccation, is another drying technique mostly employed for heat-sensitive materials such as oils and aromatic lipophilic molecules (Bakry *et al.*, 2016). The origin of freeze-drying for food preservation dates back to ancient China s in cold wintertime. Its use in scientific studies became common only in the last 80 years (Hua *et al.*, 2010). Compared to spray-drying, freeze-drying is more capable of preserving heat-sensitive and volatile compounds (Krokida & Philippopoulos, 2006). A simplified schematic diagram of a freeze-dryer is shown in Figure 2.7.

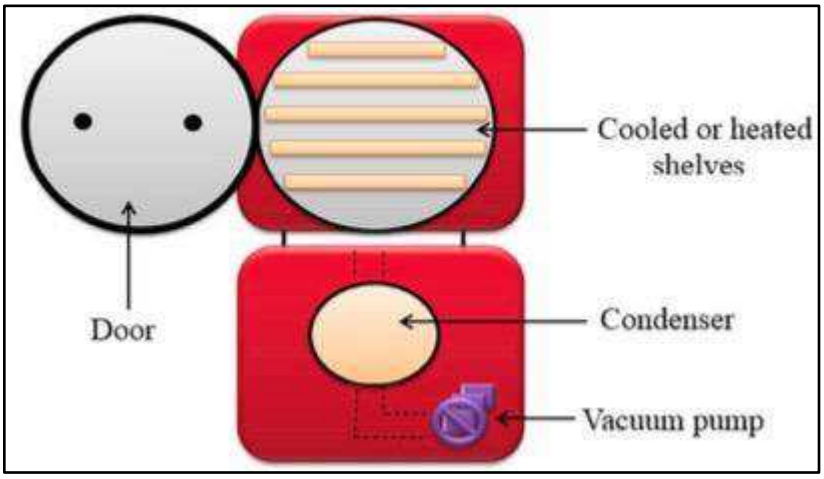

Figure 2.7. Schematic diagram of a freeze-dryer (Bakry *et al.*, 2016).

As shown in Figure 2.7, the cooling chamber is connected to a vacuum that provides low-pressure conditions. The main principle of freeze-drying is to remove water by subliming the solvent from the solid state directly to the gaseous state and by desorption of the solvent under low pressure (Liapis & Bruttini, 2007). As mentioned above, an emulsification step is usually applied before the encapsulation process by freeze-drying. Generally, the freeze-drying step involves three steps: freezing, primary drying and secondary drying (Hua *et al.*, 2010). During the freezing step (-90 ºC to -40 ºC), the emulsion becomes completely solid and phase separation begins. This step is important for microencapsulation since the cooling rate is associated with the quality and drying efficiency of the final products (Fang & Bhandari, 2012). The steps of primary and secondary drying remove water by sublimation and desorption, respectively. The primary drying step removes most of the solvent within the emulsion system in the form of free solvent, while the secondary drying step mainly removes the bound solvent (Liapis & Bruttini, 2007).

A few studies have demonstrated the significance of using freeze-drying to encapsulate oils and hydrophobic bioactive compounds such as flaxseed oils, polyphenols, limonene and extra virgin olive oil (Calvo *et al.*, 2012; Kaushik & Roos, 2007; Quispe-Condori *et al.*, 2011; Sanchez *et al.*, 2011; Wilkowska *et al.*, 2016). Apart from its capacity to retain volatile compounds, freeze-drying is a simple technique and easy to operate. This drying method gives high drying efficiency and retains the color, texture and flavor of the food matrices (Hua *et al.*, 2010). Furthermore, studies suggested that freeze-dried products exhibited higher resistance to lipid oxidation compared to products dried by other techniques (Velasco *et al.*, 2003). The disadvantages of freeze-drying include its high energy and cost input and long processing time (Desobry *et al.*, 1997). It should be noted that the high porosity of freeze-dried products results in high efficacy in drug release (Sinha *et al.*, 2007). On the other hand, the porous structure is considered to be a defect since the dried capsules are more readily subject to rehydration (Fang & Bhandari, 2012; Hua *et al.*, 2010). Currently freeze-drying is not as prevalently employed as spray-drying in food research and encapsulation technology mainly due to its high financial input. Thus, research on microencapsulation of bioactive compounds by freeze-drying is valuable.

2.2.3.3 Edible Films for Bioactive Compounds

The development of biodegradable edible films has been extensively studied by numerous scholars in the past few decades. By definition in terms of food applications, an edible film is referred as a thin layer of edible materials on a food matrix that serves to prevent environmental damage both physically and chemically, via the inhibition of migration of certain compounds such as water, oxygen, carbon dioxide, lipids and other solutes (Guilbert, 1986; Kester & Fennema, 1986). The films are usually produced from food-derived ingredients with various functions. The main advantages of using edible films in food applications are providing physical and chemical protection as well as extending the shelf life by controlling microbial growth and ingredient exchange with the environment (Han, 2003). Moreover, the edible films may offer nutritional values and convenience for specific requirements.

Edible films are usually grouped with the corresponding biopolymer materials, mainly involving polysaccharides, proteins and lipids (Falguera *et al.*, 2011). The most commonly used materials in edible films for foods are listed in Table 2.2. According to the purposes of the encapsulation and the compatibility with the core materials, the proper biopolymers are selected for edible film production, either alone or in combination. For most polysaccharides, the presence of large numbers of hydroxyl groups aids in stabilizing the edible films by creating intermolecular hydrophilic interactions within the matrix (Janjarasskul & Krochta, 2010). However, their hydrophilic nature will result in films with poor water and vapor barrier properties (Quiros-Sauceda *et al.*, 2014). Thus, polysaccharides are usually used with other macromolecules or stabilizers.

In comparison, proteins exhibit excellent emulsifying capacity that reveals great potential as the materials for edible film production. Due to the amphipathic property of proteins, they may participate in interactions with many other molecules in the film solution such as peptide and disulfide linkages, hydrogen bonding and hydrophobic interactions (Guerrero *et al.*, 2010; Hermandez-Izquierdo & Krochta, 2008). These interactions help stabilize the film structure and improve the film properties (Quiros-Sauceda *et al.*, 2014). It is usually required to denature the proteins by heat or acid/base treatments in order to obtain extended structures for film formation (Bourtoom, 2008). As it is known that the materials capable of interacting by hydrogen and ionic bonding are prone to hydration, protein-based films are good oxygen barriers when the relative humidity is low (Salame, 1986). The final quality of the films depends on both intrinsic factors

such as the amino acid composition, pI and hydrophobicity of the proteins as well as extrinsic factors such as processing temperature, pH conditions and ionic strength. (Damodaran, 1996).

The application of lipids in edible films is attributed to their hydrophobic nature that provides excellent barrier properties against moisture and gas ingress. However, this high degree of hydrophobicity will result in films with low elasticity (Bourtoom, 2008). Thus, lipids are usually used in combination with polysaccharides to obtain sufficient mechanical strength. The most effective ones are paraffin and beeswax (Debeaufort & Voilley, 2009).

Table 2.2. Common biopolymer sources used in edible films (Suput *et al.*, 2015)

Polysaccharides	**Proteins**	**Lipids**
Starch & modified starch	Soy proteins	Oils
Chitin & chitosan	Pea proteins	Free fatty acids
Pectins	Whey protein & casein	Beeswax
Cellulose & modified cellulose	Wheat gluten	Carnauba wax
Alginate	Collagen & gelatins	Paraffin
Carrageenan	Egg white protein	Shellac resin
Gums	Corn zein	Terpene resin
Pullulan	Peanut protein	Acetoglycerides

The quality and stability of edible films depend on the chemical characteristics of the selected materials as well as the interactions between molecules, such as covalent, ionic, and hydrophobic interactions and hydrogen bonding (Janjarasskul & Krochta, 2010). The cohesive strength of the edible films can be increased by various means. Usually, emulsifiers and plasticizers are added to stabilize the film system. The concept of emulsifies has been introduced in previous paragraphs in this review. Plasticizers are defined as the small molecules physically added to the film solution that stabilize the films by interacting with the polymeric network. Examples of commonly used plasticizers are glycerol, sorbitol, fatty acids and phospholipids

(Krochta & Nisperos-Carriedo, 1994). In addition, applying heat and irradiation can also induce the cohesion of the films by producing cross-linking structures (Pascall & Lin, 2013).

One of the most innovative applications of edible films is to encapsulate bioactive compounds and allow for controlled release under certain conditions to exhibit multiple functions (Pothakamury & Barbosa-Canovas, 1995). These compounds function as antioxidants, antimicrobials, probiotics and flavors etc. However, most bioactive compounds are unstable and prone to degradation and loss of functionality. Thus, the use of edible films effectively counteracts this defect (Ayala-Zavala *et al.*, 2008; Silva-Weiss *et al.*, 2013).

To be specific, the application of edible films in encapsulating bioactives exhibits several significant protective effects. Firstly, edible films can protect the bioactives from degradation under high-temperature conditions. For instance, cyclodextrin films containing vanillin, cinnamon leaf oil, garlic oil and caraway fruit oil showed great stability under high-temperature conditions such as 120-160 ^{o}C (Ayala-Zavala *et al.*, 2008; Kayaci & Uyar, 2012; Partanen *et al.*, 2002). Previous studies also revealed the capacity of edible films in protecting bioactives from UV light. Ferreira *et al.* (2007) reported that nanoparticles containing carbohydrate matrix and catechins are stable with UV light exposure. Another study indicated the effectiveness of a lipid-based film in protecting ethylhexyl methoxycinnamate (EMC) from UV light (Durand *et al.*, 2010). It is essential that some of the matrices can increase the solubility of certain bioactive compounds in food systems, especially lipophilic molecules. Studies have shown this effect by using carbohydrate-based films for encapsulating aromatic lipophilic compounds (Kolanowski *et al.*, 2004; Sereno *et al.*, 2009). Previous research also suggested that starch-based edible films efficiently prolong the flavor holding dates of panda leaf extract and peppermint (Baranauskien *et al*., 2007; Laohakunjit & Kerdchoechuen, 2007). Another important advantage of using edible films for bioactives is the controlled release, which is associated with the diffusion rates and the relative solubility of the targeted molecules. Nowadays, there are two commonly accepted mechanisms of diffusion in food matrices, free static diffusion (De Roos, 2000) and convective diffusion (Pothakamury & Barbosa-Vanovas, 1995). The former indicates that the molecules randomly move in each dimension within the matrix while the latter suggests the molecules are transported by other solutes. In aqueous solutions it was observed that the higher the relative humidity and the temperature, the higher the release rate of bioactive compounds such as D-

limonene and l-menthol (Del Toro-Sanchez *et al*., 2010; Fabra *et al.*, 2012; Soottitantawat *et al.*, 2004).

As mentioned previously, essential oils are a group of natural bioactive compounds possessing multiple significant functions in food and pharmaceutical chemistry. In recent years, many studies demonstrated the successful incorporation of essential oils into edible films prepared from different biopolymer sources. For example, chitosan films were successfully prepared to encapsulate cinnamon leaf, rosemary and thyme essential oils and exhibited higher antioxidant activity than the films without essential oil addition (Moradi *et al*., 2012; Perdones *et al.*, 2014; Ruiz-Navajas *et al.*, 2013; Xiao *et al.*, 2010; Xing *et al.*, 2011). Protein-based films containing essential oils were also prepared in several studies by employing various protein sources, such as whey protein, gelatin, triticale protein, sunflower protein and soy protein, and significant antimicrobial and antioxidant activities were shown due to the successful incorporation of oregano, clove, thyme, bergamot, lemongrass and pimento essential oils (Aguirre *et al*., 2013; Ahmad *et al.*, 2012; Emiroglu *et a;.*, 2013; Gimenez *et al.*, 2012; Oussalah *et al.*, 2004; Salgado *et al.*, 2013; Zinoviadou *et al.*, 2010). It has been confirmed that the antioxidant power of the edible films is proportional to the initial concentration of the essential oils (Jouki *et al.*, 2014; Moradi *et al.*, 2012; Tongnuanchan *et al.*, 2013). Apart from the protective effects of the films on the essential oils, on the other hand the essential oils can enhance the barrier properties of the films due to their high degree of hydrophobicity (Atares *et al*., 2010).

In conclusion, the potential of edible films in encapsulating bioactive compounds as an alternative to conventional packaging has been demonstrated by various studies. In previous studies, the incorporation of bioactives into edible films was confirmed to be a successful way to protect antimicrobials, antioxidants, probiotics and flavor compounds etc. However, the addition of bioactive compounds usually affects the overall appearance and quality of the films, and even the sensory profile of the foods. Thus, studies aimed at developing innovative edible films to overcome these problems are still ongoing in the food packaging industry.

2.3 β-Lactoglobulin

2.3.1 General Introduction

Bovine β-lactoglobulin (β-lg) is one of the major proteins in whey. It constitutes approximately 50% by weight of the whey proteins and 12% of total proteins in milk. Bovine β-lg is composed of 162 residues per monomer with a molecular weight of 18 kDa and an isoelectric point of 5.3 (Sawyer, 2013).

The tertiary structure of β-lg has been studied by previous scholars. Figure 2.8 shows the tertiary structure of β-lg monomer (Sawyer, 2013). Studies revealed that the basic three-dimensional structure of β-lg consisted of eight up-and-down antiparallel β-strands (shown in yellow in the figure), folding into a structure called a calyx. Later research found that there was a ninth β-strand connected to a three-turn α-helix (shown in red) and the β-sheets (Forrest *et al.*, 2005). The overall structure is stabilized by intramolecular disulfide bonds due to the presence of cysteine residues (Sawyer, 2013).

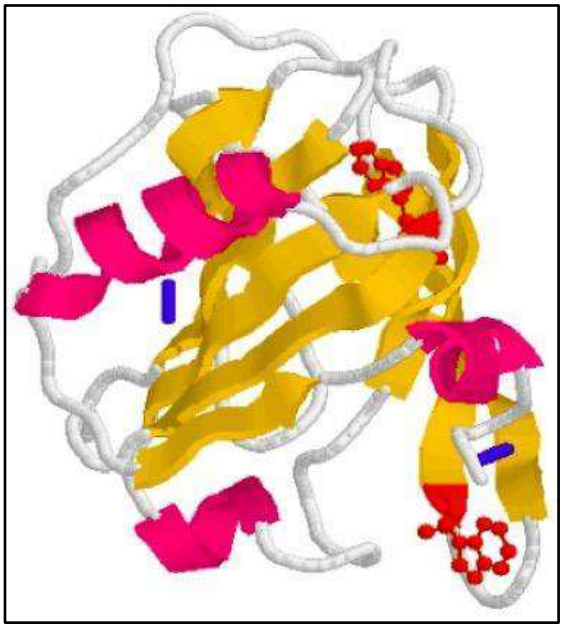

Figure 2.8. A general view of the tertiary structure of β-lg.

It is known that pH and ionic strength have significant impact on protein structure and β-lg is not an exception. First, the monomer-dimer equilibrium of β-lg is dependent on pH conditions. Generally, β-lg exists as dimers whereas it dissociates into monomers when the pH is either higher than 8.0 or lower than 3.0 (Forrest *et al.*, 2005). Between pH 3.5 and 5.2, it can form octamers, through the association of four dimers (Sawyers, 2013). Apart from pH effects,

ionic strength also influences β-lg dimerization. Previous research showed that the addition of NaCl between pH 2.0 and 9.0 promotes β-lg dimerization. In addition, the intermolecular repulsion tends to decrease as ionic strength increases (Forrest *et al.*, 2005).

2.3.2 Encapsulation Potential

Milk proteins are one group of ideal candidates for utilization in encapsulation and transport of bioactive compounds. This is mainly attributed to their excellent capacity to form stable emulsions with hydrophobic molecules. Milk proteins are also known to form covalent and electrostatic complexes with targeted molecules and entrap bioactive compounds. In addition, they can self-assemble or co-assemble into stable tertiary structures that allow the encapsulation of various types of small molecules (Tavares *et al.*, 2014). However, the binding capacity alters with different environmental conditions such as pH, temperature, and ionic strength. Thus, the proper environmental conditions must be controlled to enhance encapsulation efficiency of milk proteins.

The potential of β-lg has been studied for binding hydrophobic and amphiphilic bioactive compounds such as polyphenols, flavonoids, vitamins and fatty acids (Forrest *et al.*, 2005). The interactions of β-lg with other molecules are mainly driven by hydrophobic forces and in some cases hydrogen bonding is also involved (Tavares *et al*., 2014). Previous research has revealed that the major binding sites for hydrophobic molecules are localized in its internal calyx, plus other possible binding sites near α-helices and the external surfaces of β-barrels (Tavares *et al.*, 2014). For example, Liu *et al.* (2014) studied the controlled release of β-carotene from β-lg emulsions and found that acidic condition (pH 2.0) resulted in the highest release rate. Keppler & Schwarz (2012) found that the hydrophobic interaction between β-lg and polyphenols took place on the protein surface. The hydrophobic interaction between folic acid and β-lg was demonstrated by Perez *et al.* (2014), and each β-lg molecule could bind three folic acid molecules. The capability of β-lg to bind other molecules makes it of great potential for the transport of active agents through the digestive tract of the human body. Due to its stability at low pH, β-lg is able to protect small hydrophobic molecules, pass through the acidic stomach and eventually permit the release of those molecules in the small intestine for absorption (Sawyer, 2013).

2.4 FTIR Analysis of Protein Structure

Fourier transform infrared (FTIR) spectroscopy is considered to be a valuable tool in analyzing protein conformational changes in aqueous solutions and dried powders (Kong & Yu, 2007). Table 2.3 shows the main IR absorption bands of proteins and their corresponding assignments to the vibrational modes of protein molecules.

Table 2.3. Characteristic infrared bands of peptide linkage (Kong & Yu, 2007).

Designation	**Wavenumber (cm^{-1})**	**Assignment**
Amide A	3300	N-H stretching
Amide B	3100	N-H stretching
Amide I	1690-1600	C=O stretching
Amide II	1575-1480	C-N stretching, N-H bending
Amide III	1301-1229	C-N stretching, N-H bending
Amide IV	767-625	O=C-N bending
Amide V	800-640	Out-of-plane N-H bending
Amide VI	606-537	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

Among the various IR absorption bands of proteins, the amide I and II bands (1700 - 1480 cm^{-1}) are considered to be the most informative. The amide I band, due mostly to C=O stretching of peptide linkages, is the most sensitive to protein secondary structure. To be specific, the region between 1700 and 1600 cm^{-1} consists of overlapping bands of different protein secondary structure components such as α-helix, β-sheets, β-turns and random coils (Kong & Yu, 2007). However, to resolve the spectral information for each component, resolution enhancing methods have to be employed. Currently, the most common methods to resolve those peaks are Fourier self-deconvolution, curve fitting and second derivative analysis (Kong & Yu, 2007). By performing these methods, the relative proportions of the secondary structural components can be estimated. Previous research has shown that the results obtained by FTIR spectroscopic studies of the secondary structure of various proteins such as hemoglobin, myoglobin, trypsin,

and lysozyme are consistent with the structures determined by X-ray crystallography (Dong *et al.*, 1992; Levitt & Greer, 1977). In comparison with the amide I region, the amide II region, which represents mainly in-plane N-H bending and C-N stretching of the peptide linkages, is less sensitive to protein secondary structure but is employed to study the kinetics of H-D exchange of proteins dissolved in D_2O (Kong & Yu, 2007).

2.5 References

Abd El-Salam, M.H., & El-Shibiny, S. (2012). Formation and potential uses of milk proteins as nano delivery vehicles for nutraceuticals: A review. *International Journal of Dairy Technology, 65*(1), 13-21.

Aguirre, A., Borneo, R., & Leon, A.E. (2013). Antimicrobial, mechanical and barrier properties of triticale protein films incorporated with oregano essential oil. *Food Bioscience, 1*, 2-9.

Ahmad, M., Benjakul, S., Prodpran, T., & Agustini, T.W. (2012). Physico-chemical and antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils. *Food Hydrocolloids, 28*(1), 189-199.

Ahmed, A.M., & Ismail, T.H. (2010). Improvement of the quality and shelf-life of minced beef with soy protein by sage (*Salvia officinalis*). *African Journal of Food Science, 4*(6), 330-334.

Akhtar, M., & Dickinson, E. (2007). Whey protein-maltodextrin as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids, 21*, 607-616.

Anitha, K., Jaswitha, K., Subhashini, P., Annapurna, V., Sanjeev Rao, T., & Ashok Kumar, C.K. (2013). Wound healing activity of *Salvia officinalis* (sage). *Journal of Global Trends in Pharmaceutical Sciences, 4*(1), 1007-1012.

Asensio, C.M., Nepote, V., & Grosso, N.R. (2012). Sensory attribute preservation in extra virgin olive oil with addition of oregano essential oil as natural antioxidant. *Journal of Food Science, 77*(9), S294-S301.

Atares, L., Bonilla, J., & Chiralt, A. (2010). Characterization of sodium caseinate-based edible films incorporated with cinnamon or ginger essential oils. *Journal of Food Engineering, 100*(4), 678-687.

Augustin, M.A., & Sanguansri, L. (2008). Encapsulation of bioactives. In J.M. Aguilera, & P.J. Lillforrd (Eds.), *Food materials science - Principles and practice* (pp. 577-601). New York: Springer.

Ayala-Zavala, J., Soto-Valdez, H., Gonzalez-Leon, A., Alvarez-Parrilla, E., Martin-Belloso, O., & Gonzalez-Aguilar, G. (2008). Microencapsulation of cinnamon leaf (*Cinnamomum zeylanicum*) and garlic (*Allium sativum*) oils in β-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 60*(3), 359-368.

Badiee, P., Nasirzadeh, A.R., & Motaffaf, M. (2012). Comparison of *Salvia officinalis* L. essential oil and antifungal agents against *candida* species. *Journal of Pharmaceutical Technology and Drug Research, 1*, 1-5.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils-a review. *Food and Chemical Toxicology, 46*, 446-475.

Bakry, A.M., Abbas, S., Ali, B., Majeed, H., Abouelwafa, M.Y., Mousa, A., & Liang, L. (2016). Microencapsulation of oils: A comprehensive review of benefits, techniques, and applications. *Comprehensive Reviews in Food Science and Food Safety, 15*, 143-182.

Baranauskien, R., Bylait, E., Zukauskait, J., & Venskutonis, R.P. (2007). Flavor retention of peppermint (*Mentha piperita* L.) essential oil spray-dried in modified starches during encapsulation and storage. *Journal of Agricultural and Food Chemistry, 55*(8), 3027-3036.

Baranauskiene, R., Venskutonis, P.R., Dewettinick, K., & Verhe, R. (2006). Properties of oregano (*Origanum vulgare* L.), citronella (*Cymbopogon nardus* G.) and marjoram (*Majorana hortensis* L.) flavors encapsulated into milk protein-based matrices. *Food Research International, 39*(1), 413-425.

Baser, K.C., & Buchbauer, G. (2010). *Handbook of Essential Oils: Science, Technology, and Applications.* New York: CRC Press, Taylor & Francis Group.

Basuny, M.A., Nasef, S.L., Mahmoud, E.A.M., & Arafat, S.M. (2011). Use of medicinal and aromatic plants for increasing quality of some bakery products. *Banat's Journal of Biotechnology, 2*(4), 76-88.

Benchaar, C., Calsamiglia, S., Chaves, A.V., Fraser, G.R., Colombatto, D., McAllister, T.A., & Beauchemin, K.A. (2008). A review of plant-derived essential oils in ruminant nutrition and production. *Animal Feed Science and Technology, 145*(1), 209-228.

Bhandari, B.R., Datta, N., & Howes, T. (1997). Problems associated with spray drying of sugar-rich foods. *Drying Technology, 15*(2), 671-684.

Bhandari, B.R., Patel, K.C., & Chen, X.D. (2008). Spray drying of food materials - Process and product characteristics. In X.D. Chen, & A.S. Mujumdar, *Drying Technologies in Food Processing* (pp. 113-159). Oxford: Blackwell Publishing.

Bi, C., Li, D., Wang, L., Gao, F., & Adhikari, B. (2014). Effect of high shear homogenization on rheology, microstructure and fractal dimension of acid-induced SPI gels. *Journal of Food Engineering, 126*, 48-55.

Botreal, D.A., Borges, S.V., Fernandes, R.V., Viana, A.D., da Costa, J.M.G., & Marques, G.R. (2012). Evaluation of spray drying conditions on properties of microencapsulated oregano essential oil. *International Journal of Food Science and Technology, 47*(11), 2289-2296.

Bourtoom, T. (2008). Edible films and coatings: characteristics and properties. *International Food Research Journal, 15*(3), 237-248.

Bousbia, N., Vian, M.A., Ferhat, M.A., Petitcolas, E., Meklati, B.Y., & Chemat, F. (2009). Comparison of two isolation methods for essential oil from rosemary leaves: Hydrodistillation and microwave hydrodiffusion and gravity. *Journal of Food Chemistry, 114*(1), 355-362.

Brewer, M.S. (2011). Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety, 10*(4), 221-247.

Bringas-Lantigua, M., Valdes, D., & Pino, J.A. (2012). Influence of spray-dryer air temperatures on encapsulated lime essential oil. *International Journal of Food Science and Technology, 47*(7), 1511-1517.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. *International Journal of Food Microbiology, 94*(3), 223-253.

Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., & Ferret, A. (2007). *Invited review:* essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science, 90*(6), 2580-2595.

Calvo, P., Castano, A.L., Lozano, M., & Gonzalez-Gomez, D. (2012). Influence of the microencapsulation on the quality parameters and shelf-life of extra-virgin olive oil encapsulated in the presence of BHT and different capsule wall components. *Food Research International, 45*, 256-261.

Castillejos, L., Calsamiglia, S., Martin-Tereso, J., & Ter Wijlen, H. (2008). In vitro evaluation of effects of ten essential oils at three doses on ruminal fermentation of high concentrate feedlot-type diets. *Animal Feed Science and Technology, 145*(1), 259-270.

Cenic-Milosevic, D., Tambur, Z., Bokonjic, D., Ivancajic, S., Stanojkovic, T., Grozdanic, N., & Juranic, Z. (2013). Antiproliferative effects of some medicinal plants on hela cells. *Archives of Biological Sciences, 65*(1), 65-70.

Ceylan, A., Bayram, E., Sahbaz, N., Otan, H., & Karaman, S. (2003). Yield performance and essential oil composition of individual plants and improved clones of *Origanum onites* L. grown in the Aegean region of Turkey. *Israel Journal of Plant Sciences, 51*(4), 285-290.

Chen, H.D., Weiss, J.C., & Shahidi, F. (2006). Nanotechnology in nutraceuticals and functional foods. *Food Technology, 60*, 30-36.

Dal, B.G., Pnadin, S., Lopez, I.C., & Fabrizio, M. (2001). Laboratory evaluation of chemical-biological control of the rice weevil (*Sitophilus oryzae* L.) in stored grains. *Journal of Stored Products Research, 37*, 77-84.

Damodaran, S. (1996). Amino acids, peptides and proteins. In O. Fennema, *Food Chemistry* (pp. 321-430). New York, NY: Marcel Dekker.

De Azeredo, G.A., Stamford, T.L.M., Nunes, P.C., Neto, G., De Oliveira, M.E.G., & De Souza, E.L. (2011) Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated minimally processed vegetables. *Food Research International, 44*(5), 1541-1548.

De Roos, K.B. (2000). Physicochemical models of flavor release from foods. In *ACS symposium series* (pp. 126-141). ACS Publications.

Debeaufort, F., & Voilley, A. (2009). *Edible Films and Coatings for Food Applications*. New York: Springer.

Del Toro-Sanchez, C., Ayala-Zavala, J., Machi, L., Santacruz, H., Villegas-Ochoa, M., Alvarez-Parrilla, E., & Gonzalez-Aguilar, G. (2010). Controlled release of antifungal volatiles of thyme essential oil from β-cyclodextrin capsules. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 67*(3), 431-441.

Desai, K.G.H., & Park, H.J. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology, 23*(7), 1361-1394.

Desobry, S.A., Netto, F.M., & Labuza, T.P. (1997). Comparison of spray-drying, drum-drying and freeze-drying for β-carotene encapsulation and preservation. *Journal of Food Science, 62*(6), 1158-1162.

Domian, E., Cenkier, S.J., & Kerschke, A. (2014). Influence of agglomeration on physical characteristics and oxidative stability of spray-dried oil powder with milk protein and trehalose wall material. *Journal of Food Engineering, 125*, 34-43.

Dong, A., Huang, P., & Caughey, W.S. (1992). Redox-dependent changes in β-extended chain and turn structures of cytochrome c in water solution determined by second derivative amide I infrared spectra. *Biochemistry, 31*, 182-189.

Dorman, H.J., & Deans, S.G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology, 88*(2), 308-316.

Durand, L., Habran, N., Henschel, V., & Amighi K. (2010). Encapsulation ethylhexyl methoxycinnamate, a light-sensitive UV filter, in lipid nanoparticles. *Journal of Microencapsulation, 27*(8), 714-725.

Edris, A.E., Jirovetz, L., Buchbauer, G., Denkova, Z., Stoyanova, A., & Slavchev, A. (2007). Chemical composition, antimicrobial activities and olfactive evaluation of a *Salvia officinalis* L. (sage) essential oil from Egypt. *Journal of Essential Oil Research, 19*(2), 186-189.

Emiroglu, Z.K., Yemis, G.P., Coskun, B.K., & Candogan, K. (2013). Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties. *Meat Science, 86*, 283-288.

Fabra, M.J., Chambin, O., Voilley, A., Gary, J.P., & Debeaufort, F. (2012). Influence of temperature and NaCl on the release in aqueous liquid media of aroma compounds encapsulated in edible films. *Journal of Food Engineering, 108*(1), 30-36.

Falguera, V., Quinterob, J.P., Jimenez, A., Munoz, J.A., & Ibarz, A. (2011). Edible films and coatings: Structures, active functions and trends in their use. *Trends in Food Science & Technology, 22*(6), 292-303.

Fang, Z., & Bhandari, B. (2010). Encapsulation of polyphenols - a review. *Trends in Food Science and Technology, 21*(10), 510-523.

Fang, Z., & Bhandari, B. (2012). Spray drying, freeze drying and related processes for food ingredient and nutraceutical encapsulation. In N. Garti, & D.J. McClements, *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 73-109). Cambridge: Woodhead Publishing.

Farooqi, A.H.A., Samgwan, R.S., & Sangwan, R.S. (1999). Effect of different photoperiodic regions on growth, flowering and essential oil in Mentha species. *Plant Growth Regulation, 29*(3), 181-187.

Fernandes, R.V.B., Borges, S.V., Botrel, D.A., Silva, E.K., Costa, J.M.G., & Queiroz, F. (2013). Microencapsulation of rosemary essential oil: characterization of particles. *Drying Technology, 31*(11), 1245-1254.

Fernandez-Pan, I., Royo, M., & Mate, J.I. (2012). Antimicrobial activity of whey protein isolate edible films with essential oils against food spoilers and foodborne pathogens. *Journal of Food Science, 77*(7), M383-M390.

Ferreira, I., Rocha, S., & Coelho, M. (2007). Encapsulation of antioxidants by spray-drying. *Chemical Engineering Transactions, 11*(9), 713-717.

Filkova, I., Huang, L.X., & Mujumdar, A.S. (2007). Industrial spray drying systems. In A.S. Mujumdar, *Handbook of Industrial Drying: Third Edition* (pp. 215-256). Boca Raton: CRC Press, Taylor & Francis Group.

Forrest, S.A., Yada, R.Y., & Rousseau, D. (2005). Interactions of vitamin D_3 with bovine beta-lactoglobulin and beta-casein. *Journal of Agricultural and Food Chemistry, 53*(1), 8003-8009.

Garcia, L.C., Tonon, R.V., & Hubinger, M.D. (2012). Effect of homogenization pressure and oil load on the emulsion properties and the oil retention of microencapsulated basil essential oil (*Ocimum basilicum* L.). *Drying Technology, 30*(13), 1413-1421.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., & Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food Research International, 40*, 1107-1121.

Ghosh, S.K. (2006). Functional coatings and microencapsulation: A general perspective. In S.K. Ghosh, *Functional Coatings by Polymer Microencapsulation* (pp. 1-29). Weinheim: Wiley-VCH Verlag Gmbh & Co.

Gibbs, B.F., Kermasha, S., Alli, I., & Mulligan, C.N. (1999). Encapsulation in the food industry: a review. *International Journal of Food Science and Nutrition, 50*(3), 213-224.

Gimenez, B., Gomez-Guillen, M.C., Lopez-Caballero, M.E., & Montero, P. (2012). Role of sepiolite in the release of active compounds from gelatin-egg white films. *Food Hydrocolloids, 27*(2), 475-486.

Gouin, S. (2004). Microencapsulation: industrial appraisal of existing technologies and trends. *Trend in Food Science & Technology, 15*(7), 330-347.

Goulas, A.E., & Kontominas, M.G. (2007). Combined effect of light salting, modified atmosphere packaging and oregano essential oil on the shelf-life of sea bream (*Sparus aurata*): biochemical and sensory attributes. *Food Chemistry, 100*(1), 287-296.

Govaris, A., Solomakos, N., Pexara, A., & Chatzopoulou, P. (2010). The antimicrobial effect of oregano essential oil, nisin and their combination against *Salmonella Enteritidis* in minced sheep meat during refrigerated storage. *International Journal of Food Microbiology, 137*(2), 175-180.

Guerrero, P., Retegi, A., Gabilondo, N., & De la Caba, K. (2010). Mechanical and thermal properties of soy protein films processed by casting and compression. *Journal of Food Engineering, 100*(1), 145-151.

Guilbert, S. (1986). Technology and application of edible protective films. In Mathlouthi, M., *Food Packaging and Preservation* (pp. 371-394). London, UK: Elsevier Applied Science.

Han, J.H. (2003). Antimicrobial food packaging. In R. Ahvenainen, *Novel Food Packaging Techniques* (pp. 50-70). Cambridge, UK: Woodhead publishing.

Hermandez-Izquierdo, V.M., & Krochta, J.M. (2008). Thermoplastic processing of proteins for film formation--a review. *Journal of Food Science, 73*(2), 30-39.

Holmberg, K., Jonson, B., Kronberg, B., & Lindman, B. (2002). *Surfactants and Polymers in Aqueous Solution*. Chichester, England: John Wiley & Sons.

Hua, T., Liu, B., & Zhang, H. (2010). *Freeze-drying of Pharmaceutical and Food Products.* Cambridge: Woodhead Publishing Limited, Science Press and CRC Press LLC.

Huang, H., Hao, S., Li, L., Yang, X., Cen, J., Lin, W., & Wei, Y. (2014). Influence of emulsion composition and spray-drying conditions on microencapsulation of tilapia oil. *Journal of Food Science and Technology, 51*(9), 2148-2154.

Hussain, A.I., Anwar, F., Rasheed, S., Nigam, P.S., Janneh, O., & Sarker, S.D. (2011). Composition, antioxidant and chemotherapeutic properties of the essential oils from two *Origanum* species growing in Pakistan. *Revista Brasileira de Farmacognosia, 21*(6), 943-952.

Huynh, T.V., Caffin, N., Dykes, G.A., & Bhandari, B. (2008). Optimization of the microencapsulation of lemon myrtle oil using response surface methodology. *Drying Technology, 26*(3), 357-368.

Jafari, S.M., Assadpoor, E., Bhandari, B., & He, Y. (2008). Nanoparticles encapsulation of fish oil by spray drying. *Food Research International, 41*(2), 172-183.

Jalali-Heravi, M., Moazeni, R.S., & Sereshti, H. (2011). Analysis of Iranian rosemary essential oil: Application of gas chromatography-mass spectrometry combined with chemometrics. *Journal of Chromatography A, 1218*(18), 2569-2576.

Janjarasskul, T., & Krochta, J.M. (2010). Edible packaging materials. *Annual Review of Food Science and Technology, 1*, 415-448.

Jiang, Y., Wu, N., Fu, Y.J., Wang, W., Luo, M., Zhao, C.J., & Liu, X.L. (2011). Chemical composition and antimicrobial activity of the essential oil of rosemary. *Environmental Toxicology and Pharmacology, 32*(1), 63-68.

Jouki, M., Yazdi, F.T., Mortazavi, S.A., & Koocheki, A. (2014). Quince seed mucilage films incorporated with oregano essential oil: physical, thermal, barrier, antioxidant and antibacterial properties. *Food Hydrocolloids, 36*, 9-19.

Kahraman, Z. (2009). Herbal extracts and their usage in laying hen diets. *The Journal of Applied Poultry Research, 8*(1), 34-41.

Kaushik, V., & Roos, Y.H. (2007). Limonene encapsulation in freeze-drying of gum Arabic-sucrose-gelatin systems. *LWT - Food Science and Technology, 40*(8), 1381-1391.

Kayaci, F., & Uyar, T. (2012). Encapsulation of vanillin/cyclodextrin inclusion complex in electrospun polyvinyl alcohol (PVA) nanowebs: prolonged shelf-life and high temperature stability of vanillin. *Food Chemistry, 133*(3), 641-649.

Kenawi, M.A. (2012). The role of packaging (materials & treatments) in addition to spices extract on stability of frozen buffalo meat products. *Agronomy Series of Scientific Research, 55*(2), 23-30.

Keppler, J., & Schwarz, K. (2012). Milk proteins as nanocarriers. *EDM, 24*, 10-12.

Kester, J.J., & Fennema, O.R. (1986). Edible films and coatings: A review. *Food Technology, 40*(12), 47-59.

Kolanowski, W., Laufenberg, G., & Kunz, B. (2004). Fish oil stabilization by microencapsulation with modified cellulose. *The International Journal of Food Sciences and Nutrition, 55*(4), 333-343.

Kong, J., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica, 39*(8), 549-559.

Krochta, J.M., Nisperos-Carriedo, M.O. (1994). *Edible Coatings and Films to Improve Food Quality*. Boca Raton: CRC Press Llc.

Krokida, M.K., & Philippopoulos, C. (2006). Volatility of apples during air and freeze drying. *Journal of Food Engineering, 73*, 135-141.

Kulisic, L., Krisko, A., Dragovic-Uzelac, V., Milos, M., & Pifat, G. (2007). The effects of essential oils and aqueous tea infusions of oregano (*Origanum vulgare* L. spp. *hirtum*), thyme (*Thymus vulgaris* L.) and wild thyme (*Thymus serpyllum* L.) on the copper-induced oxidation of human low-density lipoproteins. *International Journal of Food Sciences and Nutrition, 58*(2), 87-93.

Lagouri, V., Bantouna, A., & Stathopoulos, P. (2010). A comparison of the antioxidant activity and phenolic content of nonpolar and polar extracts obtained from four endemic *Lamiaceae* species grown in Greece. *Journal of Food Processing and Preservation, 34*(5), 872-886.

Langa, E., Della Porta, G., Palavra, A.M.F., Urieta, J.S., & Mainar, A.M. (2009). Supercritical fluid extraction of Spanish sage essential oil: Optimization of the process parameters and modelling. *The Journal of Supercritical Fluids, 49*(2), 174-181.

Laohakunjit, N., & Kerdchoechuen, O. (2007). Aroma enrichment and the change during storage of non-aromatic milled rice coated with extracted natural flavor. *Food Chemistry, 101*(1), 339-344.

Larocque, N., Vincent, C., Belanger, A., & Bourassa, J.P. (1999). Effects of tansy essential oil from *Tanacetum vulgare* on biology of obliquebanded leafroller, *Choristoneura rosaceana*. *Journal of Chemical Ecology, 25*(6), 1319-1330.

Lee, S.P., Buber, M.T., Yang, Q., Cerne, R., Cortes, R.Y., Sprous, D.G., & Bryant, R.W. (2008). Thymol and related alkyl phenols activate the hTRPA1 channel. *British Journal of Pharmacology, 153*(8), 1739-1749.

Levitt, M., & Greer, J. Automatic identification of secondary structure in globular proteins. (1977). *Journal of Molecular Biology, 114*, 181-239.

Liapis, A.I., & Bruttini, R. (2007). Freeze drying. In A.S. Mujumdar, *Handbook of Industrial Drying* (pp. 257-283). Boca Raton: CRC Press Taylor and Francis Group.

Liu, F., Chen, Z., & Tang, C.H. (2014). Microencapsulation properties of protein isolates from three selected *Phaseolus* legumes in comparison with soy protein isolate. *LWT-Food Science and Technology, 55*(1), 74-82.

Liu, T., & Yang, T.S. (2011). Optimization of emulsification and microencapsulation of evening primrose oil and its oxidative stability during storage by response surface methodology. *Journal of Food Quality, 34*(1), 64-73.

Liu, Y., Lei, F., Yuan, F., & Gao, Y. (2014). Effects of milk proteins on release properties and particle morphology of β-carotene emulsions during in vitro digestion. *Food & Function, 5*(11), 2940-2947.

Liu, Z., Zhou, J., Zeng, Y., & Ouyang, X. (2004). The enhancement and encapsulation of Agaricus bisporus flavor. *Journal of Food Engineering, 65*, 391-396.

Lopez-Bote, C.J., Gray, J.I., Gomaa, E.A., & Flegal, C.J. (1998). Effect of dietary administration of oil extracts from rosemary and sage on lipid oxidation in broiler meat. *British Poultry Science*, *39*, 235-240.

Lopez-Reyes, J.G., Spadaro, D., Gullino, M.L., & Garibaldi, A. (2010). Efficacy of plant essential oils on postharvest control of rot caused by fungi on four cultivars of apples *in vivo*. *Flavour and Fragrance Journal, 25*(3), 171-177.

Lopez, A., Castro, S., Andina, M.J., Ures, X., Mungula, B., Llabot, J.M., Elder, H., Dellacassa, E., Palma, S., & Dominguez, L. (2014). Insecticidal activity of microencapsulated *Schinus molle* essential oil. *Industrial Crops and Products, 53,* 209-216.

McClements, D.J. (2005). *Food Emulsions: Principles, Practices, and Techniques, Second Edition.* Boca Raton, Florida: CRC Press.

McClements, D.J. (2012). Requirements for food ingredient and nutraceutical delivery system. In N. Garti, & D.J. McClements, *Encapsulation Technologies and Delivery Systems for Food Ingredients and Nutraceuticals* (pp. 1-70). Cambridge: Woodhead Publishing.

McClements, D.J., Decker, E.A., Park, Y., & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition, 49*(6), 577-606.

Mohammad, S.M. (2011). A study on sage (*Salvia officinalis*). *Journal of Applied Science Research, 7*(8), 1261-1262.

Moradi, M., Tajik, H., Razavi Rohani, S.M., Oromiehie, A.R., Malekinejad, H., Aliakbarlu, J., & Hadian, M. (2012). Characterization of antioxidant chitosan film incorporated with Zataria multiflora Boiss essential oil and grape seed extract. *LWT - Food Science and Technology, 46*(2), 477-484.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., & Bugarski, Branko. (2011). An overview of encapsulation technologies for food applications. *Procedia Food Science, 1*, 1806-1815.

Olmedo, R.H., Asensio, C., Nepote, V., Mestrallet, M.G., & Grosso, N.R. (2009). Chemical and sensory stability of fried-salted peanuts flavored with oregano essential oil and olive oil. *Journal of the Science of Food and Agriculture, 89*(12), 2128-2136.

Orhan, I., Aslan, S., Kartal, M., Sener, B., & Baser, K.H.C. (2008). Inhibitory effect of Turkish *Rosmarinus officinalis* L. on acetylcholinesterase and butyrylcholinesterase enzymes. Journal of *Food Chemistry, 108*(1), 663-668.

Oussalah, M., Caillet, S., Salmieri, S., Saucier, L., & Lacroix, M. (2004). Antimicrobial and antioxidant effects of milk-protein based film containing essential oils for the preservation of whole beef muscle. *Journal of Agricultural and Food Chemistry, 52*(18), 5598-5605.

Oussalah, M., Caillet, S., Salmieri, S., Saucier, L., & Lacroix, M. (2004). Antimicrobial and antioxidant effects of milk protein-based films containing essential oils for the preservation of whole beef muscle. *Journal of Agricultural and Food Chemistry, 52*(18), 5598-5605.

Partanen, R., Ahro, M., Hakala, M., Kallio, H., & Forssell, P. (2002). Microencapsulation of caraway extract in β-cyclodextrin and modified starches. *European Food Research and Technology, 214*(3), 242-247.

Pascall, M.A., & Lin, S. (2013). The application of edible polymeric films and coatings in the food industry. *Journal of Food Processing & Technology, 4*(2), e116.

Perdones, A., Vargas, M., Atares, L., & Chiralt, A. (2014). Physical, antioxidant and antimicrobial properties of chitosan-cinnamon leaf oil films as affected by oleic acid. *Food Hydrocolloids, 36*, 256-264.

Perez-Alfonso, C.O., Martinez-Romero, D., Zapata, P.J., Serrano, M., Valero, D., & Castillo, S. (2012). The effects of essential oils carvacrol and thymol on growth of *Penicillium digitatum* and *P. italicum* involved in lemon decay. *International Journal of Food Microbiology, 158*(2), 101-106.

Perez, O.E., David-Birman, T., Kesselman., E., Levi-Tal, S., & Lesmes, U. (2014). Milk protein-vitamin interactions: formulation of beta-lactoglobulin/folic acid nano-complexes and their impact on *in vitro* gastro-duodenal proteolysis. *Food Hydrocolloids, 38*, 40-47.

Pitarokili, D., Tzakou, O., & Loukis, A. (2008). Composition of the essential oil of spontaneous *Rosmarinus officinalis* from Greece and antifungal activity against phytopathogenic fungi. *Journal of Essential Oil Research, 20*(5), 457-459.

Pizzale, L., Bortolomeazzi, R., Vichi, S., Uberegger, E., & Conte, L.S. (2002). Antioxidant activity of sage (*Salvia officinalis* and *S. fruticosa*) and oregano (*Origanum onites* and *O. intercedens*) extracts related to their phenolic compound content. *Journal of the Science of Food and Agriculture, 82*, 1645-1651.

Pothakamury, U.R., & Barbosa-Canovas, G.V. (1995). Fundamentals aspects of controlled release. *Trends in Food Science & Technology, 6*(12), 397-406.

Pozzati, P., Loreto, E., Mario, D.N., Rossato, L., Santurio, J., & Alves, S. (2010). Activities of essential oils in the inhibition of *Candida albicans* and *Candida dubliniensis* germ tube formation. *Journal of Medical Mycology, 20*(3), 185-189.

Pu, J., Bankston, J.D., & Sathivel, S. (2011). Developing microencapsulated flaxseed oil containing shrimp (*Litopenaeus setiferus*) astaxanthin using a pilot spray dryer. *Biosystems Engineering, 108*(2), 121-132.

Quiros-Sauceda, A.E., Ayala-zavala, J.F., Olivas, G.I., & Gonzalez-Aguilar, G.A. (2014). Edible coatings as encapsulating matrices for bioactive compounds: a review. *Journal of Food Science and Technology, 51*(9), 1674-1685.

Quispe-Condori, S., Saldana, M.D.A., & Temelli, F. (2011). Microencapsulation of flax oil with zein using spray and freeze drying. *LWT - Food Science and Technology, 44*(9), 1880-1887.

Rasmy, M.N., Hassan, A.A., Foda, I.M., & El-Moghazy, M.M. (2012). Assessment of the antioxidant activity of sage (*Salvia officinalis* L.) extracts on the shelf life of mayonnaise. *World Journal of Dairy & Food Sciences, 7*(1), 28-40.

Roman, G.P., Neagu, E., & Radu, G.L. (2009). Antiradical activities of *Salvia officinalis* and *Viscum album* L. extracts concentrated by ultrafiltration process. *Acta Scientiarum Polonorum - Technologia Alimentaria, 8*(3), 47-58.

Rosato, A., Vitali, C., Piarulli, M., Mazzotta, M., Argentieri, M.P., & Mallamaci, R. (2009). In vitro synergic efficacy of the combination of Nystatin with the essential oils of *Origanum vulgare* and *Pelargonium graveolens* against some *Candida species. Phytomedicine, 16*(10), 972-975.

Rosenberg, M., Kopelman, I.J., & Talmon, Y. (1990). Factors affecting retention in spray-drying microencapsulation of volatile materials. *Journal of Agricultural and Food Chemistry, 38*, 1288-1294.

Royo, M., Fernandez-Pan, I., & Mate, J.I. (2010). Antimicrobial effectiveness of oregano and sage essential oils incorporated into whey protein films or cellulose-based filter paper. *Journal of the Science of Food and Agriculture, 90*(9), 1513-1519.

Ruiz-Navajas, Y.; Viuda-Martos, M., Sendra, E., Perez-Alvarez, J.A., & Fernandez-Lopez, J. (2013). In vitro antibacterial and antioxidant properties of chitosan edible films incorporated with *Thymus Moroderi* or *Thymus Piperella* essential oils. *Food Control, 30*(2), 386-392.

Russo, M., Galletti, G.C., Bocchini, P., & Carnacini, A. (1998). Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare* ssp. *hirtum* (Link) letswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis. 1. Inflorescences. *Journal of Agricultural and Food Chemistry, 46*(9), 3741-3746.

Sahin, F., Gulluce, M., Daferera, D., Sokmen, A., Polissiou, M., Agar, G., & Ozer, H. (2004). Biological activities of the essential oils and methanol extract of *Origanum vulgare* spp. *vulgare* in the Eastern Anatolia region of Turkey. *Food Control, 15*(7), 549-557.

Salager, J., Forgiarini, A., Marquez, L., Pena, A., Pizzino, A., Rodriguez, M.P., & Rondon-Gonzalez, M. (2004). Using emulsion inversion in industrial process. *Advances in Colloid and Interface Science, 108-109,* 259-272.

Salame, M. (1986). Barrier polymers. In Bakker, M., *The Wiley Encyclopedia of Packaging Technology* (pp. 48-54). New York: John Wiley and Sons.

Salem, F.M.A., & Ibrahim, H.M. (2010). Dry fermented buffalo sausage with sage oil extract: safety and quality. *Grasas Aceites, 61*(1), 76-85.

Salgado, P.R., Lopez Caballero, M.E., Gomez Guillen, M.C., Mauri, A.N., & Montero, M.P. (2013). Sunflower protein films incorporated with clove essential oil have potential application for the preservation of fish patties. *Food Hydrocolloids, 33*(1), 74-84.

Sanchez, V., Baeza, R., Galmarini, M.V., & Chirife, J. (2011). Freeze-drying encapsulation of red wine polyphenols in an amorphous matrix of maltodextrin. *Food and Bioprocess Technology, 6*(5), 1350-1354.

Sawyer, L. (2013). Beta-Lactoglobulin. In P.L.H. McSweeney & P.F. Fox, *Advanced Dairy Chemistry - 1: Proteins, Fourth Edition* (pp. 211-259). New York: Springer.

Schafroth, N., Arpagaus, C., Jadhav, U.Y., Makne, S., & Douroumis, D. (2012). Nano and microparticle engineering of water insoluble drugs using a novel spray-drying process. *Colloids and Surfaces B: Biointerfaces, 90*(1), 8-15.

Sereno, N., Hill, S., Taylor, A., Mitchell, J., & Davies, S. (2009). Aroma permeability of hydroxypropyl maize starch films. *Journal of Agricultural and Food Chemistry, 57*(3), 985-990.

Shahidi, F., & Han, X.Q. (1993). Encapsulation of food ingredients. *Critical Reviews in Food Science and Nutrition, 33*(6), 501-547.

Silva-Weiss, A., Ihl, M., Sobral, P., Gomez-Guillen, M., & Bifani, V. (2013). Natural additives in bioactive edible films and coatings: functionality and applications in foods. *Food Engineering Reviews, 5*(4), 200-216.

Sinha, V.R., Agrawal, M.K., Kumria, R., & Bhinge, J.R. (2007). Influence of operational variables on properties of piroxicam pellets prepared by extrusion-spheronization: A technical note. *AAPS PharmSciTech, 8*(1), E137-E141.

Sokmen, M., Serkedjieva, J., Daferera, D., Gulluce, M., Polissiou, M., Tepe, B., Akpulat, H.A., Sahin, F., & Sokmen, A. (2004). In vitro antioxidant, antimicrobial, and antiviral activities of the essential oil and various extracts from herbal parts and callus cultures of *Origanum acutidens*. *Journal of Agricultural and Food Chemistry, 52*(11), 3309-3312.

Soottitantawat, A., Yoshii, H., Furuta, T., Ohgawara, M., Forssell, P., Partanen, R., Poutanen, K., & Linko, P. (2004). Effect of water activity on the release characteristics and oxidative stability of d-limonene encapsulated by spray drying. *Journal of Agricultural and Food Chemistry, 52*(5), 1269-1276.

Sorensen, A.D.M., Nielsen, N.S., & Jacobsen, C. (2010). Oxidative stability of fish oil-enriched mayonnaise-based salads. *European Journal of Lipid Science and Technology, 112*(4), 476-487.

Suput, D.Z., Lazic, V.L., Popovic, S.Z., & Hromis, N.M. (2015). Edible films and coatings - sources, properties and application. *Food and Feed Research, 42*(1), 11-22.

Taarit, M.B., Msaada, K., Hosni, K., Hammami, M., Kchouk, M.E., & Marzouk, B. (2009). Plant growth, essential oil yield and composition of sage (*Salvia officinalis* L.) fruits cultivated under salt stress conditions. *Industrial Crops & Products, 30*(3), 333-337.

Tavares, G.M., Croguennec, T., Carvalho, A.F., & Bouhallab, S. (2014). Milk proteins as encapsulation devices and delivery vehicles: Applications and trends. *Trends in Foods Science & Technology, 37*(1), 5-20.

Tongnuanchan, P., Benjakul, S., & Prodpran, T. (2013). Physico-chemical properties, morphology and antioxidant activity of film from fish skin gelatin incorporated with root essential oils. *Journal of Food Engineering, 117*(3), 350-360.

Turasan, H., Sahin, S., & Sumnu, G. (2015). Encapsulation of rosemary essential oil. *Journal of Food Science and Technology, 64*(1), 112-119.

Valgimigli, L. (2012). *Essential Oils as Natural Food Additives: Composition, Applications, Antioxidant and Antimicrobial Properties.* New York: Nova Science Publishers, Inc.

Velasco, J., Dobarganes, C., & Marquez-Ruiz, G. (2003). Variables affecting lipid oxidation in dried microencapsulated oils. *Grasas Aceites, 54*(3), 304-314.

Viuda-Martos, M., Navajas, Y.R., Zapata, E.S., Fernandez-Lopez, J., & Perez-Alvarez, J. (2009). Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour and Fragrance Journal, 25*(1), 13-19.

Wang, W., Wu, N., Zu, Y.G., & Fu, Y.J. (2008). Antioxidant activity of *Rosmarinus officinalis* L. essential oil compared to its main components. *Food Chemistry, 108*(3), 1019-1022.

Wilkowska, A., Ambroziak, W., Czyzowska, A., & Adamiec, J. (2016). Effect of microencapsulation by spray-drying and freeze-drying technique on the antioxidant properties of blueberry (*Vaccinium myrtillus*) juice polyphenolic compounds. *Polish Journal of Food and Nutrition Sciences, 66*(1), 11-16.

Xiao, C., Zhu, L., Luo, W., Song, X., & Deng, Y. (2010). Combined action of pure oxygen pretreatment and chitosan coating incorporated with rosemary extracts on the quality of fresh-cut pears. *Food Chemistry, 121*(4), 1003-1009.

Xing, Y., Li., X., Xu, Q., Yun, J., Lu, Y., & Tang, Y. (2011). Effects of chitosan coating enriched with cinnamon oil on qualitative properties of sweet pepper (*Capsicum annuum* L.). *Food Chemistry, 124*(4), 1443-1450.

Yesilbag, D., Eren, M., Agel, H., kovanlikaya, A., & Balci, F. (2011). Effects of dietary rosemary, rosemary volatile oil and vitamin E on broiler performance and meat quality and serum SOD activity. *British Poultry Science, 52*(4), 472-482.

Yesilbag, D., Gezen, S.S., Biricik, H., & Bulbul, T. (2012). Effect of a rosemary and oregano volatile oil mixture on performance, lipid oxidation of meat and haematological parameters in Pharaoh quails. *British Poultry Science, 53*(1), 89-97.

Zaouali, Y., Chograni, H., Trimech, R., & Boussaid, M. (2012). Genetic diversity and population structure among *Rosmarinus officinalis* L. (Lamiaceae) varieties: var. *typicus* Batt. and var. *troglodytarum* Maire. based on multiple traits. *Industrial Crops and Products, 38,* 166-176.

Zinoviadou, K., Koutsoumanis, K., & Biliaderis, C.G. (2010). Physical and thermo-mechanical properties of whey protein isolate films containing antimicrobials, and their effect against spoilage flora of fresh beef. *Food Hydrocolloids, 24*(1), 49-59.

CHAPTER 3

MICROENCAPSULATION OF ESSENTIAL OILS (A): ENCAPSULATING OREGANO, ROSEMARY, AND SAGE ESSENTIAL OILS IN β-LACTOGLOBULIN

3.1 Abstract

The present study aimed to evaluate the microencapsulation of oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils by emulsification/freeze-drying, using β-lactoglobulin (β-lg) as the wall material. The freeze-dried samples containing oregano essential oil showed a lower hygroscopicity, revealing a better quality in terms of water sorption. Treatments using higher protein content resulted in powders with higher bulk tapped density and particle density, demonstrating that the higher amount of wall material led to a higher compression of the particles. The encapsulation efficiency was determined to be in the range of 28.35 – 42.13% and was independent of initial protein concentration. All the samples containing essential oils exhibited significant antioxidant activities in a β-carotene assay, with the percentage of inhibition ranging between 41.05% and 51.02%. The release profiles of rosemary and sage essential oils from freeze-dried microcapsules into phosphate buffer solutions were similar, while the release of oregano essential oil was much lower, suggesting significant interactions between components of oregano essential oil and protein particles. The results of Fourier transform infrared spectroscopic analysis on freeze-dried microcapsules revealed that the change of oregano essential oil concentration significantly affected the secondary structure of β-lg.

3.2 Introduction

Nowadays there is increasing awareness of health benefits and industrial applications of bioactive compounds, which is a group of substances usually referred to as nutraceuticals, encompassing antioxidants, antimicrobials, prebiotics, vitamins, fatty acids, etc. These compounds are believed to reduce the risk of specific diseases such as cancers, cardiovascular malfunction, type 2 diabetes, cataracts and many autoimmune diseases (Fearon & Faux, 2004; Huang *et al.*, 2004; Kaur & Kapoor, 2001; Lee *et al*., 2004). Thus, research on innovative methods of introducing and developing these functional compounds in the food industry tends to have a great economic potential and health benefit to the public (Kimpel & Schmitt, 2015).

Essential oils are a group of volatile liquid products usually extracted from raw plant materials by distillation or mechanical methods (Rubiolo *et al.*, 2010). Although essential oils only constitute a small fraction of a plant's weight, they are used in pharmaceutical, food and cosmetic industries, mainly due to their antibacterial, antiviral and antioxidant properties (Pourmortazavi & Hajimirsadeghi, 2007). Currently, there are over 3,000 known species of essential oils and the ones used in this study (oregano, rosemary and sage essential oils) have been shown to possess significant active functions as natural antioxidant and antimicrobial agents (Valgimigli, 2012). In general, each essential oil has a complex composition and the most abundant categories are terpenes and polyphenols (Miguel, 2010). In particular, the major components are carvacrol and thymol for oregano essential oil (Govaris *et al.*, 2010; Hussain *et al.*, 2011), 1,8-cineole and α-pinene for rosemary essential oil (Jalali-Heravi *et al.*, 2011; Orhan *et al.*, 2008), and α-thujone and camphor for sage essential oil (Langa *et al.*, 2009; Taarit *et al.,* 2009).

Microencapsulation is a technology that protects sensitive compounds from degradations by using a wall material and maintains significant biological and physicochemical properties of the core material (Bakry *et al.*, 2016). Since most essential oils are volatile, chemically unstable and susceptible to environmental factors, microencapsulation is required to counteract this problem. Among the many microencapsulation techniques, freeze-drying is one of the common ones that protects essential oils against damage and converts them into powders (Silva *et al.*, 2013). Freeze-drying, also known as lyophilization, involves freezing the water in the food followed by subliming the ice under vacuum condition. Because freeze-drying does not utilize

any high-temperature treatments, heat-labile materials are suitable to be encapsulated by freeze-drying (Wilkowska *et al.*, 2016). Moreover, this technique has been reported to have higher retention level of volatile compounds, with excellent product quality (Chranioti *et al.*, 2016; Krokida & Philippopoulos, 2006). However, for the microencapsulation of bioactive compounds freeze-drying has not been as extensively studied as spray-drying.

One of the most important steps in microencapsulation is the selection of a suitable coating material. In general, this substance is a film-forming biopolymer that possesses emulsifying capacity (Jafari *et al.*, 2008; Kim & Morr, 1996). The coating material must protect the core active compounds from loss and damage during processing, storage and transport, as well as provide convenience for controlled release and delivery. Among the various types of biopolymers, whey protein exhibits excellent emulsifying properties and has been used for microencapsulation of volatile compounds (Bernard *et al.*, 2011; Rosenberg & Lee, 2000). Being the major component in whey protein, β-lactoglobulin (β-lg) has been extensively studied for its great potential for transporting hydrophobic substances (Livney, 2010). For instance, it has been reported to be a carrier molecule for bioactives such as β-carotene (Yi *et al.*, 2015), riboflavin (Chen & Subirade, 2007), caffeine (Gunasekaran *et al.*, 2006) and catechin (Zhang *et al.*, 2009).

The present work aimed to evaluate the microencapsulation of oregano (*Origanum Vulgare* L.), rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils in β-lg by freeze-drying. The encapsulation efficiency, physical properties and antioxidant activity of the microencapsulated particles were determined. The kinetics of controlled release of essential oils in phosphate buffer solution was studied. The influence of essential oils on protein secondary structure was investigated by Fourier transform infrared spectroscopy.

3.3 Materials and Methods

Pure oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils were purchased from local grocery stores in Montreal, QC Canada, and used without further purification. Food-grade bovine β-lactoglobulin from Biopure Products (USA) was used as the coating material for microencapsulation. The organic solvent used for particle density and encapsulation efficiency determinations were toluene and *n*-hexane

from Sigma-Aldrich (Canada). Chemicals used for controlled release experiments included sodium chloride, potassium chloride, potassium phosphate and sodium phosphate dibasic dihydrate from Sigma-Aldrich (Canada). In the β-carotene bleaching test, the chemicals used were β-carotene and chloroform from Sigma-Aldrich (Canada) and conjugated linoleic acid from Trophic Canada Ltd.

3.3.1 Preparation of Emulsions

Protein solutions (10%, 15% and 20%) were prepared by adding β-lg (10 g, 15 g and 20 g) directly into a beaker containing 100 mL of distilled water with constant stirring. The solutions were left overnight to ensure β-lg was completely dissolved. On the next day, for each treatment, the essential oil was weighed and added into the prepared solution followed by immediate homogenization for 3 min using a Polytron homogenizer (Westbury, N.Y.). The homogenized solutions were filtered through a Whatman No. 1 paper before storage. The filtered emulsions were stored in the freezer (-18 ^{o}C) for approximately 48 hours before freeze-drying.

To study the effects of the essential oils on protein structure, emulsions with different essential oil concentrations (0%, 5%, 10%, 15% and 20% of the protein concentration on a weight basis) were prepared followed by the same homogenization steps and storage conditions as described above.

3.3.2 Freeze-Drying

The frozen emulsions were dried using a Labconco freeze dryer, with freezer temperature at -50 ^{o}C and internal pressure lower than 25 mmHg. The dried powder was collected after 48 hours of drying and stored in an airtight container at refrigerated temperature (4 ^{o}C) for further analyses.

3.3.3 Physical Properties

The hygroscopicity, bulk tapped density, particle density and porosity of the freeze-dried microparticles were determined by physical methods described below.

The hygroscopicity of the dried powder was determined based on the method employed by Cai & Corke (2000), with minor modifications. For each treatment, approximately 1 g of the

pre-weighed dried powder was placed in a desiccator containing saturated NaCl solution (75.29% RH) at 25 ^{o}C. The samples were reweighed after two-week storage and the hygroscopicity was calculated as grams of adsorbed moisture per 100 g of dried sample.

Bulk tapped density (ρ_b) was determined as described in the studies by Goula & Adamopoulus (2008) and Jafari *et al.* (2016) with modifications. Approximately 0.5 g of dried sample was poured into a 10-mL glass graduated cylinder, manually tapped and mixed by a mechanical mixer until there was no apparent volume difference at a vertical distance. The bulk tapped density was calculated based on the measured mass and volume of each sample and expressed in g cm^{-3} (Goula & Adamopoulus, 2008).

The particle density of the samples was determined by using pycnometers (Fernandes *et al.*, 2014). For each replicate, 0.5 g of freeze-dried powder was weighed into a 10-mL pycnometer which was filled with toluene. The particle density (ρ_p) was calculated according to Equation (1) below:

$$\text{Particle density } (\rho_p) = \frac{Sample\ weight\ (g)}{Volume\ of\ pycnometer\ (mL) - Volume\ of\ toluene\ (mL)} \quad (1)$$

where the volume of toluene can be calculated based on its density of 0.867 g mL^{-1}.

Porosity (ε_b) was derived from the values of bulk tapped density and particle density using Equation (2) below (Krokida & Maroulis, 1997):

$$\% \ \varepsilon_b = (1 - \rho_b/\rho_p) \times 100 \quad (2)$$

where ρ_b and ρ_p are the bulk tapped density and particle density of the freeze-dried microcapsules, respectively.

3.3.4 Encapsulation Efficiency

The encapsulation efficiency of essential oils was determined by direct solvent extraction (Bylaite *et al.*, 2001; Hundre *et al.*, 2015; Karim *et al.*, 2016; Naik *et al.*, 2014; Yazicioglu *et al.*, 2015), and *n*-hexane was selected as the solvent due to its excellent capacity to dissolve hydrophobic compounds. For each replicate, 1 g of freeze-dried powder was weighed into a centrifugation tube filled with 10 mL of *n*-hexane. The mixture was then vortexed for 3 min

followed by centrifugation at 5000 rpm for 5 min. After centrifugation, the supernatant was filtered through a Whatman No. 1 filter paper and transferred into a glass tube. The solution was stored in the fume hood for approximately 48 h, to allow complete solvent evaporation. The residual component was weighed and expressed as the weight of the unencapsulated fraction, also known as surface oil. By assuming minimal loss of essential oil during freeze-drying, the encapsulation efficiency was calculated as:

$$\text{Encapsulation efficiency (\%)} = \frac{Total\ oil - Surface\ oil}{Total\ oil} \times 100 \quad (3)$$

3.3.5 Antioxidant Activity

The antioxidant activity of the freeze-dried powders was assessed by the β-carotene bleaching method, with modifications (Andrade *et al.*, 2013; Kulisic *et al.*, 2004; Wang *et al.*, 2008). Into a round-bottomed flask, 6 mg of β-carotene, 60 μL of linoleic acid and 600 mg of Tween 60 were added together with boiling chips, followed by the addition of 30 mL of chloroform to dissolve all the compounds. Using a rotary evaporator with a water bath at 50 ºC, the chloroform was completely removed. Oxygenated distilled water (150 mL) was then added with vigorous stirring. For each replicate, 0.1 g of freeze-dried capsules containing essential oil was added into a small test tube, followed by the addition of 5 mL of the prepared solution. The solution was immediately vortexed for 1 min. For the control, nothing was added except for the reaction mixture. For each treatment, the absorbance was then measured at 470 nm by a spectrophotometer (Thermo Scientific Genesys 10S) at 15-min intervals for 120 min. The percentage inhibition was determined by using Equation (4) (Mallet *et al.*, 1994).

$$\text{\% inhibition} = [(A_{S(120)} - A_{C(120)}) / (A_{S(0)} - A_{C(120)})] \times 100 \quad (4)$$

where $A_{S(120)}$ is the absorbance of the essential oil sample at 120 min, $A_{C(120)}$ is the absorbance of the control at 120 min, and $A_{S(0)}$ is the absorbance of the essential oil sample at 0 min. The samples with 20% initial protein concentration and 15% (protein weight basis) essential oil were analyzed. Duplicate analyses were performed for each sample.

3.3.6 Controlled Release

The kinetics of release of essential oils from dried powder was evaluated based on previous studies (Beirao da Costa *et al.* 2012; Nastruzzi, 1999; Yin & Yates, 2009), with modifications. For each replicate, approximately 5 g of dried capsules were weighed into a dialysis bag with a molecular weight cut-off of 6000-8000 Da. The bag was subsequently placed into a beaker containing 100 mL of phosphate buffer solution (PBS). The solution was stirred constantly. At regular time intervals, 1 mL of the solution was transferred from the beaker for UV absorbance measurements by a spectrophotometer (Thermo Scientific Genesys 10S). The wavelength for each essential oil sample was selected based on the maximum absorption wavelength value of the corresponding major compound, listed in Table 3.1 below. The samples with 20% initial protein concentration and 15% (protein weight basis) essential oil were analyzed. Duplicate analyses were performed for each sample. For each essential oil, a calibration curve was constructed to determine the concentration of essential oil at time t.

Table 3.1. Major compounds of each essential oil and their maximum UV absorption wavelengths

Essential oil	**Major component**	**λ_{max} (nm)**	**Reference**
Oregano	Carvacrol	277	Beirao da Costa *et al.*, 2012
Rosemary	1,8-Cineol	254	Nigg *et al.*, 2014
Sage	α-Thujone	300	O'Neil, 2013

Assuming the essential oils migrate from the freeze-dried capsules into the PBS by diffusion, the following relationship, involving Equations (5) and (6), was used to construct the mathematical model of kinetics of controlled release (Prata *et al.*, 2008; Zhang *et al.*, 2006).

$$\frac{M_t}{M_e} = 6\left(\frac{Dt}{\pi r^2}\right)^{0.5} - \frac{3Dt}{r^2}, \qquad \text{for } \frac{M_t}{M_e} \leq 0.7 \tag{5}$$

$$\frac{M_t}{M_e} = 1 - 0.61\exp\left(-\frac{Dt\pi^2}{r^2}\right), \qquad \text{for } 0.7 \leq \frac{M_t}{M_e} \leq 1 \tag{6}$$

where M_e is the mass of the essential oil released at equilibrium, M_t is the mass of the essential oil release at time t, D is the diffusion coefficient and r is the radius of the particle. The model was fitted to the experimental data by nonlinear parameter estimation.

3.3.7 Fourier Transform Infrared Spectroscopy

Attenuated total reflectance - Fourier transform infrared (ATR-FTIR) spectroscopy was conducted on pure essential oil samples, pure β-lg powders, β-lg aqueous solutions, and freeze-dried capsules with different essential oil loadings (0%, 5%, 10%, 15% and 20% on a coating material weight basis) as well as emulsions prepared at different pH conditions (2.5, 6.6 and 8.0). The infrared spectra of samples were recorded in the wavenumber region between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1}. For each sample, spectra (128 co-added scans) were recorded in triplicate and subsequently averaged by OMNIC 7.0. Resolution of the spectra was enhanced by using Fourier self-deconvolution (FSD) in OMNIC 7.0, with a bandwidth of 44.4 and enhancement of 3.0. The area of the amide I band in the FSD spectra was normalized, and the area of each amide I band component was determined with baseline correction.

3.4 Results and Discussion

3.4.1 Physical Properties

3.4.1.1 Hygroscopicity

Moisture is an important factor affecting the shelf life and stability of microencapsulated particles since the absorption of water will lead to the loss of bioactive compounds and influence the rates of lipid oxidation. Hygroscopicity is a measure of water uptake by a sample from the surroundings over a period of storage. The hygroscopicity values of the freeze-dried protein powders containing different essential oils are shown in Figure 3.1. It can be seen that the hygroscopicity of the microcapsules slightly increased with higher initial β-lg concentrations. This may be explained by the hygroscopic nature of β-lg. The hygroscopicity results for rosemary and sage essential oils were similar, ranging from 15.61% to 18.51%. Similar values were reported in a previous study on microencapsulation of rosemary oil by spray-drying using gum Arabic, ranging between 15.87% and 18.90% (Fernandes *et al.*, 2013). However, the values

in Figure 3.1 for microcapsules containing oregano oil (8.18 to 11.97%) are lower than those for the other essential oil samples. This may be due to the interactions between the essential oil and protein molecules, which reduced the possibility for proteins to bind with water.

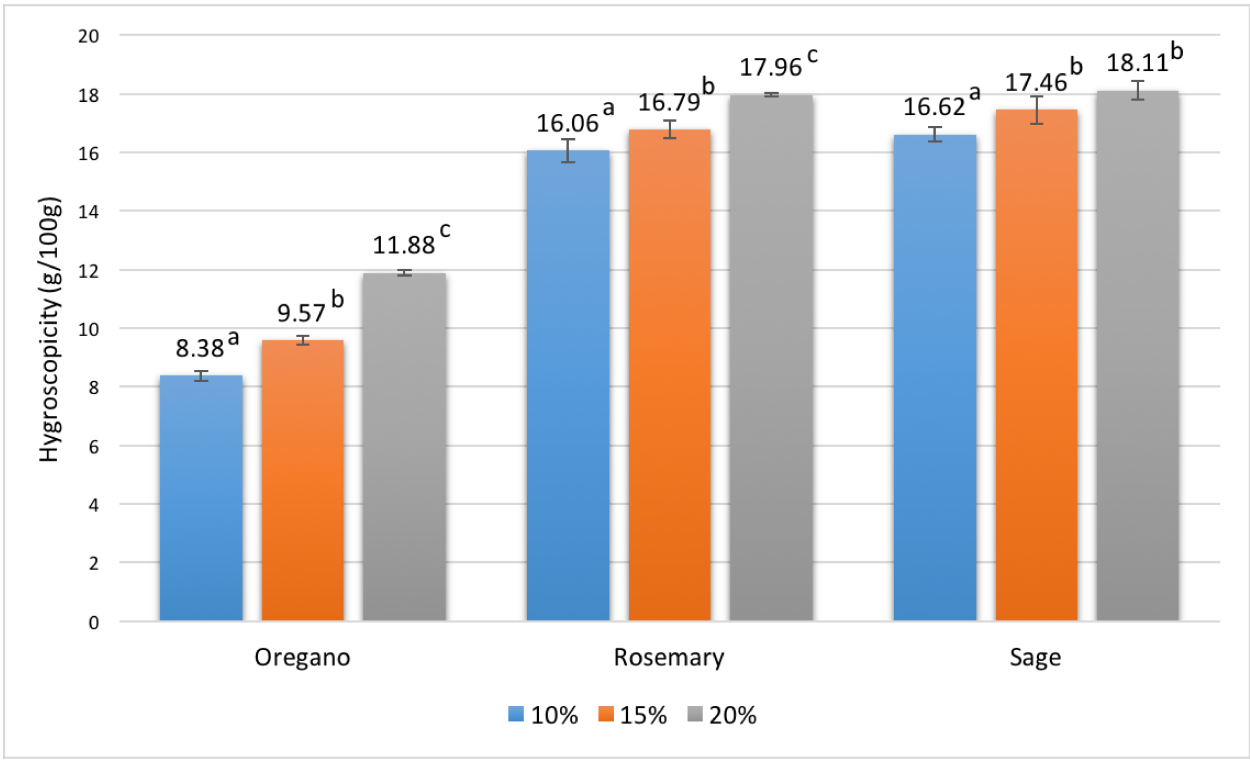

Figure 3.1. Hygroscopicity of freeze-dried protein microcapsules containing different essential oils. The initial protein concentrations are indicated by different colors of the bars. For each type of essential oil, values with different letters are significantly different ($P < 0.05$).

3.4.1.2 Bulk Tapped Density, Particle Density and Porosity

The density parameters (bulk tapped density, particle density and porosity) of all freeze-dried capsules are presented in Table 3.2.

Table 3.2. Bulk tapped density, particle density and porosity of freeze-dried protein microcapsules containing essential oils

Essential oil	Initial protein concentration (%)	Bulk tapped density (g cm^{-3})	Particle density (g cm^{-3})	Porosity (%)
Oregano	10	0.222 ± 0.004^{a}	0.938 ± 0.199^{a}	75.36 ± 4.66^{a}
	15	0.342 ± 0.010^{b}	1.484 ± 0.428^{ab}	75.04 ± 6.81^{a}
	20	0.457 ± 0.017^{c}	2.045 ± 0.383^{b}	76.74 ± 4.93^{a}
Rosemary	10	0.123 ± 0.005^{a}	0.847 ± 0.203^{a}	84.62 ± 3.60^{a}
	15	0.166 ± 0.006^{b}	1.123 ± 0.252^{b}	84.22 ± 4.36^{a}
	20	0.301 ± 0.006^{c}	1.790 ± 0.299^{c}	82.73 ± 2.82^{a}
Sage	10	0.125 ± 0.003^{a}	0.701 ± 0.086^{a}	81.90 ± 1.89^{a}
	15	0.198 ± 0.017^{b}	1.268 ± 0.278^{b}	83.75 ± 3.34^{a}
	20	0.266 ± 0.007^{c}	1.453 ± 0.096^{bc}	81.64 ± 0.91^{a}

* Values are reported as mean ± standard deviation. For each type of essential oil, values with different letters in the same column are significantly different ($P < 0.05$).

In the food processing industry, bulk tapped density is an important quality parameter in relation to packaging, distribution and storage of food powders since it reveals how much of the powder can be filled into a container with fixed volume (Ortega-Rivas, 2012). Compared to bulk density, tapped density is a better indicator of the actual compressibility of a dry product. It is known that food powders with higher tapped density can be stored in smaller containers (Quispe-

Condori *et al.*, 2011). It can be seen from Table 3.2 that the bulk tapped density increased with higher initial protein concentrations ($P < 0.05$) for the dried powders with each essential oil as the core material. Comparison of the values for different essential oil products indicates that the bulk tapped density did not differ significantly between rosemary and sage essential oil samples, whereas the bulk tapped density for the microcapsules containing oregano essential oil was apparently higher. This difference revealed the higher compressibility of the dried powders containing oregano essential oil compared to those containing the other two types of essential oil. Overall, the bulk tapped density results for all samples ranged from 0.117 g cm^{-3} to 0.477 g cm^{-3}, which are similar to the values reported in a previous study on microencapsulation of rosemary essential oil using whey protein and inulin as coating materials (Fernandes *et al.*, 2014).

Particle density of a dry product is an essential parameter since it influences the separation of different particles within a mixture (Somboonvechakarn & Barringer, 2012). Particle density is usually determined by a combination of factors such as the solid density, the internal space of the particles and the specific arrangement of particles if applicable (Dhanalakshmi *et al.*, 2011). The study by Shenoy *et al.* (2015) suggested that particle density significantly influenced the mixture quality of binary mixes. In the present study, the trend for particle density was similar to that of bulk tapped density. The microcapsules produced with higher protein concentrations showed higher particle density values ($P < 0.05$), indicating that higher amounts of coating materials would result in a compression of the particles in the final product. The particle density values did not vary greatly among different essential oil samples at the same initial protein content level. The particle density for all samples was in the range of 0.603 – 2.561 g cm^{-3}, which was acceptable based on comparison with the value (1.55 g cm^{-3}) obtained from a study on encapsulation of cardamom essential oil in mesquite gum (Beristain *et al.*, 2001).

Porosity is a derived value from tapped density and particle density of the dried powders. It is known that the porosity of milk-based powders is influenced by several factors such as drying methods, particle size and storage conditions (Moreau & Rosenberg, 1998). The significance of porosity for powder products is that it controls the rehydration rate, thereby revealing the shelf-life stability (Krokida & Maroulis, 1997). The results in Table 3.2 show that for samples containing the same essential oil, initial protein concentration did not affect the

porosity of the final products ($P > 0.05$). By comparing the values for encapsulation of different essential oils, it can be seen that the porosity for microcapsules containing oregano essential oil (66.68 – 83.36%) was slightly lower than the values for microcapsules containing rosemary (78.08 – 89.67%) and sage (79.66 – 87.86%) essential oils. This result indicated that the powders containing oregano essential oil as the core component would have a lower rehydration rate. The previous study on encapsulation of rosemary essential oil using a whey protein and inulin mixture as wall system obtained similar results (80.2 – 82.8%) for this physical property (Fernandes *et al.*, 2014).

3.4.2 Encapsulation Efficiency

For encapsulation technology, the encapsulation efficiency is an essential index which directly reveals how much of the active compounds can be protected within the coating system. The results for this parameter obtained by direct solvent extraction are shown in Table 3.3.

Table 3.3. Encapsulation efficiency (%) of essential oils (EOs) in freeze-dried microcapsules determined by solvent extraction

Initial protein concentration (%)	Encapsulation efficiency (%)		
	Oregano EO	Rosemary EO	Sage EO
10	29.50 ± 4.91^{a}	33.75 ± 1.94^{a}	30.53 ± 0.75^{a}
15	31.37 ± 2.14^{a}	35.56 ± 5.05^{a}	34.00 ± 1.62^{b}
20	36.31 ± 4.15^{a}	35.78 ± 3.75^{a}	34.76 ± 4.46^{ab}

* Values are reported as mean ± standard deviation. For each column, values with different letters are significantly different ($P < 0.05$).

The overall range for all treatments was 23.12 – 42.13%. It can be seen that the initial protein concentration did not affect the encapsulation efficiency for most samples ($P > 0.05$), as long as the ratio of the amount of essential oil to protein was fixed. Using the same method, the encapsulation efficiency of the microencapsulation of flax oil in zein protein ranged from 32.68% to 59.63% (Quispe-Condori *et al.*, 2011), while the results for encapsulating wheat germ oil in whey protein and maltodextrin mixture were between 51.29% and 89.62% (Yazicioglu *et*

al., 2015). Another study on encapsulation of rosemary essential oil by freeze-drying using whey protein and maltodextrin as the wall materials (Turansan *et al.*, 2015) employed Soxhlet extraction to determine the encapsulation efficiency and obtained much higher values (69.90 - 95.54%). In general, the encapsulation efficiency of a microencapsulation process is usually influenced by multiple factors involving the identity of the wall materials, the concentration or relative ratio of each component, the encapsulation method, and the method used to determine encapsulation efficiency, among others. The relatively low encapsulation efficiency results obtained in this study by comparison with the other studies mentioned above can be explained by differences in wall system selection and extraction methods. A mixture of coating materials will further increase the encapsulation efficiency by synergistic effects. For instance, a study using whey protein and maltodextrin as the coating materials showed reduced droplet sizes in encapsulating orange oil, due to better solubility compared to using whey protein alone (Akhta & Dickinson, 2007). The importance of carbohydrates in wall systems is attributed to their enhancement of the drying properties of the final products (Kagami *et al.*, 2003). The presence of whey protein was important due to its excellent emulsifying capacity. Previous studies have indicated that a higher amount of whey proteins would result in higher encapsulation efficiency due to the reduction in viscosity of the emulsion induced by protein structural changes (Jafari *et al.*, 2008).

Determination of encapsulation efficiency by solvent extraction and Soxhlet extraction may also result in different results. In this study, preliminary tests using Soxhlet extraction did not efficiently extract the surface oils from the microcapsules, and hence direct solvent extraction was employed. In addition, it should be noted that for the calculation of the encapsulation efficiency using Equation (3), the loss of essential oil during freeze-drying was assumed to be negligible, and therefore the oil retention in the freeze-dried powders was assumed to be 100%. The common method used for determining total oil retention in the literature is hydrodistillation in a Clevenger-type apparatus (Baranauskiene *et al.*, 2005; Turasan *et al.*, 2015). In a study on encapsulation of rosemary essential oil in a whey protein and maltodextrin mixture (3:1), the total oil retention after freeze drying ranged from 68.68% to 94.80% (Turasan *et al.*, 2015). Thus, in extending the present studies, experimental determination of oil retention during freeze drying will be an important consideration for the development of essential oil encapsulations in different wall systems.

3.4.3 Antioxidant Activity

Determination of antioxidant activity by β-carotene assay was based on the reaction between β-carotene and the radicals produced by linoleic acid oxidation, which results in gradual loss of the orange-/yellow color (Mikami *et al.*, 2009). Thus, this method actually measures the ability of antioxidants to slow down the color degradation by scavenging the free radicals (Miguel, 2010). For samples containing different essential oils, the average rate of β-carotene bleaching over 2 hours is shown in Figure 3.2 and the percentage of inhibition is calculated and listed in Table 3.4.

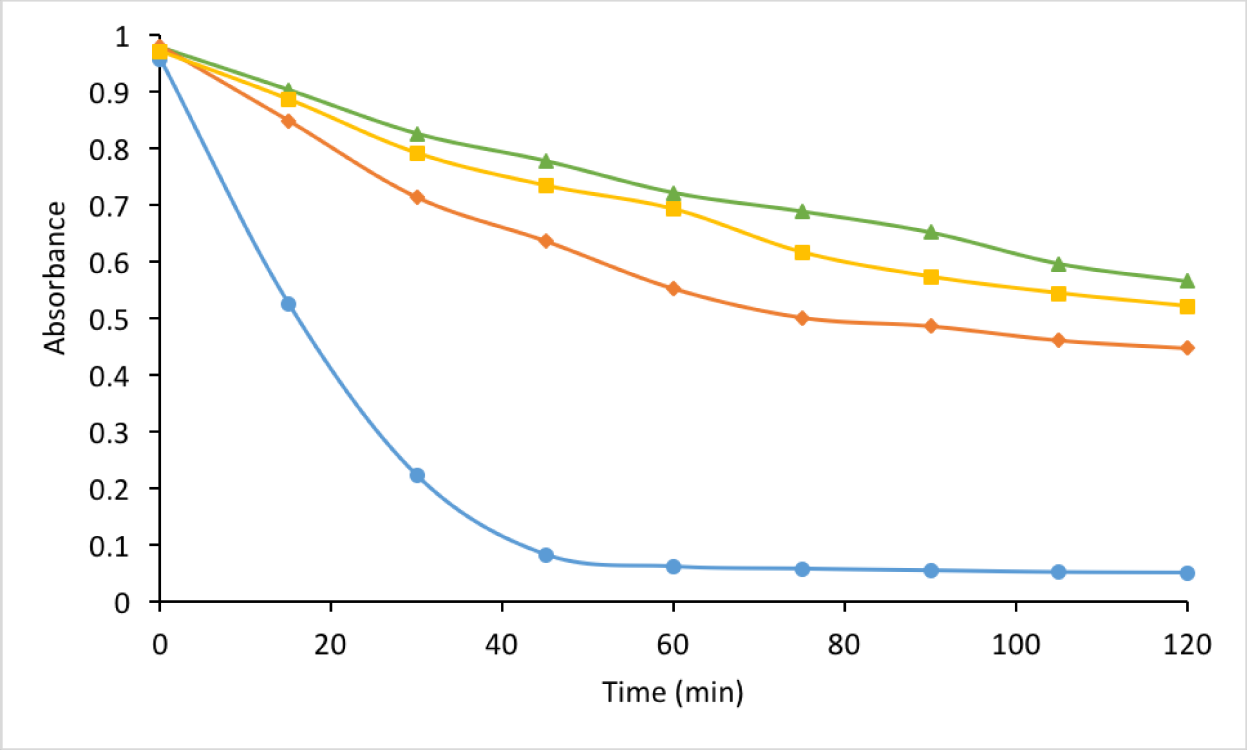

Figure 3.2. Oxidation rate of β-carotene assay over time for the treatment with microcapsules containing oregano (◆), rosemary (■) and sage (▲) essential oils, compared to control (●).

Table 3.4. Percent inhibition of β-carotene oxidation after 120-min treatment with freeze-dried microcapsules containing different essential oils

Essential oil	% Inhibition
Oregano	42.86 ± 1.82^{a}
Rosemary	51.06 ± 3.46^{b}
Sage	55.50 ± 1.52^{b}

***** Values are reported as mean ± standard deviation. Values with different letters are significantly different ($P < 0.05$).

From Figure 3.2, it can be seen that all microcapsules containing different essential oils showed significantly reduced oxidation rates, compared to the control group. Although the absorbance for all samples decreased gradually, the results demonstrated effectiveness of antioxidant activity of freeze-dried capsules containing all three types of essential oil. Table 3.4 reveals slight differences in the results of the β-carotene bleaching test obtained for powders with different essential oils. The dried products with oregano essential oil showed a lower percent inhibition than those with the other two essential oils ($P < 0.05$). This may be explained by multiple reasons. First, the various chemical compositions of different essential oils are fundamental factors that affect their antioxidant activity. The study by Kulisic *et al.* (2004) on evaluation of oregano essential oil obtained more than 50% inhibition using β-carotene bleaching assay. Using the same method, another study on the essential oil of *Hymenocrater longiflorus* Benth. found the antioxidant activity to be 54.6%, 50.0% and 64.7%, for total phenols, polar fraction and non-polar fraction, respectively (Ahmadi, *et al.*, 2010). The major chemical compounds in the essential oil of *Hymenocrater longiflorus* Benth. include α-pinene, 1,8-cineol, β-eudesmol, and spathulenol, some of which are also present in rosemary and sage essential oils (Ahmadi, *et al.*, 2010; Bousbia *et al.*, 2009; Taarit *et al.*, 2009). Second, since microcapsules were tested instead of pure essential oils, the difference in powder solubility is another factor that may have had an impact on the results. In addition, the possible difference in the amount of

available essential oil per unit weight of microcapsules should be also taken into consideration. Previous studies also demonstrated antioxidant activity of essential oil extracts or in the form of microcapsules by other methods such as oxygen radical absorbance capacity assay (Beirao da Costa *et al.*, 2012), conjugated diene assay (Wei & Shibamoto, 2010), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Schultze, *et al.*, 2010), thiobarbituric acid reactive species (TBARS) test (Viuda-Martos *et al.*, 2010) and ferric reducing/antioxidant power (FRAP) assay (Gourine *et al.*, 2010). These methods will give different results in terms of antioxidant activities due to different reactants and detection techniques. Therefore, for further studies, multiple techniques can be applied to evaluate the antioxidant activities of microcapsules containing different essential oils.

3.4.4 *In vitro* Controlled Release

In addition to protecting bioactive compounds from degradation, controlled release into specific environments is one of the purposes of microencapsulation technology in the food industry. In general, the movement of microencapsulated essential oils to the exterior of the micropores is usually achieved by their diffusion via the polymer matrix (Edwards-Jones *et al.*, 2004). The diffusion rate often declines gradually over time, and thus it is of value to study the kinetics in order to evaluate the efficacy of a polymeric matrix for releasing essential oils (Siepmann & Siepmann, 2008). Among the many factors that may affect the diffusion process from the same matrix, the vapor pressure is considered to be the most important driving force (Maderuelo *et al.*, 2011; Thakhiew *et al.*, 2011). In the present study, the kinetics of controlled release for the microcapsules containing different essential oils in PBS was evaluated and is shown in Figure 3.3. Using Equations (5) and (6) based on Fickian diffusion, a mathematical model was generated by non-linear parameter estimation and is also shown in Figure 3.3. The values of the sum of the squared deviations (SSD) indicate how close the model-generated data fit to the experimental results (the smaller the value, the better the fit). The comparison of the release profiles obtained for the three microencapsulated essential oils is shown in Figure 3.4.

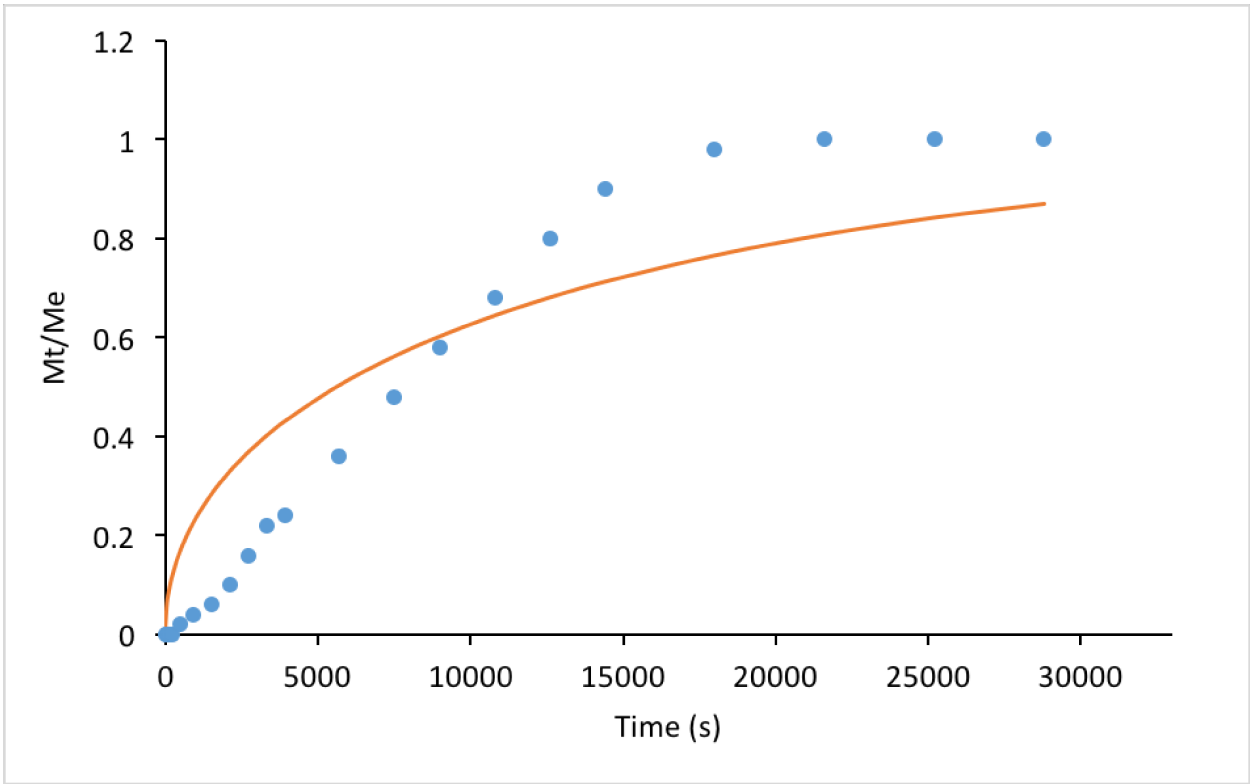

Figure 3.3(a). Kinetics of release of oregano essential oil from freeze-dried β-lg microcapsules over 8 hours. M_t is the mass of the essential oil released at time t and M_e is the mass of the essential oil released at equilibrium. Scatter plot = experimental results; smooth solid line = model-generated data. Sum of squared deviations = 0.49.

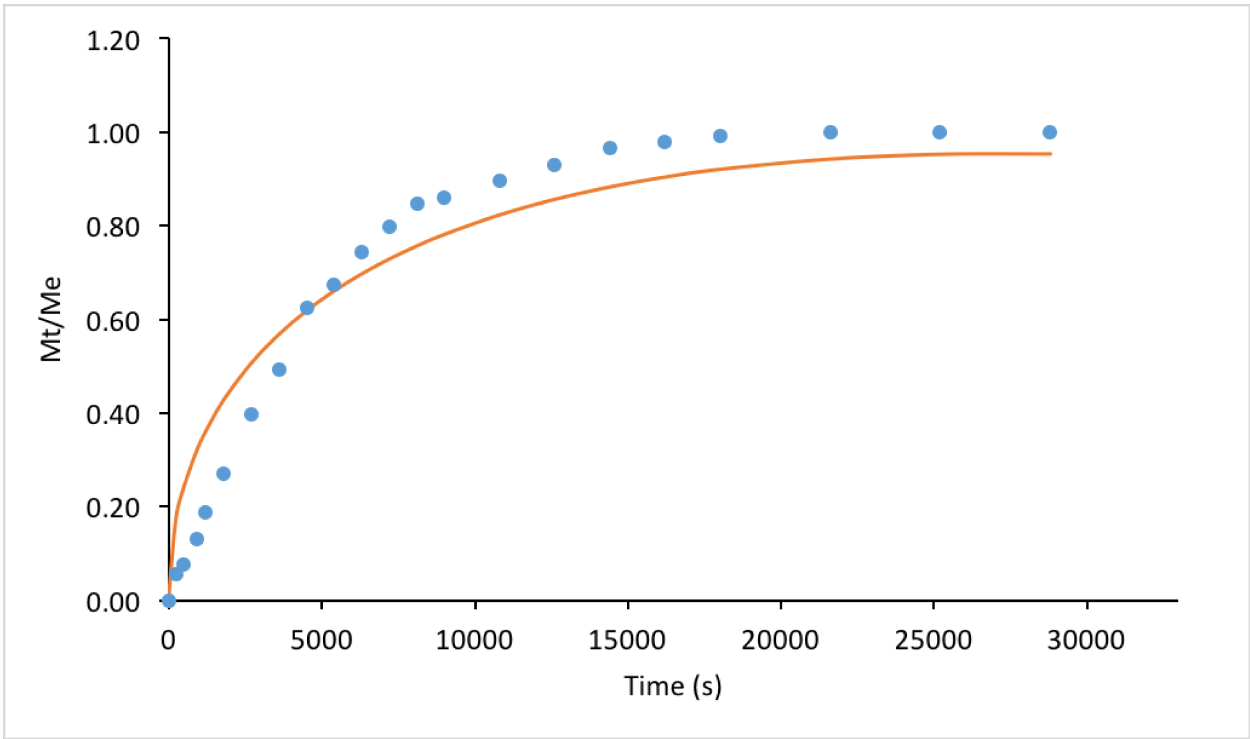

Figure 3.3(b). Kinetics of release of rosemary essential oil from freeze-dried β-lg microcapsules over 8 hours. M_t is the mass of the essential oil released at time t and M_e is the mass of the essential oil released at equilibrium. Scatter plot = experimental results; smooth solid line = model-generated data. Sum of squared deviations = 0.19.

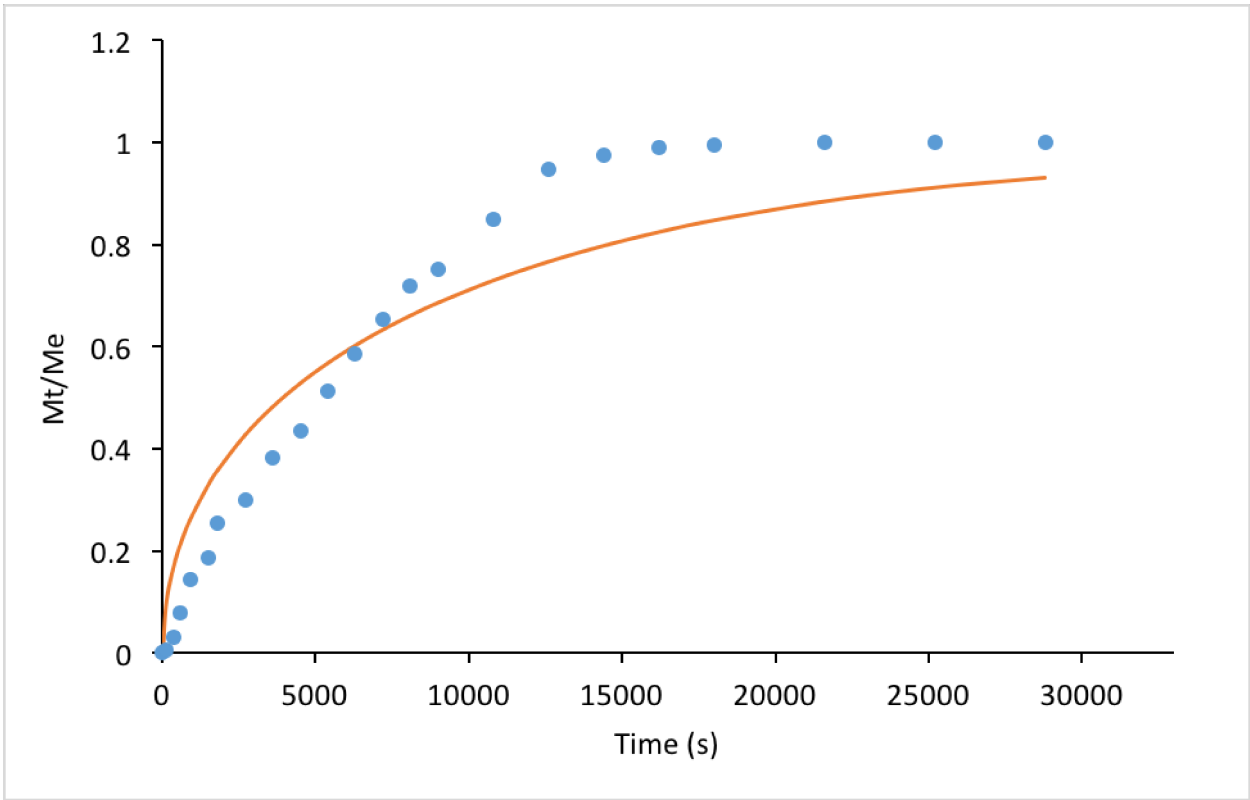

Figure 3.3(c). Kinetics of release of sage essential oil from freeze-dried β-lg microcapsules over 8 hours. M_t is the mass of the essential oil released at time t and M_e is the mass of the essential oil released at equilibrium. Scatter plot = experimental results; smooth solid line = model-generated data. Sum of squared deviations = 0.29.

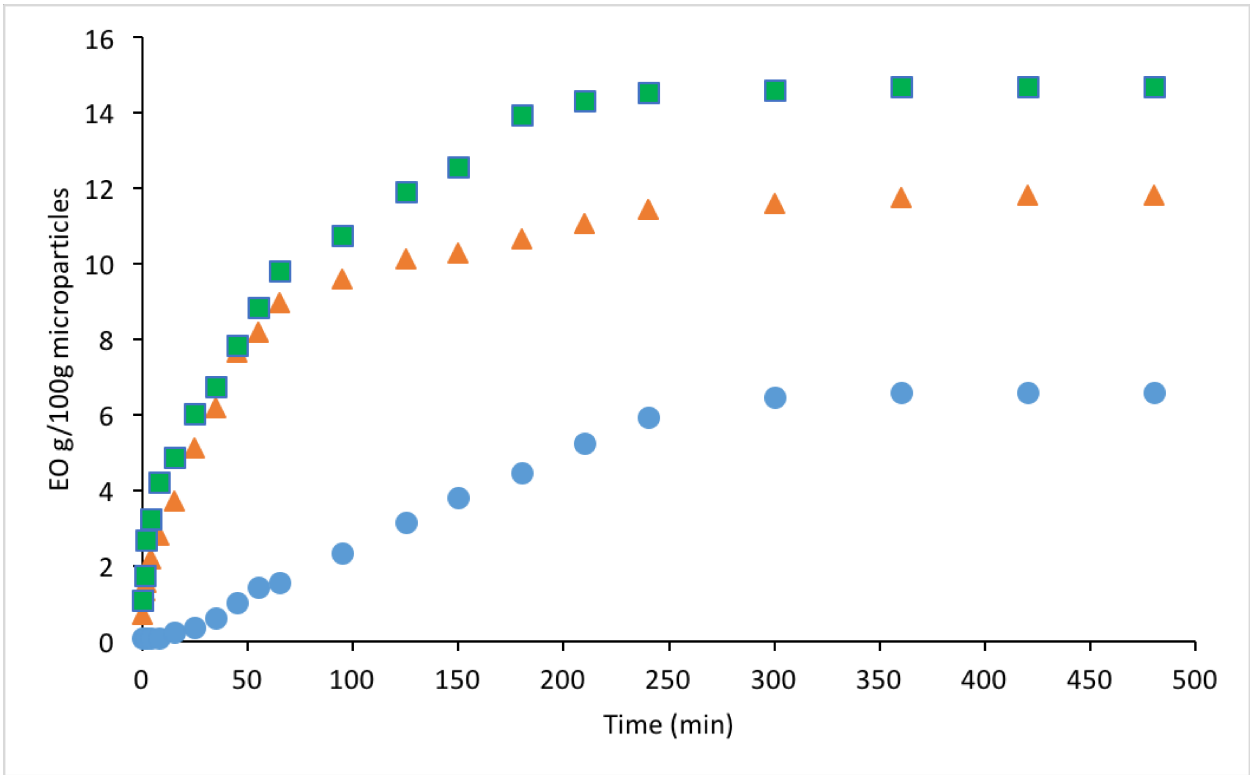

Figure 3.4. Release profiles of freeze-dried microparticles containing different essential oils: oregano (●), rosemary (▲), sage (■) over 8-hour controlled release in PBS. EO= essential oil.

From Figure 3.3, it can be seen that the release of all the essential oils can be fitted to the model-generated values, among which the samples containing rosemary essential oil showed the best fit with the lowest SSD value (0.19). From Figure 3.4 it can be seen that the common trend for all the samples was that most of the essential oils were released during the first 3 hours; after that, the "lag" phase where the essential oil concentrations in PBS became equilibrium was reached. This general trend and the model-fitted data in Figure 3.3 are consistent with the results obtained by another study on microparticles containing oregano essential oil using various types of polymeric matrices (Beirao da Costa *et al.*, 2012). By comparing with different essential oils, it can be seen that the release of rosemary and sage essential oils from the protein matrix was significantly higher than that of oregano essential oil. This may be attributed to the differences in their vapor pressures. Based on literature information on the major components, the vapor pressures of each essential oil at room temperature (~25 ^{o}C) are approximately 0.0296 – 0.0376

mmHg for oregano (Wang *et al.*, 2016), 1.90 mmHg for rosemary (Riddick *et al.*, 1985), and 1.90 mmHg for sage (Barceloux, 2008) essential oil, respectively. Based on this comparison, the vapor pressure of oregano essential oil is significantly lower than that of the other two essential oils, indicating a smaller driving force to initiate the diffusion process. It should be noted that other factors may also influence the diffusion process, such as the interactions between the core and coating materials, the size of the microparticles, and the homogeneity of the essential oils distributed in the micropores, as well as the consistency of the particle dimensions over time (Maderuelo *et al.*, 2011).

3.4.5 FTIR Analysis

3.4.5.1 Freeze-Dried Microcapsules

In order to characterize the freeze-dried microcapsules by FTIR spectroscopy, the FTIR spectra of pure essential oil samples were first recorded and are shown in Figure 3.5. Based on previous studies, the assignments of the major IR bands associated with chemical components unique to each essential oil are presented in Table 3.5 (Beirao da Costa *et al.,* 2012; Gudi *et al.,* 2015; Nowak *et al.,* 2013). The bands in the region between 3000 and 2900 cm^{-1}, assigned to C-H stretching vibrations, are common to all of these essential oils and thus are not listed in the table.

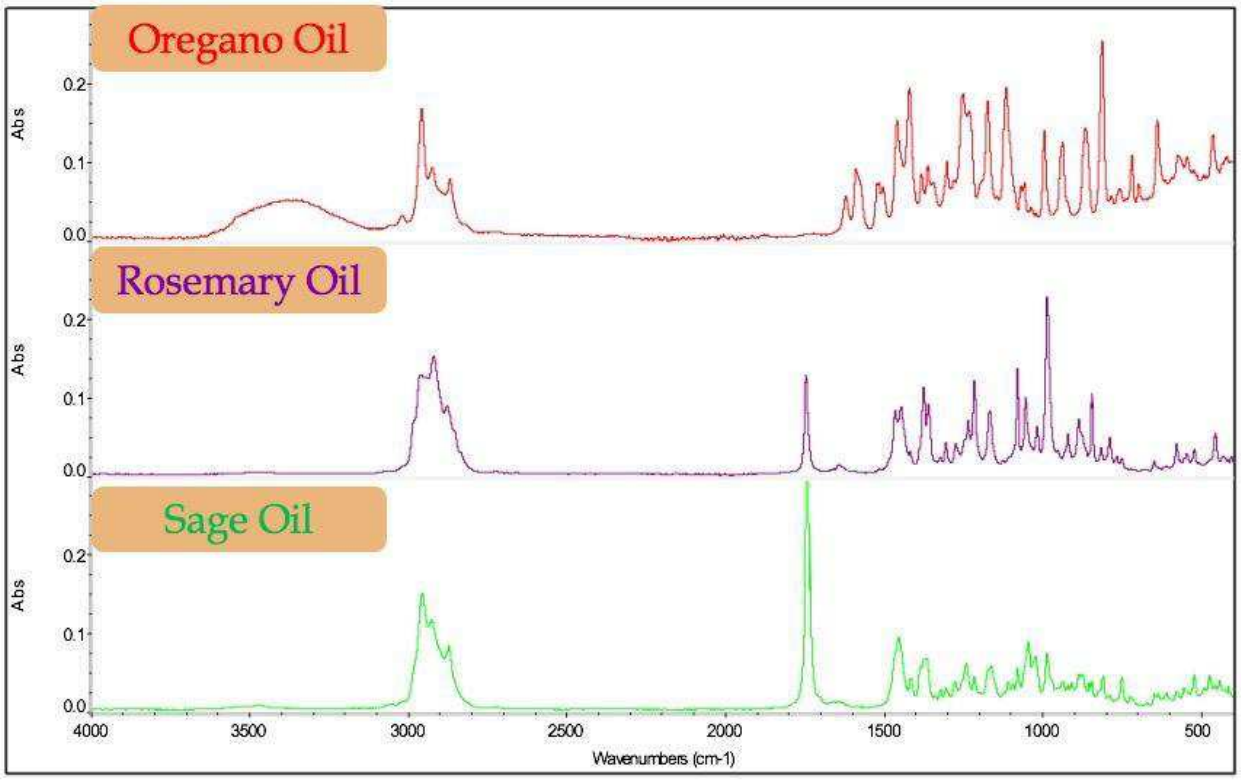

Figure 3.5. Stacked ATR-FTIR spectra of pure essential oil samples. (Abs = absorbance)

Table 3.5. Assignments of major IR peaks of each essential oil sample

Essential oil	**Wavenumber (cm^{-1})**	**Assignment**	**Chemical component**
Oregano	811	C-H out-of-plane bending	Carvacrol
Rosemary	1745	C=O stretching	Camphor
	984	C-H out-of-plane bending	1,8-Cineol
Sage	1745	C=O stretching	α-Thujone, camphor

FTIR spectra of freeze-dried β-lg samples containing each of the three essential oils are presented in Figure 3.6. The bands of the essential oils that are listed in Table 3.5 are marked with asterisks.

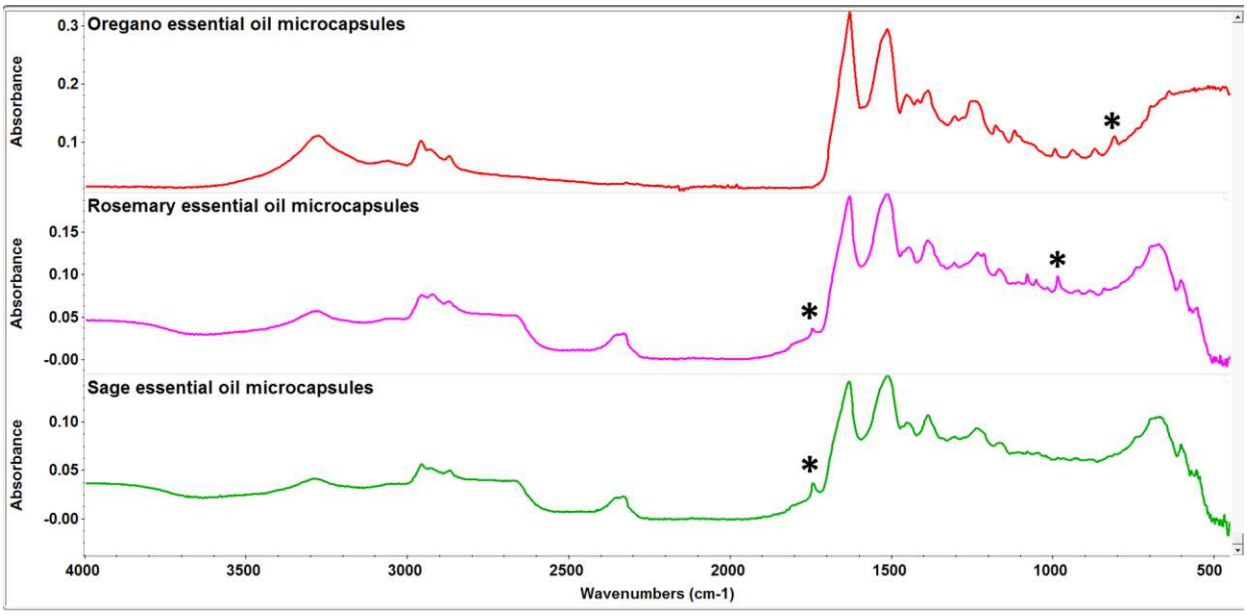

Figure 3.6. Stacked ATR-FTIR spectra of freeze-dried β-lg samples containing essential oils at a concentration of 20%.

The two most intense bands in the spectra in Figure 3.6 are the amide I and amide II bands of β-lg at 1628 and 1514 cm^{-1}, respectively. The amide I band of proteins, which is due mostly to C=O stretching of peptide linkages, consists of overlapping bands of different protein secondary structure components such as α-helix, β-sheets, β-turns and random coils, which can be resolved by Fourier self-deconvolution or other resolution-enhancing methods (Kong & Yu, 2007). Thus, in order to examine the interaction between essential oils and β-lg, analysis of the Fourier self-deconvoluted (FSD) amide I band in the spectra of the freeze-dried β-lg samples containing different concentrations of essential oils was conducted. As the basis for comparison, the FSD amide I band in the spectrum of pure β-lg powder is shown in Figure 3.7, with assignments of secondary structure components. Table 3.6 shows the percent contribution of each secondary-structure component estimated from the relative areas of the component bands in the FSD spectrum. The FSD amide I band in the spectra of the samples containing different concentrations of essential oils is shown in Figure 3.7 and the estimated percentages of

secondary structure components are shown in Table 3.6. It should be noted that the absolute band intensities in these spectra cannot be compared because they are affected by the contact between the sample and the ATR surface, which is variable from sample to sample. On the other hand, the values in Table 3.6 reflect the relative intensities of the amide I component bands and hence can be interpreted in terms of changes in the relative proportions of the different secondary structure components.

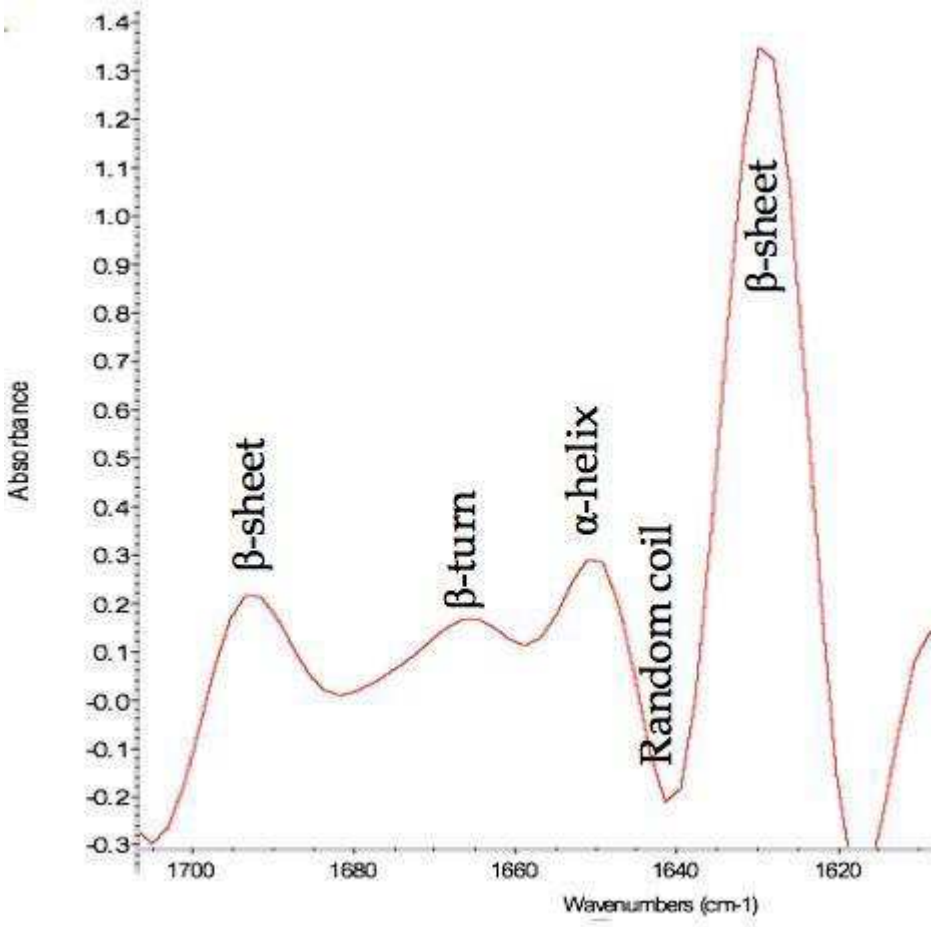

Figure 3.7. Fourier self-deconvoluted amide I region (1700-1600 cm^{-1}) of averaged IR spectra of pure β-lg powder.

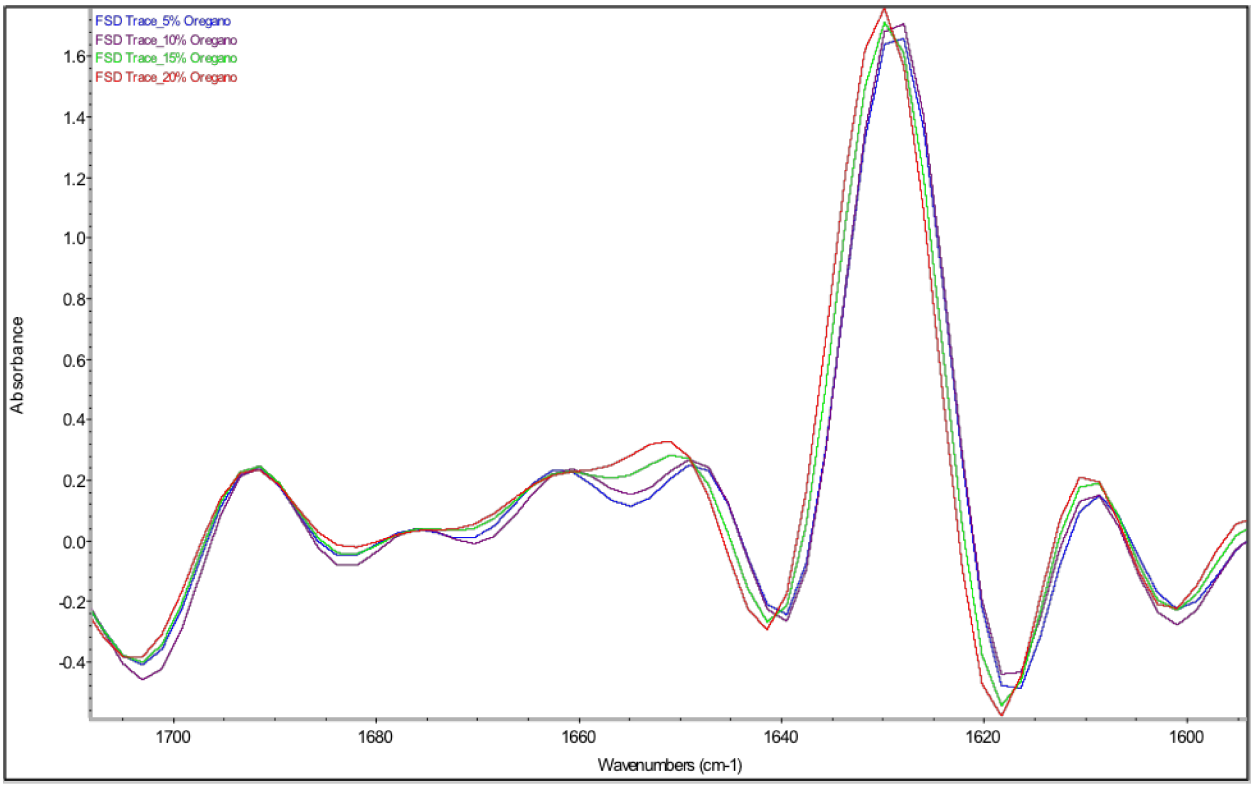

Figure 3.8(a). Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg containing oregano essential oil at different concentrations.

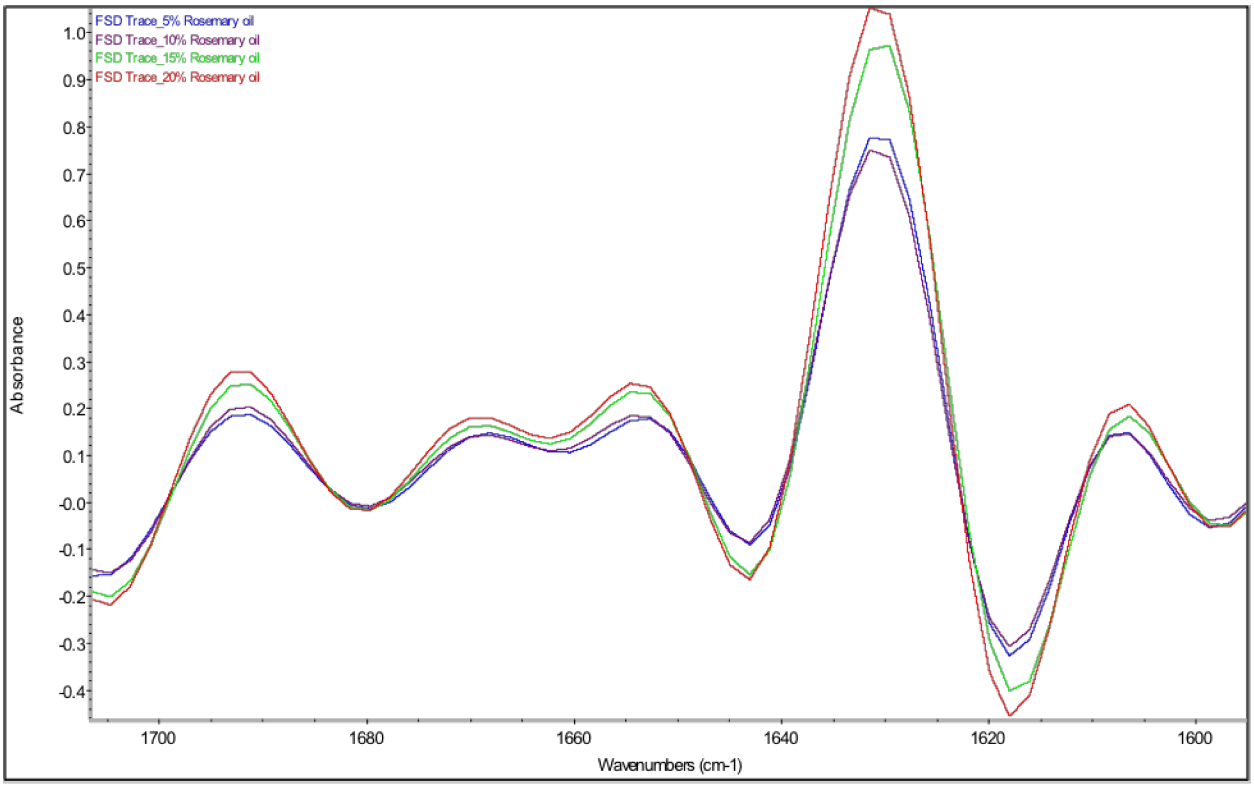

Figure 3.8(b). Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg containing rosemary essential oil at different concentrations.

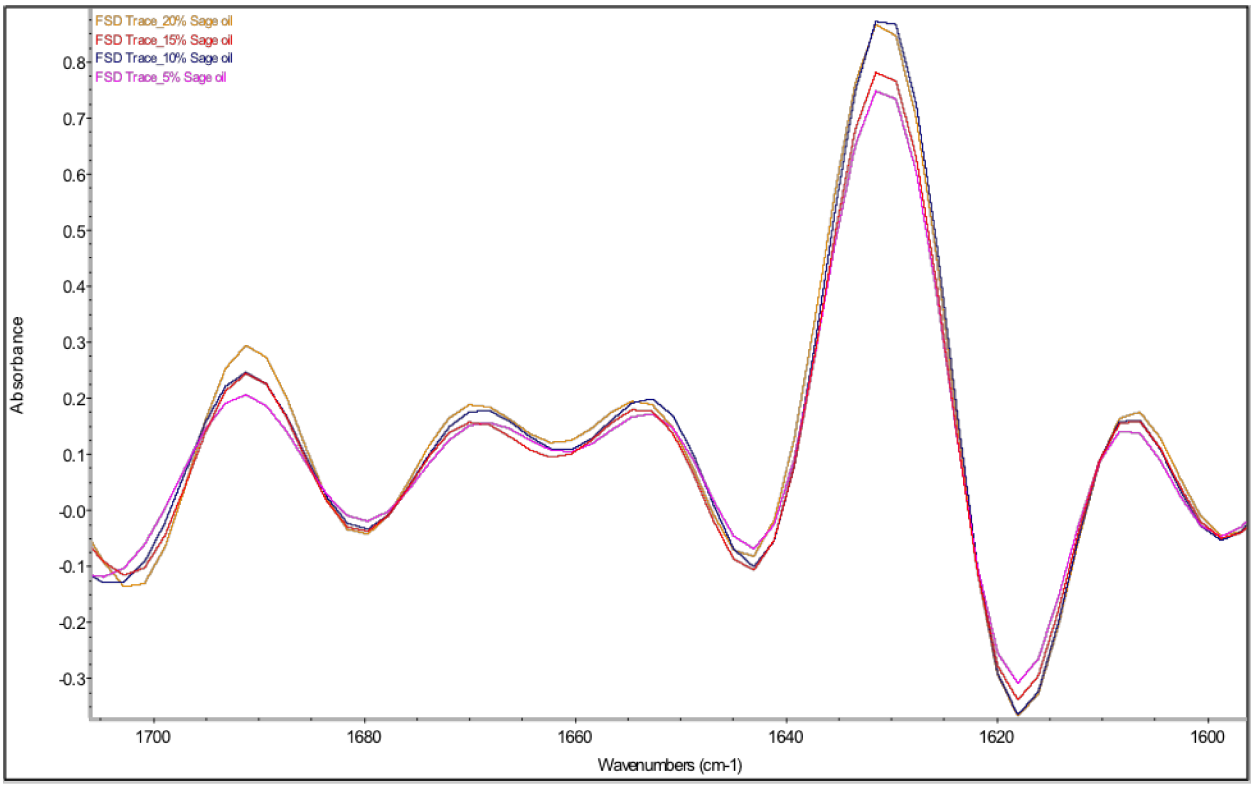

Figure 3.8(c). Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg containing sage essential oil at different concentrations.

Table 3.6. FTIR secondary-structure analysis of freeze-dried β-lg samples containing different essential oils (EOs)

Sample	Percentage of secondary structure (%)		
	α-Helix	**β-Turn**	**β-Sheet**
β-lg (powder)	11.7	3.9	84.4
5% Oregano EO	7.5	5.4	87.0
10% Oregano EO	7.4	5.3	87.2
15% Oregano EO	6.9	2.6	90.4
20% Oregano EO	9.5	1.2	89.3
5% Rosemary EO	8.0	4.3	87.6
10% Rosemary EO	8.4	4.5	87.2
15% Rosemary EO	9.5	4.0	86.4
20% Rosemary EO	9.3	4.1	86.6
5% Sage EO	8.1	5.5	86.3
10% Sage EO	8.7	6.0	85.3
15% Sage EO	8.8	6.6	84.6
20% Sage EO	7.6	7.0	85.5

Comparison of the spectra in Figure 3.8 with the spectrum of β-lg in Figure 3.7 shows little effect of rosemary or sage essential oil on the amide I band profile. In contrast, the spectra of the samples containing oregano essential oil show a different amide I band profile, which is dependent on the concentration of the essential oil. Specifically, in the spectra of the samples containing 5% and 10% oregano essential oil, all the bands with the exception of the band at 1692 cm^{-1} are slightly shifted to lower frequencies, and an additional band at 1675 cm^{-1} is observed. These observations suggest that the protein interacts strongly with components of oregano essential oil, resulting in perturbation of its secondary structure, similar to findings for drug-protein interactions from previous studies by Tajmir-Riahi (2007). However, as the

concentration of oregano essential oil is increased to 15% and 20%, the bands progressively shift back toward their original positions in the spectrum of β-lg and the intensity of the band at 1675 cm^{-1} is substantially diminished.

Additional information on the effects of the essential oils on the secondary structure of β-lg can be provided by examination of the FTIR estimates of the percentages of the secondary structure components in Table 3.6. Based on these estimates, the presence of oregano, rosemary and sage essential oils caused significant decrease in the percentage of α-helical structure and increase in the percentage of β-sheet structure. These results may indicate that the interaction of essential oil components with β-lg occurred at the surface of the protein between the α-helix and β-barrel, as reported in the literature for hydrophobic ligand binding with β-lg (Forrest *et al.*, 2005). It may be noted that the lowest percentages of α-helical structure were obtained for samples containing oregano essential oil at concentrations of 5%, 10%, and 15% while the highest percentages of β-sheet structure were obtained for samples containing oregano essential oil at concentrations of 15% and 20%. The latter samples also exhibited a substantial decrease in the percentage of β-turn structure. Thus, both the band shifts discussed above and the estimated percentages of secondary structure components based on relative band intensities indicate that β-lg interacted more strongly with components of oregano essential oil than with the components of the other two essential oils employed in preparing freeze-dried microcapsules. These findings are consistent with the release profiles presented in Figure 3.4, which showed that the release of oregano essential oil from the β-lg microcapsules was significantly lower than that of rosemary and sage essential oils. For future studies, the effects of varying the protein/essential oil ratio on the release profile should be investigated, as the FTIR studies presented here indicated that the nature of the interactions between β-lg and components of oregano essential oil depended on the concentration of the essential oil.

3.4.5.2 Emulsions

FTIR analysis of the secondary structure of β-lg in emulsions of essential oils prepared at different pH conditions was also performed, to complement the results obtained on the dried samples. Figure 3.9 shows the FSD amide I region in the spectra of these emulsions.

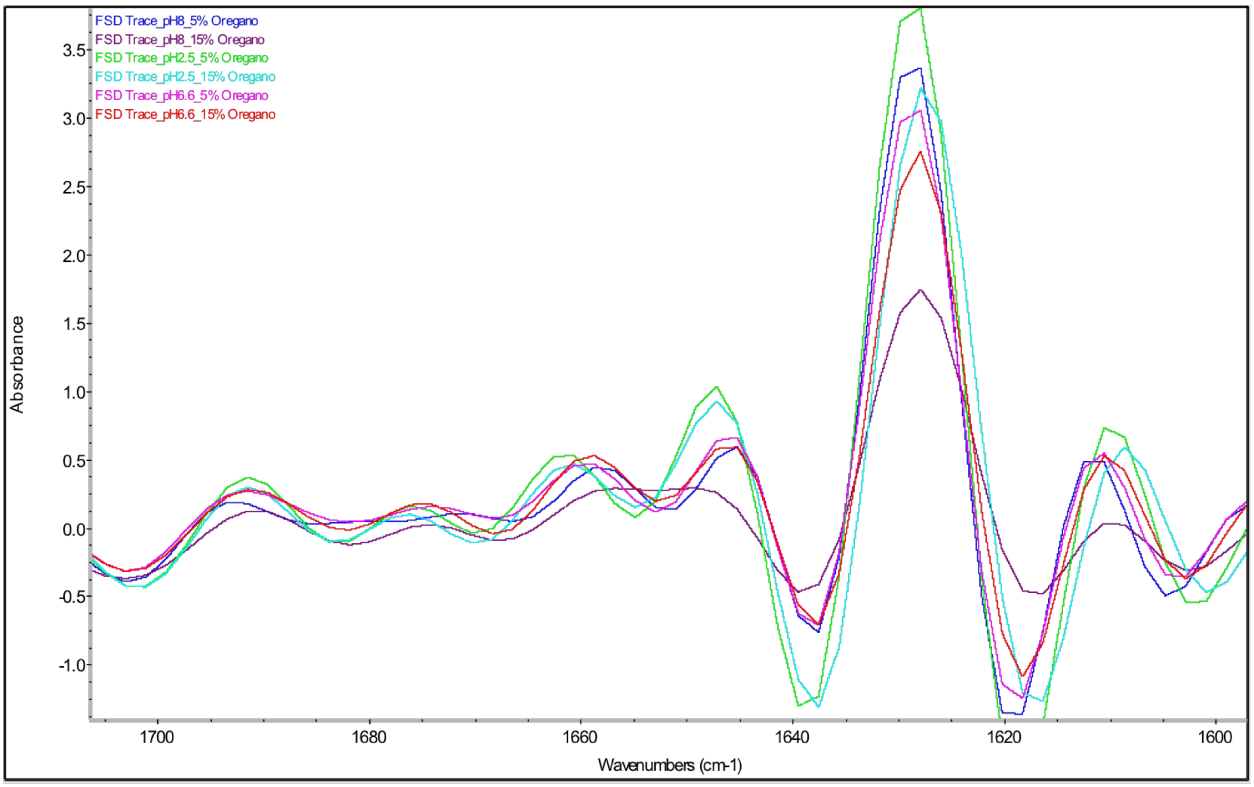

Figure 3.9(a). Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg in emulsions containing oregano essential oil at different pH (2.5, 6.6 and 8.0) conditions.

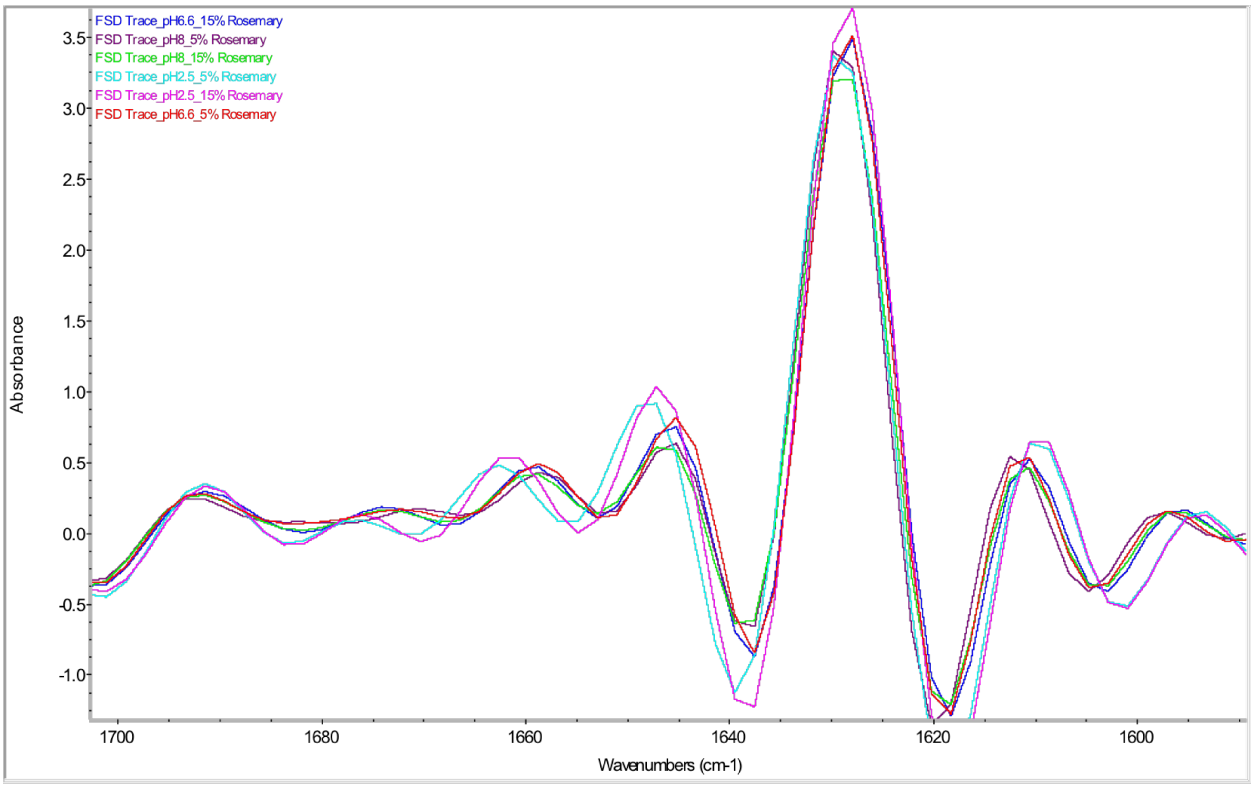

Figure 3.9(b). Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg in emulsions containing rosemary essential oil at different pH (2.5, 6.6 and 8.0) conditions.

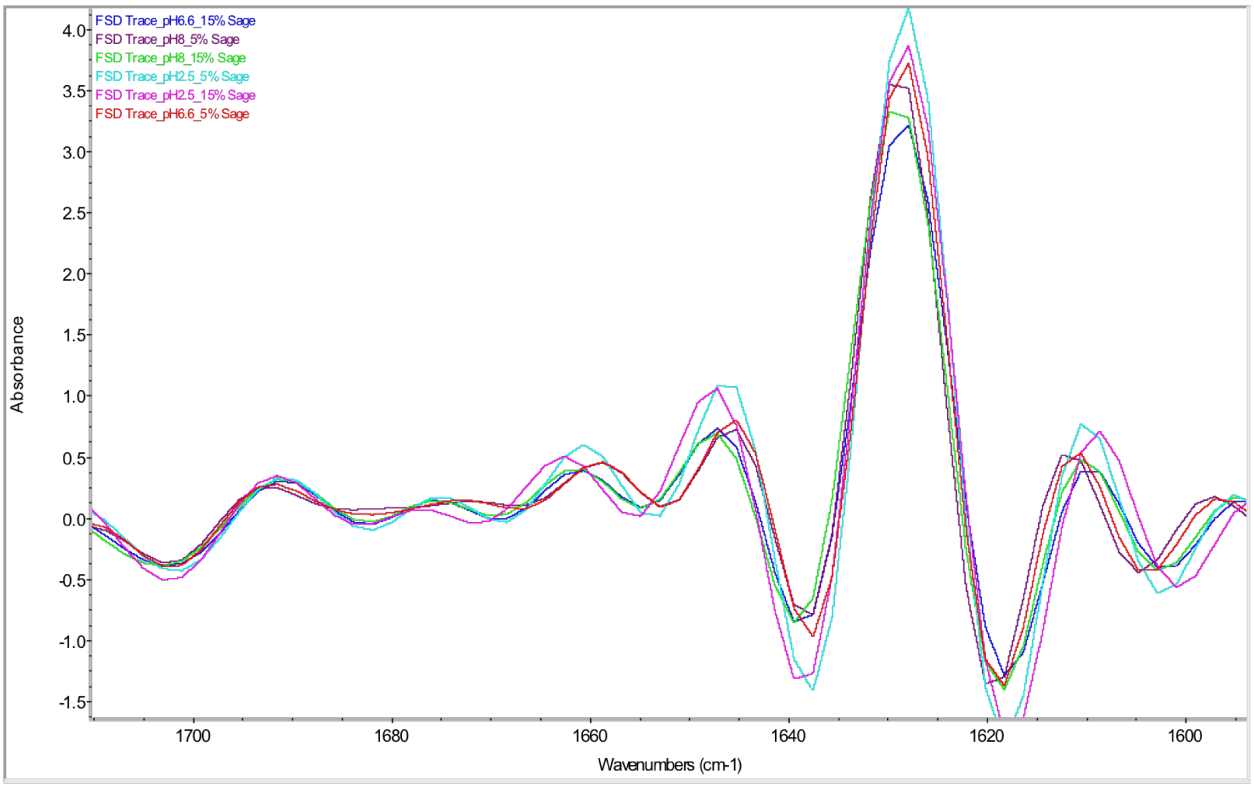

Figure 3.9(c). Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg in emulsions containing sage essential oil at different pH (2.5, 6.6 and 8.0) conditions.

Among the spectra in Figure 3.9, the spectrum of the pH 8 emulsion containing 15% oregano essential oil exhibits a unique amide I band profile characterized by a broad absorption band in the region (1665 – 1640 cm^{-1}) in which the other samples exhibit two well-defined peaks attributed to β-turn and α-helical structures. The absence of these peaks may be indicative of partial denaturation of the protein, although the β-sheet structure appears to remain intact. This emulsion also exhibited higher viscosity than all the other emulsions that were prepared.

Apart from this anomaly, it can be seen from Figure 3.9 that no significant band shifts were observed when comparing the spectra of the pH 6.6 and pH 8.0 emulsions. However, for all emulsions at pH 2.5 except for the one containing 5% sage essential oil, there was an apparent shift in the bands attributed to α-helical and β-turn structures to higher frequencies by comparison with the spectra of the pH 6.6 and pH 8.0 emulsions. No such band shifts as a function of pH were observed for β-lg solutions. Moreover, the positions of the bands attributed

to α-helical and β-turn structures in the spectra of the β-lg solutions were more closely matched in the spectra of the pH 2.5 emulsions than in the spectra of the pH 6.6 and pH 8.0 emulsions, suggesting more extensive interaction between β-lg and the essential oils at the higher pH conditions. This may be related in some manner to the higher proportions of monomeric forms of β-lg when the pH is lower than 3.0, whereas the protein is usually present as dimers or octamers in the pH range between 3.0 and 8.0 (Zsila, 2003).

The percentages of the secondary structure components estimated from the areas of the amide I band components in the spectra shown in Figure 3.9 are presented in Table 3.7. In order to facilitate examination of these data, the bar graph presented in Figures 3.10 was plotted.

Table 3.7(a). Secondary structure analysis of β-lg (20%) in emulsions containing different essential oils (5% and 15% on a protein weight basis) at pH 6.6

Sample	**Percentage of secondary structure (%)**		
	α-Helix	**β-Turn**	**β-Sheet**
β-lg Solution	12.8	3.7	83.4
5% Oregano EO	11.6	5.3	83.0
15% Oregano EO	12.0	6.8	81.2
5% Rosemary EO	12.8	4.9	82.4
15% Rosemary EO	12.2	5.0	82.8
5% Sage EO	12.6	4.6	82.8
15% Sage EO	14.3	4.5	81.1

Table 3.7(b). Secondary structure analysis of β-lg (20%) in emulsions containing different essential oils (5% and 15% protein weight basis) at pH 2.5

Sample	Percentage of secondary structure (%)		
	α-Helix	**β-Turn**	**β-Sheet**
β-lg Solution	17.7	5.0	77.2
5% Oregano EO	15.8	5.1	79.0
15% Oregano EO	16.0	4.8	79.2
5% Rosemary EO	16.1	5.0	78.9
15% Rosemary EO	16.9	6.3	76.7
5% Sage EO	18.5	6.0	75.5
15% Sage EO	19.6	5.3	75.0

Table 3.7(c). Secondary structure analysis of β-lg (20%) in emulsions containing different essential oils (5% and 15% protein weight basis) at pH 8.0.

Sample	Percentage of secondary structure (%)		
	α-Helix	**β-Turn**	**β-Sheet**
β-lg Solution	12.8	4.1	83.1
5% Oregano EO	12.0	4.9	83.1
15% Oregano EO	7.5[a]	3.5[a]	89.1
5% Rosemary EO	11.1	3.6	85.3
15% Rosemary EO	10.2	4.2	85.6
5% Sage EO	11.9	4.5	83.6
15% Sage EO	10.9	4.5	84.5

[a]No well-defined peak observed; see text.

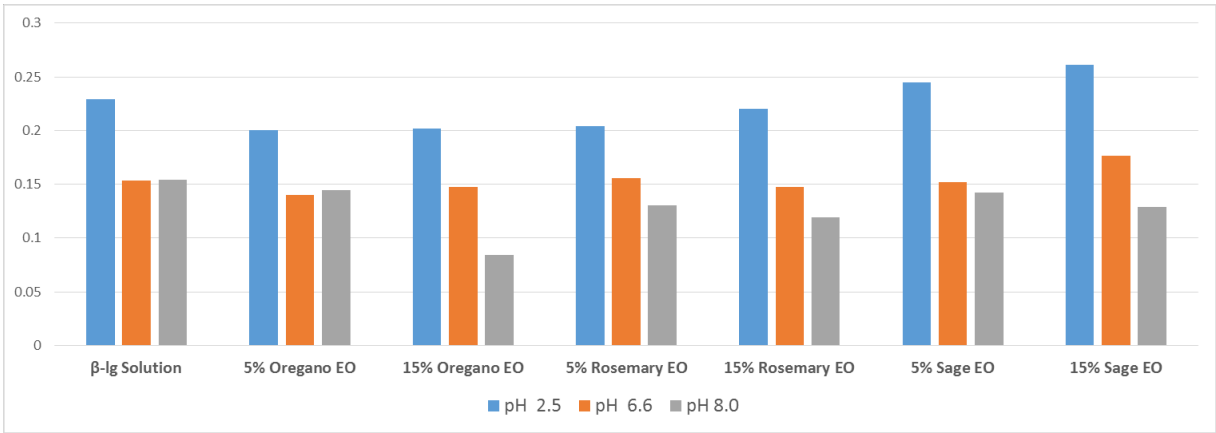

Figure 3.10. Ratios of % α-helical structure to % β-sheet structure estimated from the Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) in IR spectra of β-lg in solution and in emulsions containing essential oils at different pH (2.5, 6.6 and 8.0) conditions.

Figure 3.10 shows that the ratio of % α-helical structure to % β-sheet structure, as estimated by FTIR analysis, was higher at pH 2.5 than at pH 6.6 and pH 8.0 for β-lg solutions as well as for all the essential oil emulsions. By comparison with this effect of acidic pH, the type of essential oil and its concentration (5% or 15% of the protein concentration on a weight basis) had minor effects on this ratio. The sole exception is the anomalously low value of this ratio in the case of the pH 8 emulsion containing 15% oregano essential oil. In fact, as noted above, the FSD amide I band in the spectrum of this emulsion did not exhibit any well-defined peaks in the frequency ranges associated with α-helical structure and β-turns. In contrast to the results presented in Section 3.4.5.1 for the freeze-dried microcapsules, which showed that the presence of essential oil resulted in a significant decrease in the percentage of α-helical structure and increase in the percentage of β-sheet structure, no such trend was consistently obtained for the emulsions at any pH. Similarly, the changes in the estimated percentage of β-turns observed among microcapsules prepared with different types and concentrations of essential oil shown in in Section 3.4.5.1 (Table 3.6) were not apparent in the case of the emulsion spectra. However inhomogeneous distribution of the essential oils in these emulsions was a potential source of variability in these experiments.

3.5 Conclusion

In conclusion, the microencapsulation of oregano, rosemary and sage essential oils in β-lg by freeze-drying was successfully performed in this study. The encapsulation efficiency and oil retention were determined to be in the range of 28.35-42.13% and 55.91-75.45%, respectively. The microparticles containing oregano essential oil showed a lower hygroscopicity, higher bulk and particle density and lower porosity, compared to those containing rosemary essential oil and sage essential oil, revealing a lower rehydration rate and higher shelf-life stability. All dried powders containing the essential oils showed significant antioxidant activities via a β-carotene bleaching test. In a phosphate buffer solution, rosemary and sage essential oils were released readily and the amount released was much higher than that for oregano essential oil. FTIR analysis on protein conformational changes demonstrated there were significant interactions between essential oil components and β-lg, particularly in the case of oregano essential oil. Potential binding sites were proposed to be in the cleft on the surface of the protein between the α-helix and the β-barrel.

For future studies, the encapsulation process of essential oils can be further developed by improving the homogenization step or using a secondary coating material. The controlled release study of essential oil microcapsules in organic solvents or *in vivo* study may be of interest. The hydrophobic ligand binding between the major essential oil components with β-lg can be further investigated to determine the binding sites and binding affinities by other techniques such as fluorescence spectroscopy and isothermal titration calorimetry. Additionally, studying other essential oils for which there is less information available in the literature will also be of interest.

3.6 References

Ahmadi, F., Sadeghi, S., Modarresi, M., Abiri, R., & Mikaeli, A. (2010). Chemical composition, *in vitro* anti-microbial, antifungal and antioxidant activities of the essential oil and methanolic extract of *Hymenocrater longiflorus* Benth. of Iran. *Food and Chemical Toxicology, 48*(5), 1137-1144.

Akhta, M., & Dickinson, E. (2007). Whey protein-maltodextrin conjugates as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids, 21*(4), 607-616.

Andrade, M.A., Cardoso, M.G., Andrade, J., Silva, L.F., Teixeira, M.L., Resende, J.M.V., Figueiredo, A.C., & Barroso, J.G. (2013). Chemical composition an antioxidant activity of essential oils from *Cinnamodendron dinisii* Schwacke and *Siparuna guianensis* Aublet. *Antioxidants, 2*(4), 384-397.

Bakry, A.M., Abbas, S., Ali, B., Majeed, H., Abouelwafa, M.Y., Mousa, A., & Liang, L. (2016). Microencapsulation of oils: A comprehensive review of benefits, techniques and applications. *Comprehensive Reviews in Food Science and Food Safety, 15*(1), 143-182.

Baranauskiene, R., Venskutonis, P.R., Dewettinck, K., & Verhe, R. (2005). Properties of oregano (*Origanum vulgare* L.), citronella (*Cymbopogon nardus* G.) and marjoram (*Majorana hortensis* L.) flavors encapsulated into milk protein-based matrices. *Food Research International, 39*, 413-425.

Barceloux, D.G. (2008). *Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals.* Hoboken, NJ: John Wiley and Sons.

Beirao da Costa, S., Duarte, C., Bourbon, A.I., Pinheiro, A.C., Serra, A.T., Martins, M.M., Januario, M.I.N., Vicente, A.A., Delgadillo, I., Duarte, C., & Beirao da Costa, M.L. (2012). Effect of the matrix system in the delivery and in vitro bioactivity of microencapsulated oregano essential oil. *Journal of Food Engineering, 110*(2), 190-199.

Beristain, C.I., Garcia, H.S., & Vernon-Carter, E.J. (2001). Spray-dried encapsulation of cardamom (*Elettaria cardamomum*) essential oil with mesquite (*Prosopis juliflora*) gum. *LWT-Food Science and Technology, 34*(6), 398-401.

Bernard, C., Regnault, S., Gendreau, S., Charbonneau, S., & Relkin, P. (2011). Enhancement of emulsifying properties of whey proteins by controlling spray-drying parameters. *Food Hydrocolloids, 25*(4), 758-763.

Bousbia, N., Vian, M.A., Ferhat, M.A., Petitcolas, E., Meklati, B.Y., & Chemat, F. (2009). Comparison of two isolation methods for essential oil from rosemary leaves: Hydrodistillation and microwave hydrodiffusion and gravity. *Journal of Food Chemistry, 114*(1), 355-362.

Bylaite, E., Venskutonis, P.R., & Mazdzieriene, R. (2001). Properties of caraway (*Carum carvi* L.) essential oil encapsulated into milk protein-based matrices. *European Food Research and Technology, 212*(6), 661-670.

Cai, Y.Z., & Corke, H. (2000). Production and properties of spray-dried *Amaranthus betacyanin* pigments. *Journal of Food Science, 65*(7), 1248-1252.

Chen, L., & Subirade, M. (2007). Effect of preparation conditions on the nutrient release properties of alginate-whey protein granular microspheres. *European Journal of Pharmaceutics and Biopharmaceutics, 65*(3), 354-362.

Chranioti, C., Karaberi, A., Tsakanika, L-A., & Tzia, C. (2016). Freeze-dried fennel oleoresin products formed by biopolymers: Storage stability and characterization. *Food and Bioprocess Technology, 9*(12), 2002-2011.

Dhanalakshmi, K., Ghosal, S., & Bhattacharya, S. (2011). Agglomeration of food powder and applications. *Critical Reviews in Food Science and Nutrition, 51*(5), 432-441.

Edward-Jones, V., Buck, R., Shawcross, S.G., Dawson, M.M., & Dunn, K. (2004). The effect of essential oils on methicillin-resistant *Staphylococcus aureus* using a dressing model. *Burns, 30*(8), 772-777.

Fearon, I.M., & Faux, S.P. (2009). Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. *Journal of Molecular and Cellular Cardiology, 47*(3), 372-381.

Fernandes, R.V.B., Borges, S.V., & Botrel, D.A. (2013). Influence of spray drying operating conditions on microencapsulated rosemary oil properties. *Ciencia e Tecnologia de Alimentos, 33*(1), 171-178.

Fernandes, R.V.B., Borges, S.V., Botrel, D.A., & Rodrigues, C. (2014). Physical and chemical properties of encapsulated rosemary essential oil by spray drying using whey protein-inulin blends as carriers. *International Journal of Food Science and Technology, 49*(6), 1522-1529.

Forrest, S.A., Yada, R.Y., & Rousseau, D. (2005). Interactions of vitamin D_3 with bovine beta-lactoglobulin and beta-casein. *Journal of Agricultural and Food Chemistry, 53*(1), 8003-8009.

Goula, A.M., & Adamopoulus, K.G. (2008). Effect of maltodextrin addition during spray drying of tomato pulp in dehumidified air: II. Powder properties. *Drying Technology, 26*(6), 726-737.

Gourine, N., Yousfi, M., Bombarda, I., Nadjemi, B., Stocker, P., & Gaydou, E.M. (2010). Antioxidant activities and chemical composition of essential oil of *Pistacia atlantica* from Algeria. *Industrial Crops and Products, 31*(2), 203-208.

Govaris, A., Solomakos, N., Pexara, A., & Chatzopoulou, P. (2010). The antimicrobial effect of oregano essential oil, nisin and their combination against *Salmonella Enteritidis* in minced sheep meat during refrigerated storage. *International Journal of Food Microbiology, 137*(2), 175-180.

Gudi, G., Krahmer, A., Kruger, H., & Schulz, H. (2015). ATR-FTIR spectroscopy on intact dried leaves of sage (*Salvia officinalis* L.) - Accelerated chemotaxonomic discrimination and analysis of essential oil composition. *Journal of Agricultural and Food Chemistry, 63*(1), 8743-8750.

Gunasekaran, S., Xiao, L., & Ould Eleya, M.M. (2006). Whey protein concentrate hydrogels as bioactive carriers. *Journal of Applied Polymer Science, 99*(5), 2470-2476.

Huang, M-T., Ghai, G., & Ho, C-T. (2004). Inflammatory process and molecular targets for anti-inflammatory nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety, 3*(4), 127-139.

Hundre, S.Y., Karthik, P., & Anandharamakrishnan, C. (2015). Effect of whey protein isolate and β-cyclodextrin wall systems on stability of microencapsulated vanillin by spray-freeze drying method. *Food Chemistry, 174*(1), 16-24.

Hussain, A.I., Anwar, F., Rasheed, S., Nigam, P.S., Janneh, O., & Sarker, S.D. (2011). Composition, antioxidant and chemotherapeutic properties of the essential oils from two *Origanum* species growing in Pakistan. *Revista Brasileira de Farmacognosia, 21*(6), 943-952.

Jafari, S-M., Mahdavi-Khazaei, K., & Hemmati-Kakhki, A. (2016). Microencapsulation of saffron petal anthocyanins with cress seed gum compared with Arabic gum through freeze drying. *Carbohydrate Polymers, 140*, 20-25.

Jafari, S.M., Assadpoor, E., He, Y., & Bhandari, B. (2008). Encapsulation efficiency of food flavours and oils during spray drying. *Drying Technology, 26*(7), 816-835.

Jalali-Heravi, M., Moazeni, R.S., & Sereshti, H. (2011). Analysis of Iranian rosemary essential oil: Application of gas chromatography-mass spectrometry combined with chemometrics. *Journal of Chromatography A, 1218*(18), 2569-2576.

Kagami, Y., Sugimura, S., Fujishima, N., Matsuda, K., Kometani, T., & Matsumura, Y. (2003). Oxidative stability, structure, and physical characteristics of microcapsules formed by spray drying of fish oil with protein and dextrin wall materials. *Journal of Food Science, 68*(7), 2248-2255.

Karim, F.T., Sarker, Z.M., Ghafoor, K., Al-Juhaimi, F.Y., Jalil, R., Awang, M.B., Amid, M., Hossain, M.D.S., & Khalil, H.P.S.A. (2016). Microencapsulation of fish oil using hydroxypropyl methylcellulose as a carrier material by spray drying. *Journal of Food Processing and Preservation, 40*(2), 140-153.

Kaur, C., & Kapoor, H.C. (2001). Antioxidants in fruits and vegetables: the millennium's health. *International Journal of Food Science and Technology*, *36*(7), 703-725.

Kim, Y.D., & Morr, C.V. (1996). Microencapsulation properties of gum arabic and several food proteins: spray-dried orange oil emulsion properties. *Journal of Agricultural and Food Chemistry, 44*(5), 1314-1320.

Kimpel, F., & Schmitt, J.J. (2015). Review: Milk proteins as nanocarrier systems for hydrophobic nutraceuticals. *Journal of Food Science, 80*(11), 2361-2366.

Kong, J., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochimica et Biophysica Sinica, 39*(8), 549-559.

Krokida, M.K., & Maroulis, Z.B. (1997). Effect of drying method on shrinkage and porosity. *Drying Technology, 15*(10), 2441-2458.

Krokida, M.K., & Philippopoulos, C. (2006). Volatility of apples during air and freeze drying. *Journal of Food Engineering, 73*, 135-141.

Kulisic, T., Radonic, A., Katalinic, V., & Milos, M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chemistry, 85*(4), 633-640.

Langa, E., Della Porta, G., Palavra, A.M.F., Urieta, J.S., & Mainar, A.M. (2009). Supercritical fluid extraction of Spanish sage essential oil: Optimization of the process parameters and modelling. *The Journal of Supercritical Fluids, 49*(2), 174-181.

Lee, J., Koo, N., & Min, D.B. (2004). Reactive oxygen species, aging and antioxidative nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety, 3*(1), 21-33.

Liveney, Y.D. (2010). Milk proteins as vehicles for bioactives. *Current Opinion in Colloids & Interface Science, 15*(1), 73-83.

Maderuelo, C., Zarzuelo, A., & Lanao, J.M. (2011). Critical factors in the release of drugs from sustained release hydrophilic matrices. *Journal of Controlled Release, 154*(1), 2-19.

Mallet, J.F., Cerati, C., Ucciani, E., Gamisana, J., & Gruber, M. (1994). Antioxidant activity of fresh pepper (*Capsicum annuum*) cultivars. *Food Chemistry, 49*, 61-65.

Miguel, M.G. (2010). Antioxidant and anti-inflammatory activities of essential oils: A short review. *Molecules, 15*(12), 9252-9287.

Mikami, I., Yamaguchi, M., Shinmoto, H., & Tsuchida, T. (2009). Development and validation of a microplate-based beta-carotene bleaching assay and comparison of antioxidant activity (AOA) in several crops measured by beta-carotene bleaching, DPPH and ORAC assays. *Food Science and Technology Research, 15*(2), 171-178.

Moreau, D.L., & Rosenberg, M. (1998). Porosity of whey protein-based microcapsules containing anhydrous milkfat measured by gas displacement pycnometry. *Journal of Food Science, 63*(5), 819-823.

Naik, A., Meda, V., Lele, S.S. (2014). Freeze drying for microencapsulation of α-linolenic acid rich oil: A functional ingredient from *Lepidium sativum* seeds. *European Journal of Lipid Science and Technology, 116*(7), 837-846.

Nastruzzi, C. (1999). Dextran cross-link gelatin microspheres as a drug delivery system. *European Journal of Pharmaceutics and Biopharmaceutics, 47*(2), 153-160.

Nigg, J., Strobel, G., Knighton, W.B., Hilmer, J., Geary, B., Riyaz-UI-Hassan, S., Harper, J.K., Valenti, D., & Wang, Y. (2014). Functionalized *para*-substituted benzenes as 1,8-cineole production modulators in an endophytic *Nodulisporium* species. *Microbiology*, *160*(8), 1772-1782.

Nowak, A., Kalemba, D., Piotrowska, M., & Czyzowska. (2013). Effects of thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) essential oils on growth of *Brochothrix thermosphacta. African Journal of Microbiology Research, 7*(26), 3396-3404.

O'Neil, M.J. (2013). *The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals*, (pp. 1739). Cambridge, UK: Royal Society of Chemistry.

Orhan, I., Aslan, S., Kartal, M., Sener, B., & Baser, K.H.C. (2008). Inhibitory effect of Turkish *Rosmarinus officinalis* L. on acetylcholinesterase and butyrylcholinesterase enzymes. *Journal of Food Chemistry, 108*(1), 663-668.

Ortega-Rivas, E. (2012). Characterization and processing relevance of food particulate materials. *Particle & Particle Systems Characterization, 29*(3), 192-203.

Pourmortazavi, S.M., & Hajimirsadeghi, S.S. (2007). Supercritical fluid extraction in plant essential and volatile oil analysis. *Journal of Chromatography, 1163*(1), 2-24.

Prata, A.S., Zanin, M.H.A., Re, M.I., & Grosso, C.R.F. (2008). Release properties of chemical and enzymatic crosslinked gelatin-gum Arabic microparticles containing a fluorescent probe plus vetiver essential oil. *Colloids and Surfaces B: Biointerfaces, 67*(2), 171-178.

Quispe-Condori, S., Saldana, D.A., & Temelli, F. (2011). Microencapsulation of flax oil with zein using spray and freeze drying. *LWT-Food Science and Technology, 44*(9), 1880-1887.

Riddick, J.A., Bunger, W.B., & Sakano, T.K. (1985). Techniques of chemistry 4th ed. In *Organic Solvents* (pp. 307). New York, NY: John Wiley and Sons.

Rosenberg, M., & Lee, S.J. (2000). Preparation and some properties of water-insoluble, whey protein-based microcapsules. *Journal of Microencapsulation, 17*(1), 29-44.

Rubiolo, P., Sgorbini, B., Liberto, E., Cordero, E., & Bicchi, C. (2010). Essential oils and volatiles: sample preparation and analysis. A review. *Flavour and Fragrance Journal, 25*(5), 282-290.

Schultze, W., Al-Rehaily, A.J., & Mothana, R.A. (2010). Chemical analysis and biological activity of the essential oils of two endemic Soqotri Commiphora species. *Molecules, 15*(2), 689-698.

Shenoy, P., Viau, M., Tammel, K., Innings, F., Fitzpatrick, J., & Ahrne, L. (2015). Effect of powder densities, particle size and shape on mixture quality of binary food powder mixtures. *Powder Technology, 272*, 165-172.

Siepmann, J., & Siepmann, F. (2008). Mathematical modeling of drug delivery. *International Journal of Pharmaceutics, 364*(2), 328-343.

Silva, K.A., Coelho, M.A., Calado, V.M.A., & Rocha-Leao, M.H.M. (2013). Olive oil and lemon salad dressing microencapsulated by freeze-drying. *LWT - Food Science and Technology, 50*(2), 569-574.

Somboonvechakarn, C., & Barringer, S.A. (2012). Effect of particle density and composition on mixtures during nonelectrostatic and electrostatic powder coating. *Journal of Food Process Engineering, 35*(2), 236-249.

Taarit, M.B., Msaada, K., Hosni, K., Hammami, M., Kchouk, M.E., & Marzouk, B. (2009). Plant growth, essential oil yield and composition of sage (*Salvia officinalis* L.) fruits cultivated under salt stress conditions. *Industrial Crops & Products, 30*(3), 333-337.

Tajmir-Riahi, H.A. (2007). An overview of drug binding to human serum albumin: protein folding and unfolding. *Scientia Iranica, 14*(2), 87-95.

Thakhiew, W., Waisayawan, P., & Devahastin, S. (2011). Comparative evaluation of mathematical models for release of antioxidant from chitosan films prepared by different drying methods. *Drying Technology, 29*(12), 1396-1403.

Turasan, H., Sahin, S., & Sumnu, G. (2015). Encapsulation of rosemary essential oil. *LWT-Food Science and Technology, 64*(1), 112-119.

Valgimigli, L. (2012). *Essential Oils as Natural Food Additives: Composition, Applications, Antioxidant and Antimicrobial Properties.* New York: Nova Science Publishers, Inc.

Viuda-Martos, M., Navajas, Y.R., Zapata, E.S., Fernandez-Lopez, J., & Perez-Alvarez, J.A. (2010). *Flavour and Fragrance Journal, 25*(1), 13-19.

Wang, T.H., Hsia, S.M., Wu, C.H., Ko, S.Y., Chen, Y.H., Shieh, T.M., Chuang, L.C., & Wu, C.Y. (2016). Evaluation of the antibacterial potential of liquid and vapor phase phenolic essential oil compounds against oral microorganisms. *PLOS ONE, 11*(9), e0163147.

Wang, W., Wu, N., Zu, Y.G., & Fu, Y.J. Antioxidant activity of *Rosmarinus officinalis* L. essential oil compared to its main components. (2008). *Food Chemistry, 108*(3), 1019-1022.

Wei, A., & Shibamoto, T. (2010). Antioxidant/lipoxygenase inhibitory activities and chemical compositions of selected essential oils. *Journal of Agricultural and Food Chemistry, 58*(12), 7218-7225.

Wilkowska, A., Ambroziak, W., Czyzowska, A., & Adamiec, J. (2016). Effect of microencapsulation by spray-drying and freeze-drying technique on the antioxidant properties of blueberry (*Vaccinium myrtillus*) juice polyphenolic compounds. *Polish Journal of Food and Nutritional Sciences, 66*(1), 11-16.

Yazicioglu, B., Sahin, S., & Sumnu, G. (2015). Microencapsulation of wheat germ oil. *Journal of Food Science and Technology, 52*(6), 3590-3597.

Yi, J., Lam, T.I., Yokoyama, W., Cheng, L.W., & Zhong, F. (2015). Beta-carotene encapsulated in food protein nanoparticles reduces peroxyl radical oxidation in Caco-2 cells. *Food Hydrocolloids, 43*, 31-40.

Yin, W., & Yates, M.Z. (2009). Encapsulation and sustained release from biodegradable microcapsules made by emulsification/freeze drying and spray/freeze drying. *Journal of Colloid and Interface Science, 336*(1), 155-161.

Zhang, P., Dudhani, A., Lundin, L., & Kosaraju, S.L. (2009). Macromolecular conjugate based particulates: preparation, characterisation and evaluation of controlled release properties. *European Polymer Journal, 45*(7), 1960-1969.

Zhang, Z., Grijpma, D.W., & Feijen, J. (2006). Poly (trimethylene carbonate) and monomethoxy poly (ethylene glycol) - block - poly (trimethylene carbonate) nanoparticles for the controlled release of dexamethasone. *Journal of Controlled Release, 111*(3), 263-270.

Zsila, F. (2003). A new ligand for an old lipocalin: induced circular dichroism spectra reveal binding of bilirubin to bovine beta-lactoglobulin. *FEBS Letters, 539*(1), 85-90.

CHAPTER 4

MICROENCAPSULATION OF ESSENTIAL OILS (B): STUDY ON POTENTIAL OF INULIN FOR ENCAPSULATING OREGANO ESSENTIAL OIL

4.1 Abstract

The present study aimed to investigate the microencapsulation of oregano essential oil in inulin by freeze-drying. The evaluation of physical properties of the freeze-dried microparticles showed that the increase in initial inulin concentration from 10% to 20% resulted in higher hygroscopicity and higher bulk tapped density and particle density values, due to the increase in drying efficiency. This also revealed that the microcapsules produced from 20% inulin solution tended to be a better option in terms of packaging, transport and distribution purposes. The porosity of the dried powders ranged from 77.09% to 90.21%, indicating a high level of rehydration rate. On the basis of comparison with the results obtained using β-lactoglobulin (β-lg) as the wall material from the previous chapter, the encapsulation efficiency of inulin microcapsules (11.22% – 33.86%) tended to be lower than that of the β-lg microcapsules. However, as previous studies have demonstrated that the addition of a carbohydrate into a protein matrix resulted in higher encapsulation efficiency for volatile compounds, further research to optimize a protein/inulin mixture as the carrier in which the protein is the primary wall material will be valuable. Fourier transform infrared analysis was performed on the freeze-dried inulin microcapsules and it was found the intensities of predominant bands of oregano essential oil increased with higher initial inulin content.

4.2 Introduction

Oregano (*Origanum vulgare* L.) essential oil, extracted from the aromatic herb usually present in the Mediterranean area, has been found to possess significant antimicrobial (Burt 2004) and antioxidant (Dobre *et al.*, 2011; Karpinska *et al.*, 2001) activities which make it of great potential to preserve foods. Besides, due to its favorable aroma, oregano essential oil is also used as a spice in cooking and a natural flavoring agent in the perfumery industry (Fournomiti *et al.*, 2015; Holley & Patel, 2005). Many previous studies have shown that oregano essential oil can help prolong the shelf-life and enhance the stability of food commodities, such as seafood (Goulas & Kontominas, 2007), cooked chicken meat (Lemay *et al.*, 2002) and beef (Skandamis *et al.*, 2002). Due to the high degree of volatility and instability of oregano essential oil, certain processing techniques must be applied to protect it.

Microencapsulation is a technology that helps to protect unstable active compounds by physically entrapping the chemicals into a wall matrix composed of inert materials (Calvo *et al.*, 2011). In the food industry, microencapsulation of active compounds provides a suitable means of packaging, transporting, and distributing those compounds for further usage. It is known that most bioactive compounds commonly used in the food industry, such as vitamins, fatty acids, antioxidants and prebiotics, are susceptible to environmental factors. Microencapsulation is a solution that not only provides a way to protect the actives but also aids their controlled release and delivery (El-Salam & El-Shibiny, 2012). Among the many encapsulation techniques, freeze-drying is a common method that minimizes thermal degradation reactions during the encapsulation process.

The most common wall materials that have served to encapsulate active compounds are inert biopolymers such as maltodextrin (Gandia-Herrero *et al.*, 2010; Sanchez *et al.*, 2013), starch (Glenn *et al.*, 2010; Felker *et al.*, 2013), chitosan (Abreu *et al.*, 2012; Klaypradit & Huang, 2008), gelatin (Kaushik & Roos, 2007; Wang *et al.*, 2012), whey protein (Na *et al.*, 2011; Silva *et al.*, 2016), and soy protein (Brito-Oliveira *et al.*, 2017; Nasrin & Anal, 2015). In the food industry, the use of inulin as the major wall material has not been extensively reported yet, but a few studies have indicated a great potential of inulin for encapsulating essential oils (Beirao-da-Costa *et al.*, 2013; Fernandes *et al.*, 2016). Inulin is a natural fructooligosaccharide that is composed of fructose monomers linked by β(2→ 1) bonds, with a glucose unit at the end (Silva

& Meireles, 2015). The main natural sources of inulin are chicory root, garlic, salisfy, dahlia tubers and Jerusalem artichoke, where chicory and Jerusalem artichoke are the most commonly used in the food industry (Zaur & Gupta, 2002). Inulin is considered to be a functional ingredient for promoting human health such that it can decrease the incidence of osteoporosis by increasing calcium bioavailability and reduce the risk of colon cancer (Saenz *et al.*, 2009).

In this study, the microencapsulation of oregano essential oil by freeze-drying using inulin as the wall material was examined. The physical properties, namely, hygroscopicity and density-related variables, of the microparticles were determined. The encapsulation efficiency was determined so as to compare the results with those obtained by using β-lactoglobulin as the wall material from Chapter 3. In addition, Fourier transform infrared (FTIR) spectroscopy was performed for a qualitative study of the freeze-dried microcapsules.

4.3 Materials and Methods

Pure oregano (*Origanum vulgare* L.) essential oil was purchased from a local grocery store in Montreal, QC, Canada, and used without further purification. Frutafit® inulin, a natural extract from chicory, was obtained from a local company in Lachine, QC, Canada. The organic solvents used for particle density and encapsulation efficiency determination were toluene and *n*-hexane from Sigma-Aldrich (Canada).

4.3.1 Microencapsulation Process

Inulin solutions (10%, 15% and 20%) were prepared by adding inulin powder directly into a beaker containing distilled water with constant stirring. The solutions were left overnight to allow full hydration. On the next day, the oregano essential oil was added into the prepared solutions in amounts corresponding to 15% of the inulin concentration on a weight basis, followed by immediate homogenization for 3 min, using a Polytron homogenizer (Westbury, N.Y.). The homogenized solutions were filtered through Whatman No. 1 filter paper before storage. The filtered solutions were stored in the freezer (-18 ^{o}C) for approximately 48 hours before freeze-drying.

The frozen solutions were dried using a Labconco freeze dryer, with the freezer temperature at -50 ^{o}C and internal pressure lower than 25 mmHg. The dried powder was

collected after 48 hours of drying and stored in airtight containers at refrigerated temperature conditions for further analyses.

4.3.2 Physical Properties

The hygroscopicity, bulk tapped density, particle density and porosity of the freeze-dried microparticles were determined by physical methods described below.

The hygroscopicity of the dried powder was determined based on the method employed by Cai & Corke (2000), with minor modifications. For each treatment, approximately 1 g of the pre-weighed dried powder was placed in a desiccator containing a saturated NaCl solution (75.29% RH) at 25°C. The samples were reweighed after two-week storage and the hygroscopicity was calculated as grams of adsorbed moisture per 100 g of dried sample.

Bulk tapped density (ρ_b) was determined according to the studies by Goula & Adamopoulus (2008) and Jafari *et al.* (2016) with modifications. Approximately 0.5 g of dried sample was poured into a 10-mL glass graduated cylinder, manually tapped and mixed by a mechanical mixer, until there was no apparent volume difference at a vertical distance. The bulk tapped density was calculated based on the mass and measured volume of each sample and expressed in g cm^{-3} (Goula & Adamopoulus, 2008).

The particle density of the samples was determined by using a pycnometer (Fernandes *et al.*, 2014). For each replicate, 0.5 g of freeze-dried powder was weighed into a 10-mL pycnometer which was filled with toluene. The particle density (ρ_p) was calculated according to Equation (1) below:

$$\text{Particle density } (\rho_p) = \frac{Sample\ weight\ (g)}{Volume\ of\ pycnometer\ (mL) - Volume\ of\ toluene\ (mL)} \quad (1)$$

where the volume of toluene can be calculated based on its density of 0.867 g mL^{-1}.

Porosity (ε_b) was derived from the values of bulk tapped density and particle density using Equation (2) below (Krokida & Maroulis, 1997):

$$\% \varepsilon_b = (1 - \rho_b/\rho_p) \times 100 \quad (2)$$

where ρ_b and ρ_p are the bulk tapped density and particle density, respectively, of the freeze-dried microcapsules.

4.3.3 Encapsulation Efficiency

The encapsulation efficiency of essential oil was determined by direct solvent extraction (Bylaite *et al.*, 2001; Hundre *et al.*, 2015; Karim *et al.*, 2016; Naik *et al.*, 2014; Yazicioglu *et al.*, 2015), and *n*-hexane was selected as the solvent due to its excellent capacity to dissolve hydrophobic compounds. For each replicate, 1 g of freeze-dried powder was weighed into a centrifugation tube filled with 10 mL of *n*-hexane. The mixture was then vortexed for 3 min followed by centrifugation at 5000 rpm for 5 min. After centrifugation, the supernatant was filtered through a Whatman No. 1 filter paper and transferred into a glass tube. The solution was stored in the fume hood for approximately 48 hours, to allow complete solvent evaporation. The residual component was weighed and expressed as the weight of the unencapsulated fraction, also known as surface oil. By assuming minimal loss of essential oil during freeze-drying, the encapsulation efficiency was calculated using Equation (3):

$$\text{Encapsulation efficiency (\%)} = \frac{Total\ Oil - Surface\ Oil}{Total\ Oil} \times 100 \quad (3)$$

4.3.4 Fourier Transform Infrared Spectroscopy

Attenuated total reflectance (ATR) - Fourier transform infrared (FTIR) spectroscopy was employed to acquire infrared spectra of both pure inulin powder and freeze-dried microcapsules. The infrared spectra of samples were recorded in the wavenumber region between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1}. For each sample, spectra (128 co-added scans) were recorded in triplicate and subsequently averaged by OMNIC 7.0. Resolution of the spectra was enhanced by Fourier self-deconvolution (FSD) in OMNIC 7.0, with a bandwidth of 44.4 cm^{-1} and enhancement of 3.0.

4.4 Results and Discussion

4.4.1 Physical Properties

The physical characteristics of the freeze-dried inulin microcapsules are listed in Table 4.1, and the significance of each property has been discussed in the previous chapter. It is apparent that the hygroscopicity, bulk tapped density and particle density increased significantly with higher inulin concentrations as expected, because higher drying rates were achieved when inulin was initially present in relatively higher concentrations. The hygroscopicity for all samples ranged from 9.11% to 16.15%, which is slightly lower than the values reported in a study on encapsulation of rosemary essential oil by gum arabic using spray drying (Fernandes *et al.*, 2013). This difference can be explained by the different drying techniques, nature of wall materials and initial wall material concentrations. In comparison with the other study using whey protein/inulin mixture as the coating material (Fernandes *et al.*, 2014), the values of bulk tapped density (0.185 – 0.297 g cm^{-3}), particle density (1.04 – 2.90 g cm^{-3}) and porosity (77.09 – 90.21 %) in this study were similar and acceptable. The results showed that using an initial inulin concentration of 20% could result in a higher density product that was easier for further packaging and transportation, but with a relatively higher rehydration rate due to its higher porosity ($P < 0.05$).

Table 4.1 Hygroscopicity, bulk tapped density, particle density and porosity of freeze-dried inulin microparticles containing oregano essential oil

Initial inulin concentration (%)	Hygroscopicity (g/100 g)	Bulk tapped density (g cm^{-3})	Particle density (g cm^{-3})	Porosity (%)
10	9.73 ± 0.44^{a}	0.212 ± 0.022^{a}	1.18 ± 0.14^{a}	81.79 ± 3.34^{ab}
15	11.76 ± 0.27^{b}	0.260 ± 0.022^{b}	1.42 ± 0.19^{a}	81.57 ± 1.34^{a}
20	15.63 ± 0.71^{c}	0.293 ± 0.007^{c}	2.29 ± 0.51^{b}	86.50 ± 3.34^{b}

* Values are reported as mean ± standard deviation. Values with different letters in the same column are significantly different ($P < 0.05$).

4.4.2 Encapsulation Efficiency

Encapsulation efficiency is one of the most important criteria for evaluation of a microencapsulation process and the compatibility between the coating and core materials. Figure 4.1 shows a comparison between the encapsulation efficiencies obtained by using inulin and β-lg as the coating material for encapsulating oregano essential oil. The encapsulation efficiency values for inulin capsules ranged from 11.22% to 33.86%. It can be seen that for both inulin and protein samples, the encapsulation efficiency did not vary significantly ($P > 0.05$) when the initial concentration of the wall material changed. Comparison of the values for inulin and protein samples at the same concentration level shows that the value for 20% inulin capsules was significantly lower than that for 20% protein microparticles *(*$P < 0.05$), suggesting a higher efficiency of using β-lg for encapsulating oregano essential oil. The nature of the carrier molecule is a critical factor influencing the encapsulation efficiency. Due to the lack of emulsifying capacity, using inulin independently as the coating material for encapsulating hydrophobic volatiles is less effective than using proteins. For the 10% and 15% samples, the encapsulation efficiency of inulin microcapsules was slightly lower than that of β-lg microparticles, but the differences are not statistically significant ($P > 0.05$). Another finding from Figure 4.1 was that the standard deviations for inulin samples were larger than for β-lg samples, indicating that the essential oil was relatively more uniformly distributed in the protein matrix. This may be attributed to the emulsifying capacity of the protein molecules that resulted in smaller oil droplet size. However, research has demonstrated that incorporating a carbohydrate into a protein-based emulsion can increase the encapsulation efficiency mainly by enhancing the drying efficiency (Kagami *et al.*, 2003). Studies by Fernandes *et al.* (2014) illustrated that using whey protein-inulin blends (3:1 weight ratio) could improve the encapsulation efficiency of rosemary essential oil. Thus, for future studies, consideration of using a protein as the primary coating material combined with a small portion of carbohydrate as the secondary coating material may be a better solution for encapsulation of essential oils.

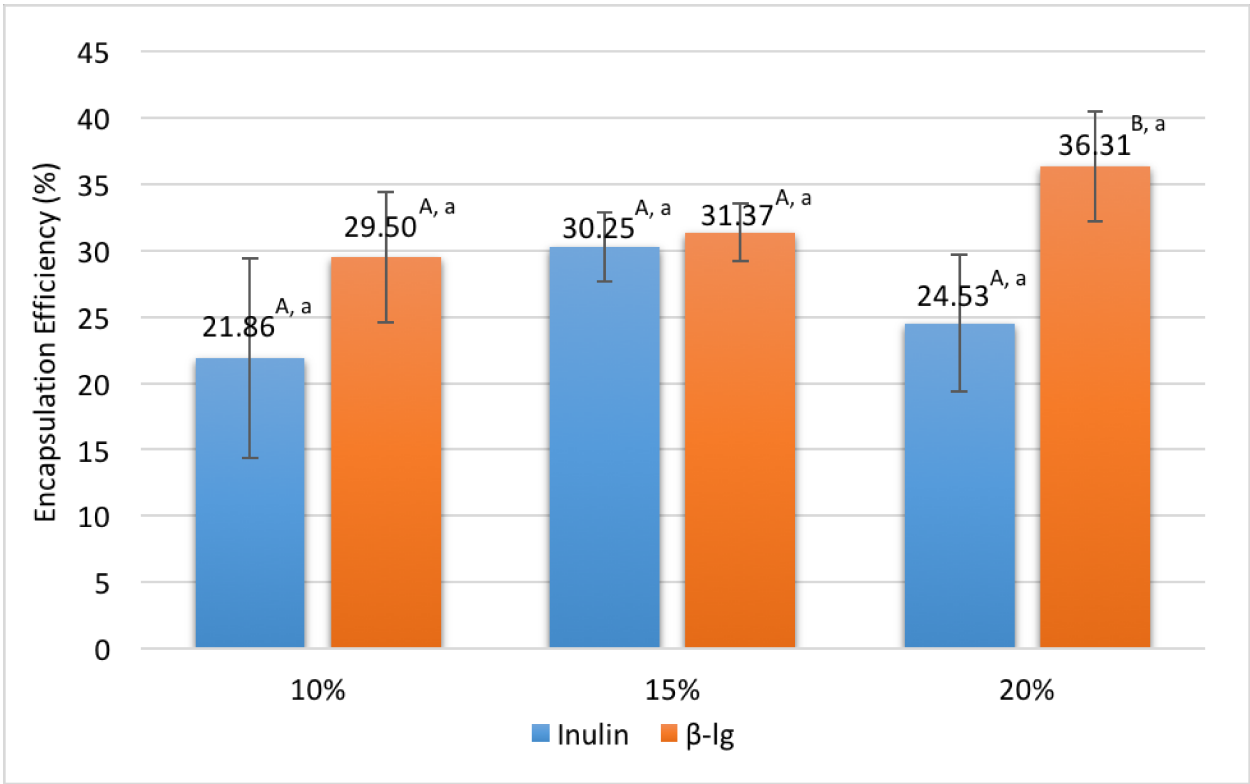

Figure 4.1. Comparison of encapsulation efficiency values for freeze-dried inulin and β-lg microcapsules containing oregano essential oil. Values with different letters (uppercase letter: between inulin and protein samples at the same concentration; lowercase letter: between different initial concentrations using the same coating material) are significantly different ($P < 0.05$).

4.4.3 Fourier Transform Infrared Spectroscopy

Preliminary FTIR analysis of pure oregano essential oil showed that the two major bands were in the regions of 3000-2900 cm^{-1} and 815-810 cm^{-1}, corresponding to C-H stretching and C-H out-of-plane bending, respectively. The FTIR spectra acquired from the inulin powder employed in the preparation of the microcapsules did not exhibit any absorption bands in these regions. Thus, absorption in these regions in the FTIR spectra of inulin microcapsules containing oregano essential oil is due to the essential oil. Figures 4.2 and 4.3 show these regions of the spectra of microcapsules containing oregano essential oil, prepared from solutions of different inulin concentrations.

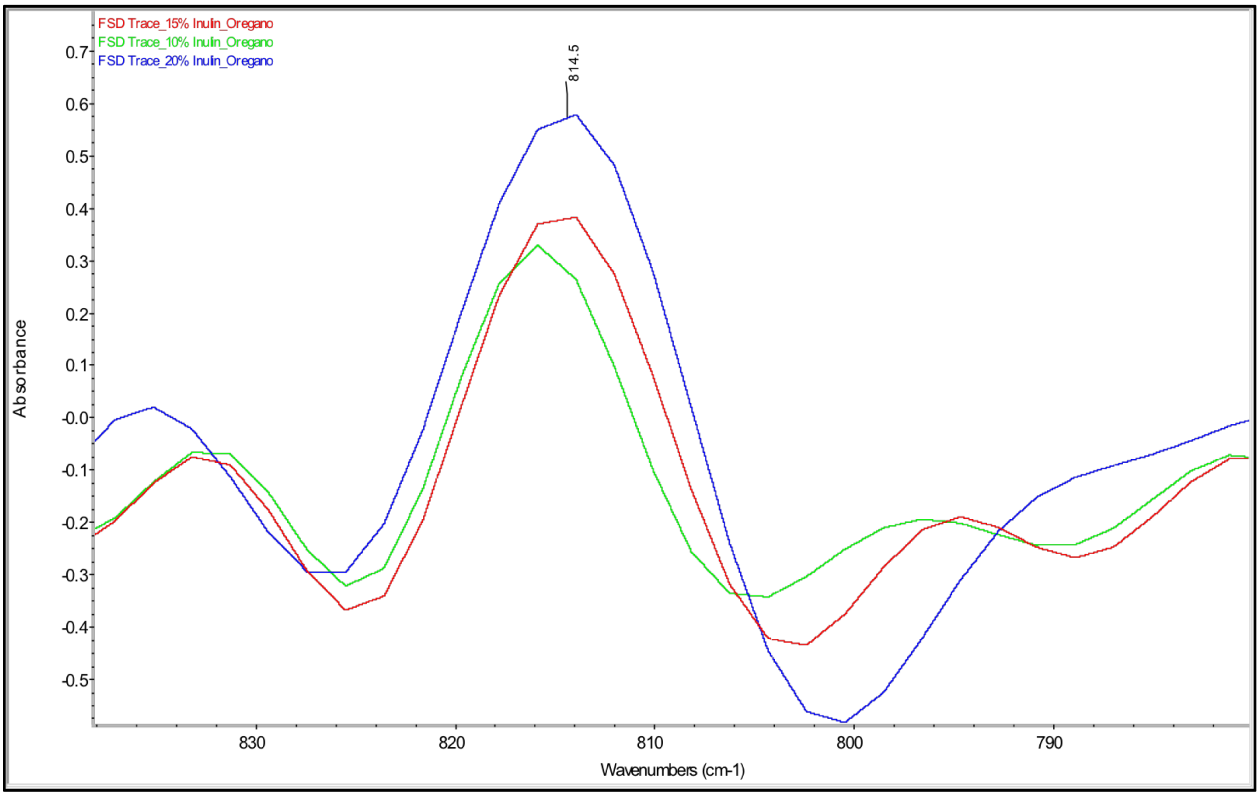

Figure 4.2. Fourier self-deconvoluted IR spectra (region 850-750 cm^{-1}) of freeze-dried inulin microcapsules containing oregano essential oil, prepared with different initial inulin concentrations (10%, 15% and 20%).

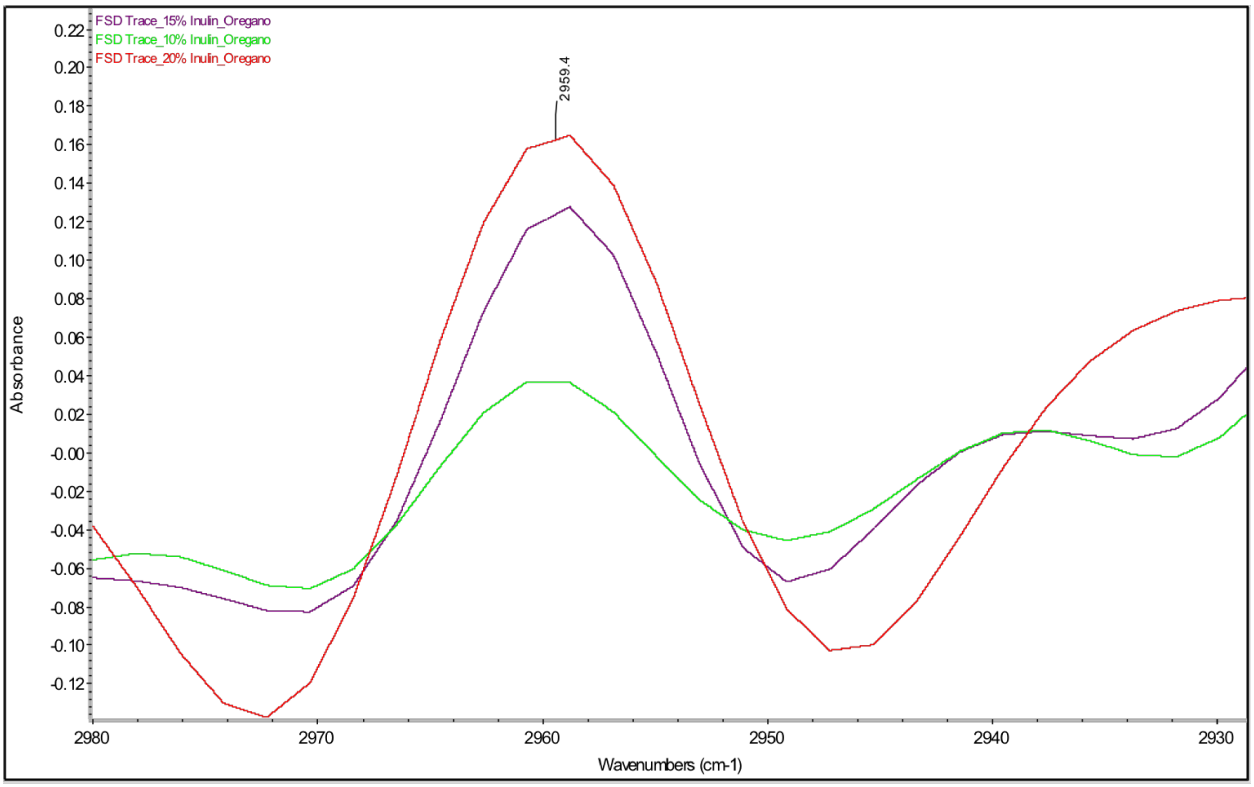

Figure 4.3. Fourier self-deconvoluted IR spectra (region 2980-2930 cm^{-1}) of freeze-dried inulin microcapsules containing oregano essential oil prepared with different initial inulin concentrations (10%, 15% and 20%).

It is evident in Figures 4.2 and 4.3 that the intensities of the bands in both regions increased with increasing initial inulin concentration from 10% to 20%. As the ratio of oregano essential oil to inulin was kept the same (15% on a weight basis) for all the solutions from which the freeze-dried samples were prepared, this observation may be attributed to a higher percentage of essential oil retention with higher initial inulin content. This may indicate higher drying efficiency with increasing initial inulin concentration. Further experiments such as gas chromatography can be employed to confirm the results by determining the amount of essential oil retention in the dried powders.

4.5 Conclusion

In conclusion, employing the same techniques as described in the previous chapter, this study focused on the use of inulin independently as the wall material for encapsulating oregano essential oil. Evaluation of the physical properties of the microcapsules indicated that their preparation by freeze-drying of a 20% inulin solution was a good option from the perspective of storage and transportation of the microcapsules. Inulin exhibited lower encapsulation efficiency values than those obtained by using β-lg as the wall material for encapsulating essential oils. FTIR analysis indicated a possible increase in the percentage of essential oil retention with an increase in the initial inulin concentration.

For further experiments, considering inulin as a secondary wall material will be a better option for encapsulating essential oils. Proteins such as milk proteins, soy protein, and corn zein tend to be the best choice for the primary wall material owing to their emulsifying capacity. The relative ratio of the individual ingredients will be valuable to determine to achieve the highest encapsulation efficiency and total oil retention rate. Furthermore, the encapsulation process can be further modified by improving the emulsification process, adding antifoaming agents or combining two or more encapsulation techniques.

4.6 References

Abreu, F.O.M.S., Oliveira, E.F., Paula, H.C.B., & de Paula, C.M. (2012). Chitosan/cashew gum nanogels for essential oil encapsulation. *Carbohydrate Polymers, 89*(4), 1277-1282.

Beirao-da-Costa, S., Duarte, C., Bourbon, A.I., Pinheiro, A.C., Januario, M.I.N., Vicente, A.A., Beirao-da-Costa, M.L., & Delgadillo, I. (2013). Inulin potential for encapsulation and controlled delivery of oregano essential oil. *Food Hydrocolloids, 33*(2), 199-206.

Brito-Oliveira, T.C., Molina, C.V., Netto, F.M., & Pinho, S. (2017). Encapsulation of beta-carotene in lipid microparticles stabilized with hydrolyzed soy protein isolate: production parameters, alpha-tocopherol coencapsulation and stability under stress conditions. *Journal of Food Science, 82*(3), 659-669.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. *International Journal of Food Microbiology, 94*(3), 223-253.

Bylaite, E., Venskutonis, P.R., & Mazdzieriene, R. (2001). Properties of caraway (*Carum carvi* L.) essential oil encapsulated into milk protein-based matrices. *European Food Research and Technology, 212*(6), 661-670.

Cai, Y.Z., & Corke, H. (2000). Production and properties of spray-dried *Amaranthus betacyanin* pigments. *Journal of Food Science, 65*(7), 1248-1252.

Calvo, P., Castano, L.A., Hernandez, M.T., & Gonzalez-gomez, D. (2011). Effects of microcapsule constitution on the quality of microencapsulated walnut oil. *European Journal of Lipid Science and Technology, 113*(10), 1273-1280.

Dobre, A.A., Gagiu, V., & Petru, N. (2011). Antimicrobial activity of essential oils against food-borne bacteria evaluated by two preliminary methods. *Romanian Biotechnological Letters, 16*(6), 119-125.

El-Salam, M.H.A., & El-Shibiny, S. (2012). Formation and potential uses of milk proteins as nanodelivery vehicles for nutraceuticals: A review. *International Journal of Dairy Technology, 65*(1), 13-21.

Felker, F.C., Singh, M., & Fanta, G.F. (2013). Effect of gluten on soybean oil droplets in jet-cooked starch-oil composites. *Journal of Food Processing and Preservation, 37*(2), 152-162.

Fernandes, R.V., Botrel, D.A., Silva, E.K., Borges, S.V., Oliveira, C.R., Yoshida, M.I., Feitosa, J.P., & de Paula, R.C. (2016). Cashew gum and inulin: New alternative for ginger essential oil microencapsulation. *Carbohydrate Polymers, 153*, 133-142.

Fernandes, R.V.B., Borges, S.V., & Botrel, D.A. (2013). Influence of spray drying operating conditions on microencapsulated rosemary oil properties. *Ciencia e Tecnologia de Alimentos, 33*(1), 171-178.

Fernandes, R.V.B., Borges, S.V., Botrel, D.A., & Rodrigues, C. (2014). Physical and chemical properties of encapsulated rosemary essential oil by spray drying using whey protein-inulin blends as carriers. *International Journal of Food Science and Technology, 49*(6), 1522-1529.

Fournomiti, M., Kimbaris, A., Mantzourani, I., Plessas, S., Theodoridou, I., Papaemmanouil, V., Kapsiotis, I., Panopoulou, M., Stavropoulou, E., Bezirtzoglou, E.E., & Alexopoulos, A. (2015). Antimicrobial activity of essential oils of cultivated oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) against clinical isolates of *Escherichia coli*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*. *Microbial Ecology in Health and Disease, 26*, 23289.

Gandia-Herrero, F., Jimenez-Atienzar, M., Cabanes, J., Garcia-Carmona, F., & Escribano, J. (2010). Stabilization of the bioactive pigment of opuntia fruits through maltodextrin encapsulation. *Journal of Agricultural and Food Chemistry, 58*(19), 10646-10652.

Glenn, G.M., Klamczynski, A.P., Woods, D.F., Chiou, B., Orts, W.J., & Imam, S.H. (2010). Encapsulation of plant oils in porous starch microspheres. *Journal of Agricultural and Food Chemistry, 58*(7), 4180-4184.

Goula, A.M., & Adamopoulus, K.G. (2008). Effect of maltodextrin addition during spray drying of tomato pulp in dehumidified air: II. Powder properties. *Drying Technology, 26*(6), 726-737.

Goulas, A.E., & Kontominas, M.G. (2007). Combined effect of light salting, modified atmosphere packaging and oregano essential oil on the shelf-life of sea bream (*Sparus aurata*): biochemical and sensory attributes. *Food Chemistry, 100*(1), 287-296.

Holley, R.A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology, 22*(4), 273-292.

Hundre, S.Y., Karthik, P., & Anandharamakrishnan, C. (2015). Effect of whey protein isolate and β-cyclodextrin wall systems on stability of microencapsulated vanillin by spray-freeze drying method. *Food Chemistry, 174*(1), 16-24.

Kagami, Y., Sugimura, S., Fujishima, N., Matsuda, K., Kometani, T., & Matsumura, Y. (2003). Oxidative stability, structure, and physical characteristics of microcapsules formed by spray drying of fish oil with protein and dextrin wall materials. *Journal of Food Science, 68*(7), 2248-2255.

Karim, F.T., Sarker, Z.M., Ghafoor, K., Al-Juhaimi, F.Y., Jalil, R., Awang, M.B., Amid, M., Hossain, M.D.S., & Khalil, H.P.S.A. (2016). Microencapsulation of fish oil using hydroxypropyl methylcellulose as a carrier material by spray drying. *Journal of Food Processing and Preservation, 40*(2), 140-153.

Karpinska, M., Borowski, J., & Danowska-Oziewicz, M. (2001). The use of natural antioxidants in ready-to-serve food. *Food Chemistry, 72*(1), 5-9.

Kaur, N., & Gupta, A.K. (2002). Applications of inulin and oligofructose in health and nutrition. *Journal of Biosciences, 27*(7), 703-714.

Kaushik, V., & Roos, Y.H. (2007). Limonene encapsulation in freeze-drying of gum Arabic-sucrose-gelatin systems. *LWT-Food Science and Technology, 40*(8), 1381-1391.

Klaypradit, W., & Huang, Y. (2008). Fish oil encapsulation with chitosan using ultrasonic atomizer. *LWT-Food Science and Technology, 41*(6), 1133-1139.

Lemay, M.J., Choquette, J., Delaquis, P.J., Gariepy, C., Rodrigue, N., & Saucier, L. (2002). Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model. *International Journal of Food Microbiology, 78*(3), 217-226.

Mallet, J.F., Cerati, C., Ucciani, E., Gamisana, J., & Gruber, M. (1994). Antioxidant activity of fresh pepper (*Capsicum annuum*) cultivars. *Food Chemistry, 49*, 61-65.

Na, H., Kim, J., Kim, J., & Lee, K. (2011). Encapsulation of fish oil using cyclodextrin and whey protein concentrate. *Biotechnology and Bioprocess Engineering, 16*(6), 1077-1082.

Naik, A., Meda, V., Lele, S.S. (2014). Freeze drying for microencapsulation of α-linolenic acid rich oil: A functional ingredient from *Lepidium sativum* seeds. *European Journal of Lipid Science and Technology, 116*(7), 837-846.

Nasrin, T.A., & Anal, A.K. (2015). Enhanced oxidative stability of fish oil by encapsulating in culled banana resistant starch-soy protein isolate based microcapsules in functional bakery products. *Journal of Food Science and Technology, 52*(8), 5120-5128.

Saenz, C., Tapia, S., Chavez, J., & Robert, P. (2009). Microencapsulation by spray drying of bioactive compounds from cactus pear (*Opuntia ficus-indica*). *Food Chemistry, 114*(2), 616-622.

Sanchez, V., Baeza, R., Galmarini, M.V., Zamora, M.C., & Chirife, J. (2013). Freeze-drying encapsulation of red wine polyphenols in an amorphous matrix of maltodextrin. *Food and Bioprocess Technology, 6*(5), 1350-1354.

Silva, E.K., & Meireles, M.A.A. (2015). Influence of the degree of inulin polymerization on the ultrasound-assisted encapsulation of annatto seed oil. *Carbohydrate Polymers, 133*, 578-566.

Silva, E.K., Azevedo, V.M., Cunha, R.L., Hubinger, M.D., & Meireles, M.A.A. (2016). Ultrasound-assisted encapsulation of annatto seed oil: Whey protein isolate versus modified starch. *Food Hydrocolloids, 56*, 71-83.

Skandamis, P., Tsigarida, E., & Nychas, G.J.E. (2002). The effect of oregano essential oil on survival/death of *Salmonella typhimurium* in meat stored at 5 ºC under aerobic, VP/MAP conditions. *Food Microbiology, 19*(1), 97-103.

Wang, Y., Ye, H., Zhou, C., Lv, F., Bie, X., & Lu, Z. (2012). Study on the spray-drying encapsulation of lutein in the porous starch and gelatin mixture. *European Food Research and Technology, 234*(1), 157-163.

Yazicioglu, B., Sahin, S., & Sumnu, G. (2015). Microencapsulation of wheat germ oil. *Journal of Food Science and Technology, 52*(6), 3590-3597.

CHAPTER 5

PRELIMINARY STUDY ON INCORPORATION OF ESSENTIAL OILS IN PROTEIN-BASED EDIBLE FILMS

5.1 Abstract

The preparation of β-lactoglobulin (β-lg) based edible films when an essential oil or carvacrol was added to the film-forming solution, using glycerol as the plasticizer, was investigated. It was found that the optimum composition of the film-forming solution was subject to change due to seasonal effects. The incorporation of higher concentrations of essential oils and carvacrol increased the brittleness of the films, causing them to crack. It was observed the addition of 1.0% oregano essential oil and 0.5% and 1.0% carvacrol resulted in films that could be readily cast with apparent elasticity, and this concentration level was consistent with other studies on essential oil incorporation in milk protein based edible films. The edible films containing oregano essential oil and carvacrol showed significantly lower hygroscopicity and were considered to possess better moisture barrier properties, compared to the films prepared without essential oil addition. Attenuated total reflectance (ATR) – Fourier transform infrared (FTIR) spectroscopic analysis of β-lg edible films prepared with use of glycerol as a plasticizer demonstrated significant interactions between glycerol and β-lg but also revealed that the changes in the β-sheet structure of β-lg induced by the presence of glycerol were counteracted by the addition of oregano essential oil.

5.2 Introduction

Historically, edible films and coatings were initially invented to enhance the appearance and shelf life stability of foods. In the present-day food packaging industry, the development of protein-based edible films has been extensively studied, where the commonly used protein matrices are gelatin (Lopez *et al.*, 2017; Mu *et al.*, 2012), peanut protein (Jangchud & Chinnan, 1999; Sun *et al.*, 2013), corn zein (Cho *et al.*, 2010), soy protein (Nandane & Jain, 2015; Sivarooban *et al.*, 2008), casein (Sohail *et al.*, 2006) and whey protein (Han *et al.*, 2008; Ket-On *et al.*, 2016). In general, milk protein based edible films tend to have excellent oxygen barrier properties and strong mechanical strength but are susceptible to water sorption due to their hydrophilic nature (Seydim & Sarikus, 2006). It is known that in the cheese industry, there is a considerable amount of processing waste, mainly attributed to whey protein (Lent *et al.*, 1998). Therefore, the application of whey protein fractions in edible film production creates an alternative solution to reduce food waste and promote food sustainability. Whey protein accounts for almost 20% of bovine milk protein, and β-lactoglobulin (β-lg) is the most abundant component of whey. At neutral pH and room temperature conditions, β-lg is present mostly in dimeric form, which dissociates into monomers when the pH is increased 8.0 or is decreased to 3.0 (Zsila, 2003).

In order to further develop the functionality of edible films, several functional ingredients can be added, such as fatty acids (Ma *et al.*, 2016; Thakur *et al.*, 2016), vitamins (Janjarasskul *et al.*, 2011; Lin & Pascall, 2014), spice powders (Ket-on *et al.*, 2016; Meenatchisundaram *et al.*, 2016) and essential oils (Jouki *et al.*, 2014; Randazzo *et al.*, 2016; Soni *et al.*, 2016) etc. Edible films in which essential oils have been successfully incorporated have been shown in several studies to possess antifungal (Avila-Sosa *et al.*, 2010), antibacterial (Randazzo *et al.*, 2016) and antioxidant (Moradi *et al.*, 2016) activities. Such edible films can be used as a form of active packaging actively inhibiting microbial growth and prolonging the food's shelf-life. However, the number of essential oil species studied in edible film formation is limited and the biological origin of essential oil extracts varied between studies. Thus, more studies on producing active packaging materials containing essential oils are ongoing.

The main objective of this study was to determine the best formula (concentrations of glycerol and essential oils) for preparing edible films using β-lg as the carrier material and

glycerol as the plasticizer, when different essential oils were incorporated into the film solution. The effects of adding oregano, rosemary and sage essential oils into the film solution on the final products were compared. The influence of adding carvacrol on film formation was also studied. The self-supporting films that were successfully cast were subject to hygroscopicity determination and qualitative studies by Fourier transform infrared (FTIR) spectroscopy.

5.3 Materials and Methods

Pure oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) essential oils were purchased from local grocery stores in Montreal, QC Canada. Carvacrol (≥98%) was purchased from Sigma Aldrich (Canada) and used without further purification. Food-grade bovine β-lactoglobulin from Biopure Products (USA) was used as the carrier material. Glycerol (≥99.9) from Fisher Scientific (Canada) was used as the plasticizer without further purification.

5.3.1 Film Preparation

The experimental protocols for film preparation were based on the study by Kim and Ustunol (2011), with modifications. A 5% (w/v) β-lg solution was prepared by directly dissolving the protein in distilled water and left overnight to allow full hydration. On the next day, glycerol was added to the prepared β-lg solution, in different concentrations (1.0%, 1.5%, 2.0%, 2.5% and 3.0% w/v in summer; 3.0%, 3.5%, 4.0%, 4.5% and 5.0% w/v in winter). The pH of the solution was 6.94 ± 0.10, which was then adjusted to 8.00 ± 0.05 by adding 2M NaOH solution. The film solution was then heated to 90 ± 2 ^{o}C with constant stirring. A filtration step was applied using a cheesecloth followed by cooling at room temperature for approximately 2 hours. Once the solution had cooled down, oregano, rosemary, or sage essential oil or carvacrol was added (0.5%, 1.0%, 1.5% and 2.0% w/v) followed by 2-min high-speed homogenization by a Polytron homogenizer (Westbury, N.Y.). The prepared film solution (10 mL) was immediately transferred into a Petri dish and allowed to dry for approximately 48 hours under ambient conditions. Finally, the cast films were peeled off and subjected to further analyses.

5.3.2 Hygroscopicity

The weight of each film was directly obtained on an analytical balance. The hygroscopicity of the dried powder was determined based on the method employed by Cai & Corke (2000), with minor modifications. For each treatment, approximately a quarter of each film was placed in a desiccator containing saturated NaCl solution (75.29% RH) at 25 ^{o}C. The samples were reweighed after two-week storage and the hygroscopicity was calculated as grams of adsorbed moisture per 100 g of dried sample.

5.3.3 Fourier Transform Infrared Spectroscopy

Attenuated total reflectance (ATR) - Fourier transform infrared (FTIR) spectroscopy was performed on the films prepared with or without addition of oregano essential oil. The infrared spectra of samples were recorded in the wavenumber region between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1}. For each sample, spectra (128 co-added scans) were acquired in triplicate. Average spectra of triplicates were generated by OMNIC 7.0. Resolution of the spectra was enhanced by using Fourier self-deconvolution (FSD) in OMNIC 7.0, with bandwidth of 44.4 and enhancement of 3.0. The area of the amide I band was normalized after baseline correction.

5.4 Results and Discussion

5.4.1 Optimization of the Composition of the Film-Forming Solution

It is known that the composition of the film-forming solution has a significant impact on the quality of protein-based edible films. For the optimization of β-lg edible films incorporating essential oils or carvacrol in the present study, the concentration of β-lg in the film-forming solution was kept at 5% (w/v) whereas the glycerol content was varied between 1.0% and 3.0% in summer, and between 3.0% and 5.0% in winter. The concentration of essential oils and carvacrol (0.5 – 2.0%) was the other variable. Generally, edible films of good quality were considered to be the ones that could be readily and completely peeled off from the Petri dishes with apparent elasticity.

Some pictures showing the general appearance of β-lg films prepared from solutions of different compositions are presented in Figure 5.1. From Figure 5.1(b) and (c) we can see that at higher concentrations of oregano essential oil (1.5% and 2.0%) and carvacrol (1.5% and 2.0%), the films were fragmented, indicating that the addition of the essential oil or its principal

component significantly increased the brittleness of the edible films. Similar findings were reported in a previous study: the addition of cinnamon essential oil to whey protein concentrate edible film decreased the tensile strength and resulted in a cracked structure (Bahram *et al.*, 2014).

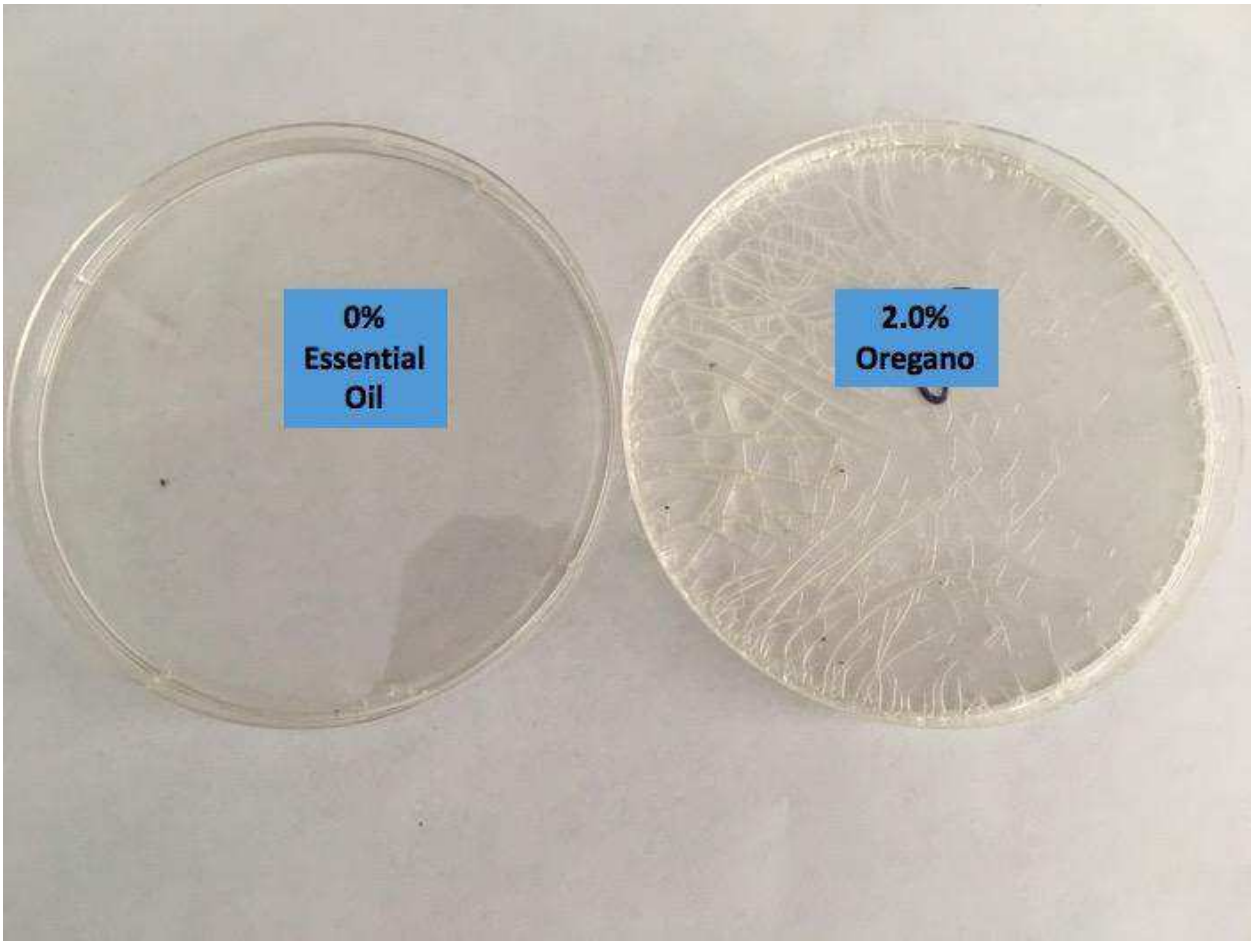

Figure 5.1(a). Comparison of the general appearance of β-lg films prepared without (left) and with the addition of 2.0% oregano essential oil with the same level of glycerol content.

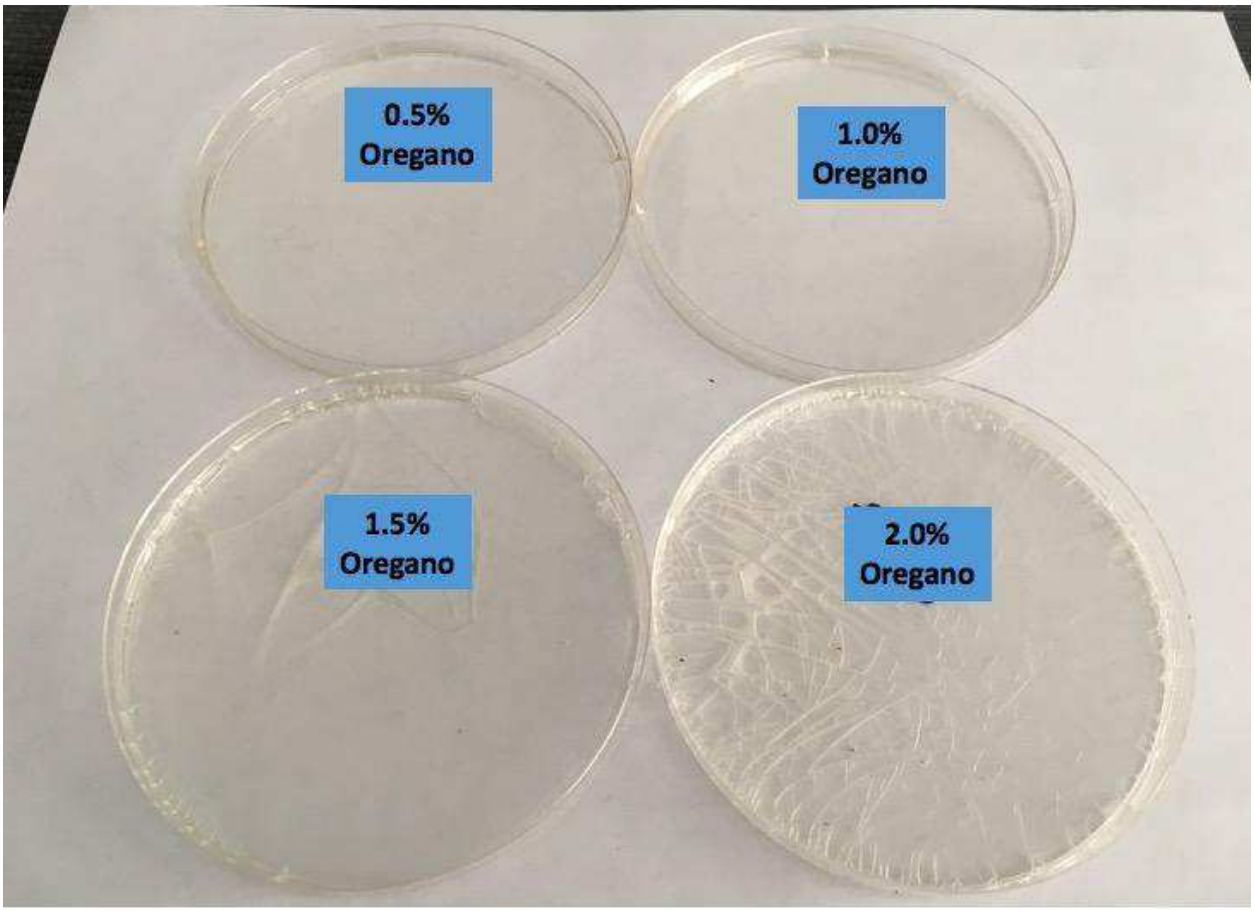

Figure 5.1(b). General appearance of β-lg films prepared with addition of different levels of oregano essential oil (0.5%, 1.0%, 1.5% and 2.0%).

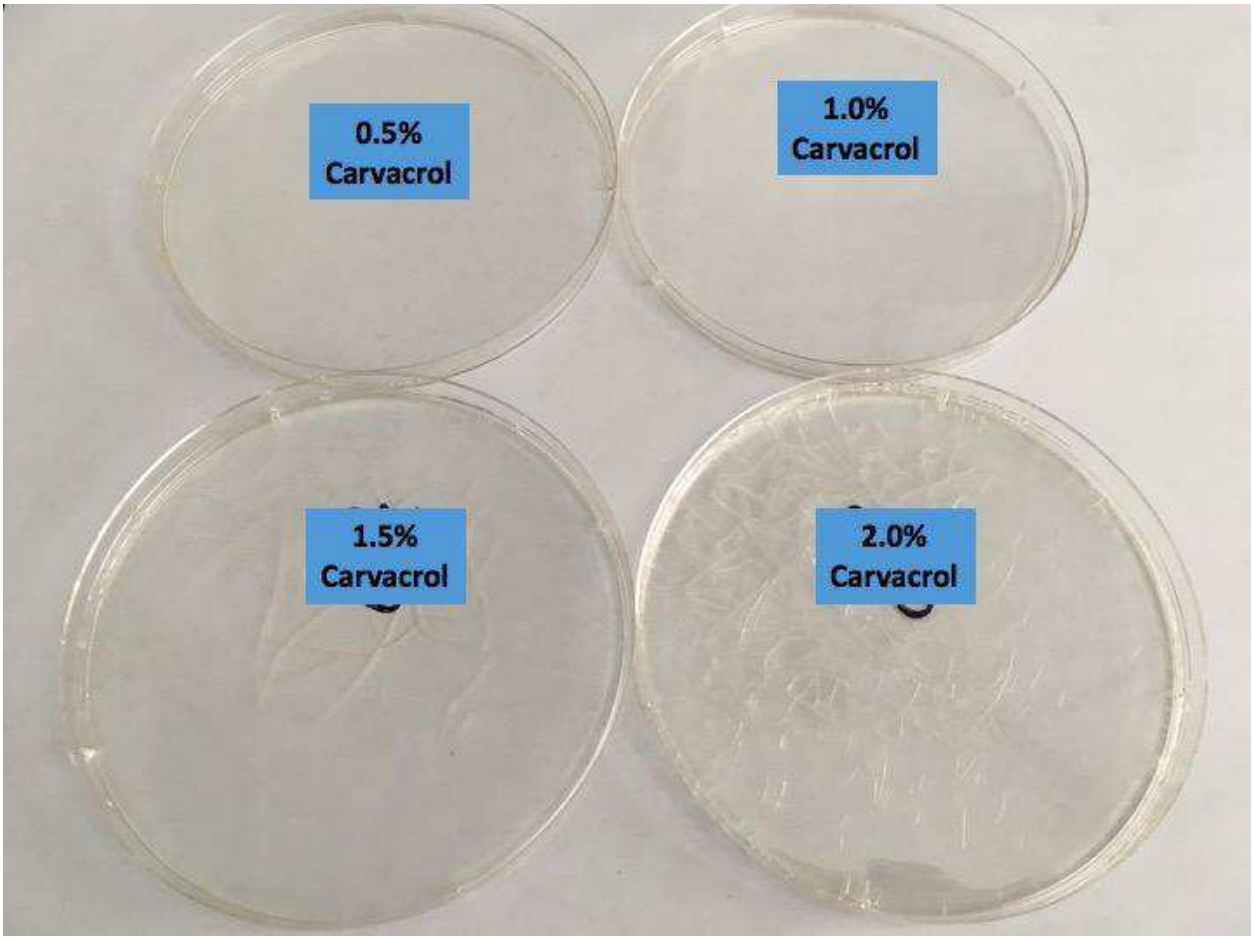

Figure 5.1(c). General appearance of β-lg films prepared with addition of different levels of carvacrol (0.5%, 1.0%, 1.5% and 2.0%).

Table 5.1. Summary of glycerol concentrations (% w/v) needed to produce self-supporting β-lg edible films with or without addition of other ingredients, including seasonal effects

Other ingredients	Glycerol concentration (% w/v)	
	Summer	**Winter**
None	1.5, 2.0 and 2.5	3.5, 4.0 and 4.5
1.0% Oregano essential oil	2.5	5.0
0.5% Carvacrol	2.5	5.0
1.0% Carvacrol	2.5	5.0
Rosemary essential oil	N/A	N/A
Sage essential oil	N/A	N/A

* The concentrations listed for oregano essential and carvacrol are the ones that yielded edible films with apparent elasticity. In the case of rosemary and sage essential oils, none of the concentrations tested (ranging between 0.5% and 2.0%) yielded edible films with acceptable appearance. The concentration of β-lg for each film-forming solution was fixed at 5% (w/v).

The compositions of film-forming solutions that yielded self-supporting β-lg edible films are shown in Table 5.1. It was observed that β-lg films could be readily cast from solutions containing 1.0% (w/v) oregano essential oil and from solutions containing 0.5% and 1.0% (w/v) carvacrol, which is the principal component of oregano essential oil. In a previous study on incorporation of cinnamon essential oil in whey protein concentrate edible film (Bahram *et al.*, 2014), the concentrations of essential oil were 0.8% and 1.5% (v/v), similar to the present study. Another study using sodium and calcium caseinate as the carrier molecule obtained functional edible films by adding 0.5% (w/v) carvacrol (Arrieta *et al.*, 2014). For the treatments with addition of rosemary and sage essential oils, no self-supporting edible films could be produced, indicating the incompatibility of these essential oils with β-lg films. It is notable that the production of edible films during different seasons required different levels of plasticizer. This may be mainly attributed to the difference in the relative humidity of the environment, which significantly impacts the water evaporation rates during film formation. Therefore, the amount of

glycerol required during wintertime was much higher than that in the summer. The data also revealed that more glycerol (plasticizer) was required when adding oregano essential oil to the film-forming solution in winter, demonstrating that the incorporation of essential oil increased the film's brittleness.

5.4.2 Hygroscopicity

Hygroscopicity of the edible films represents the ability to absorb water and can be considered as one of the indicators of film quality. In general, whey protein based edible films tend to have poor moisture barrier properties due to the presence of numerous hydrophilic groups (McHugh *et al.*, 1994). Previous studies have demonstrated that the addition of nonpolar molecules significantly reduced the water vapor permeability of whey protein based edible films (Cagri *et al.*, 2001). Thus, the addition of oregano essential oil and carvacrol in β-lg films was expected to decrease the hygroscopicity. The experimental results shown in Figure 5.2 confirmed this hypothesis. The hygroscopicity of the β-lg films incorporating oregano essential oil (5.58 g/100 g) and carvacrol (6.71 g/ 100 g) was significantly lower ($P < 0.05$) than that of the films without these ingredients (8.97 g/100 g) . Therefore, the edible films containing oregano essential oil and carvacrol exhibited improved stability in terms of water sorption.

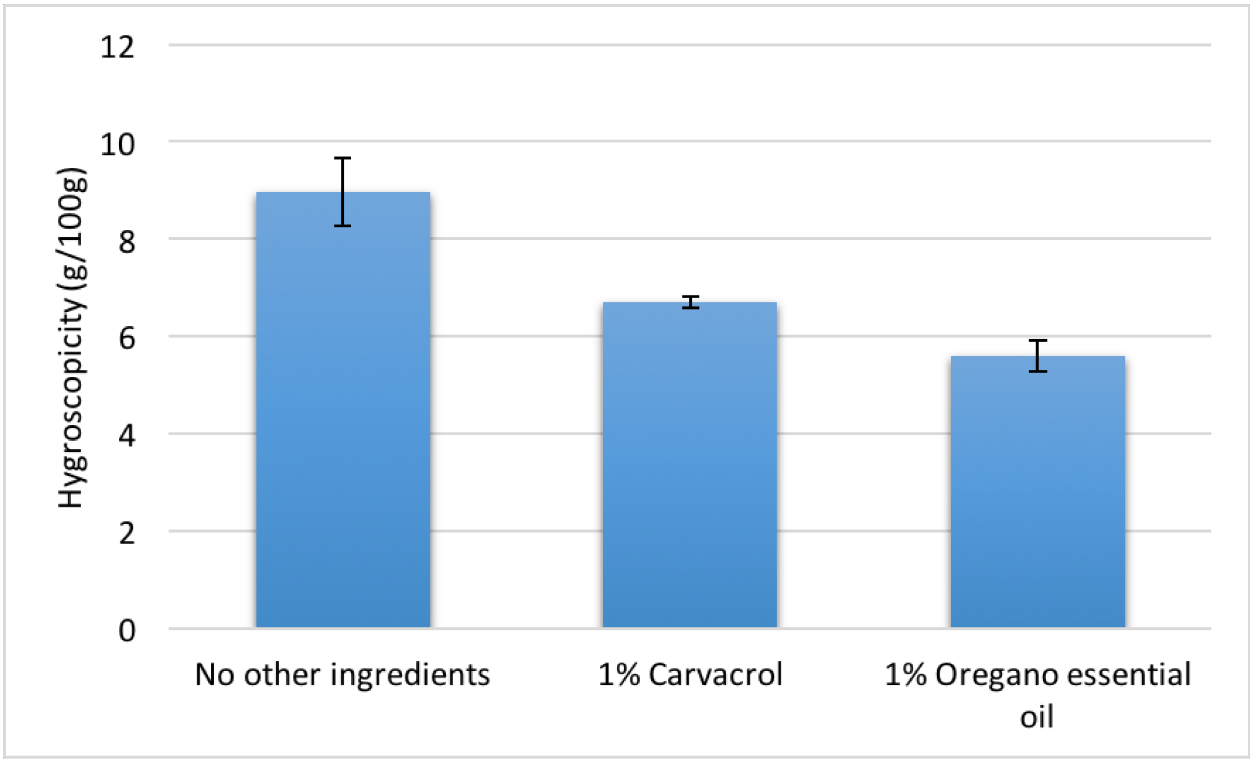

Figure 5.2. Comparison of hygroscopicity of β-lg films prepared with 5.0% glycerol and other ingredients.

5.4.3 Fourier Transform Infrared Spectroscopy

5.4.3.1 Edible Films Prepared with 1% Oregano Essential Oil

FTIR spectra of the edible films prepared with the addition of 1% oregano essential oil were acquired to ascertain whether the absorption bands of oregano essential oil could be identified. Figure 5.3(a) shows the FTIR spectra of pure oregano essential oil and of edible films prepared with or without addition of oregano essential oil. Since the predominant band in the spectrum of oregano essential oil is observed at 815-810 cm^{-1}, the comparison of the IR spectra in the 1000-750 cm^{-1} region is shown in Figure 5.3(b). When the spectra of edible films with and without oregano essential oil were subtracted from each other, the absorption in this region was close to zero. Thus, it could be concluded that in this experiment, the addition of 1.0% oregano essential oil in β-lg edible films could not be detected by ATR-FTIR analysis.

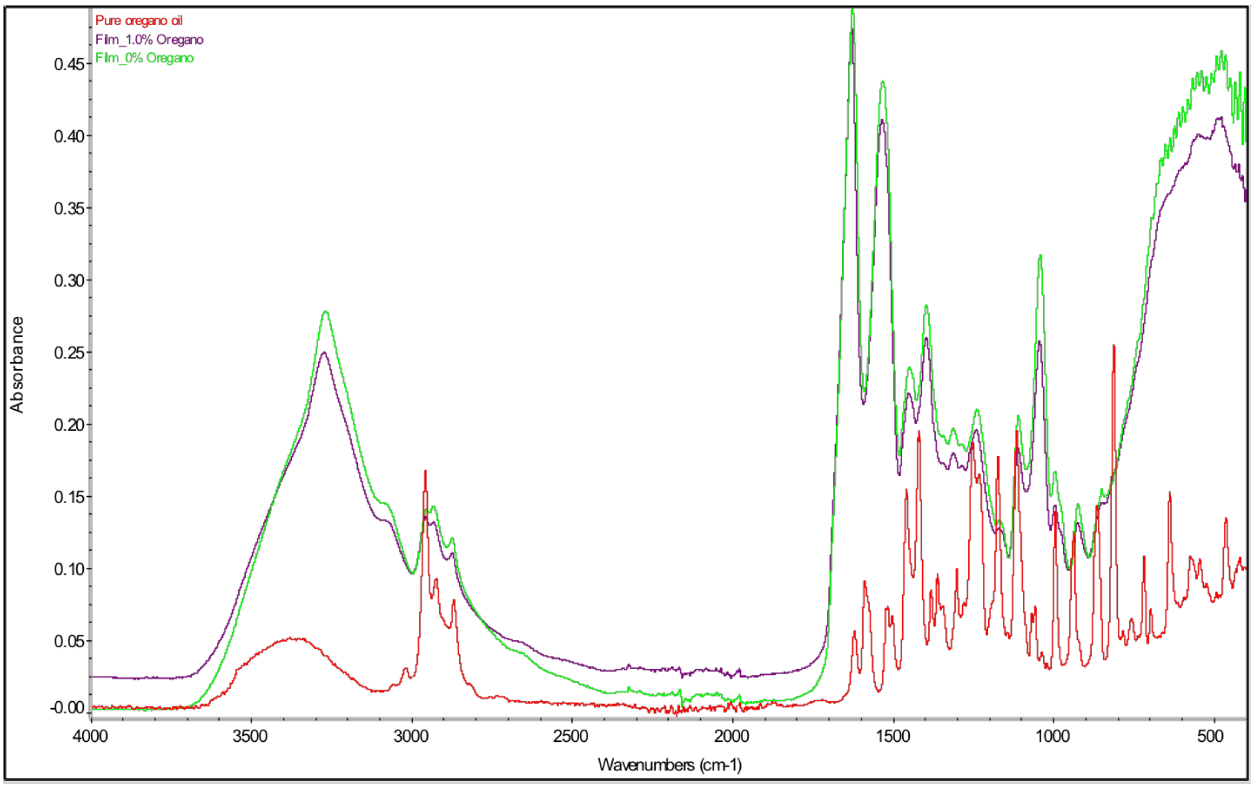

Figure 5.3(a). FTIR spectra (4000-600 cm^{-1}) of pure oregano essential oil (red) and of edible films prepared with addition of 1% oregano essential oil (purple) and without addition of oregano essential oil (green).

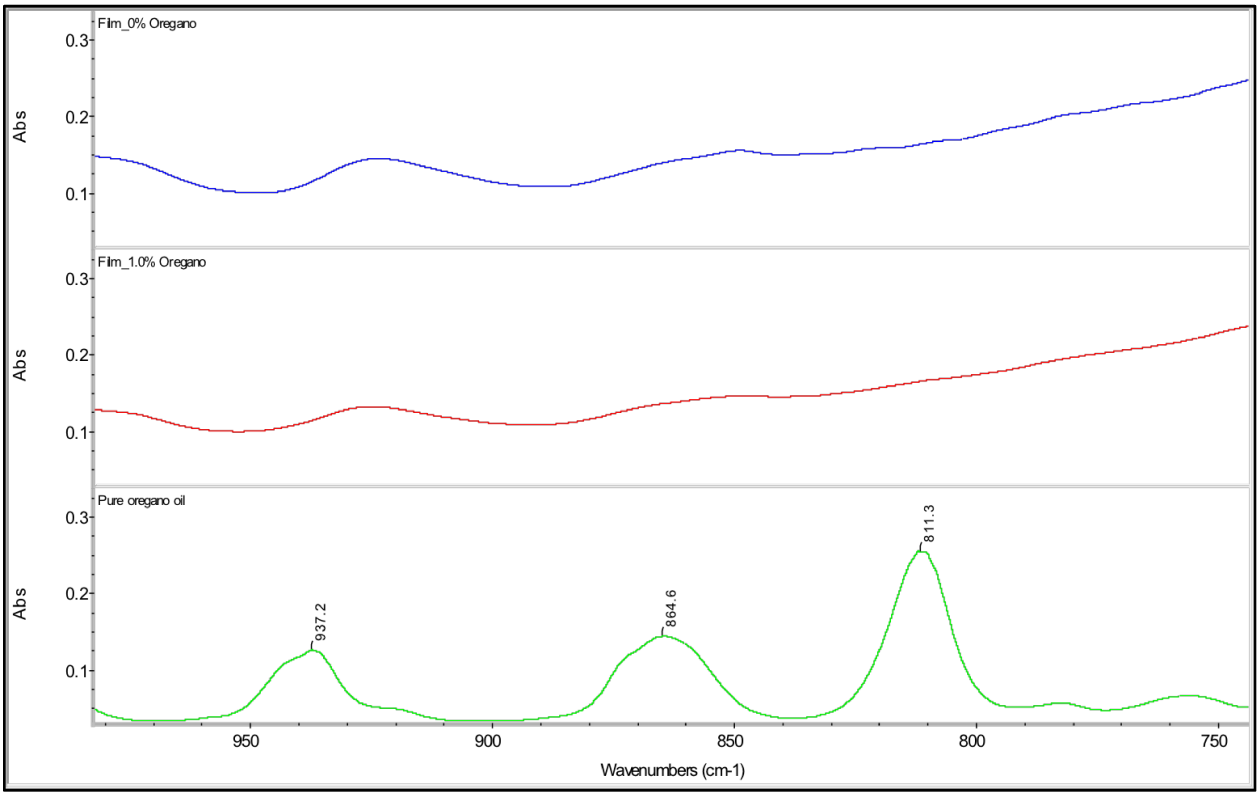

Figure 5.3(b). Expanded view of the 1000-750 cm^{-1} region in the FTIR spectra of pure oregano essential oil (green) and of edible films prepared with addition of 1% oregano essential oil (red) and without addition of oregano essential oil (blue). (Abs = absorbance)

5.4.3.2 Protein Structural Analysis

FTIR analysis of edible films was also performed to study potential interactions between the other ingredients (glycerol and oregano essential oil) and β-lg by examination of the protein amide I region, shown in Figures 5.4 and 5.5.

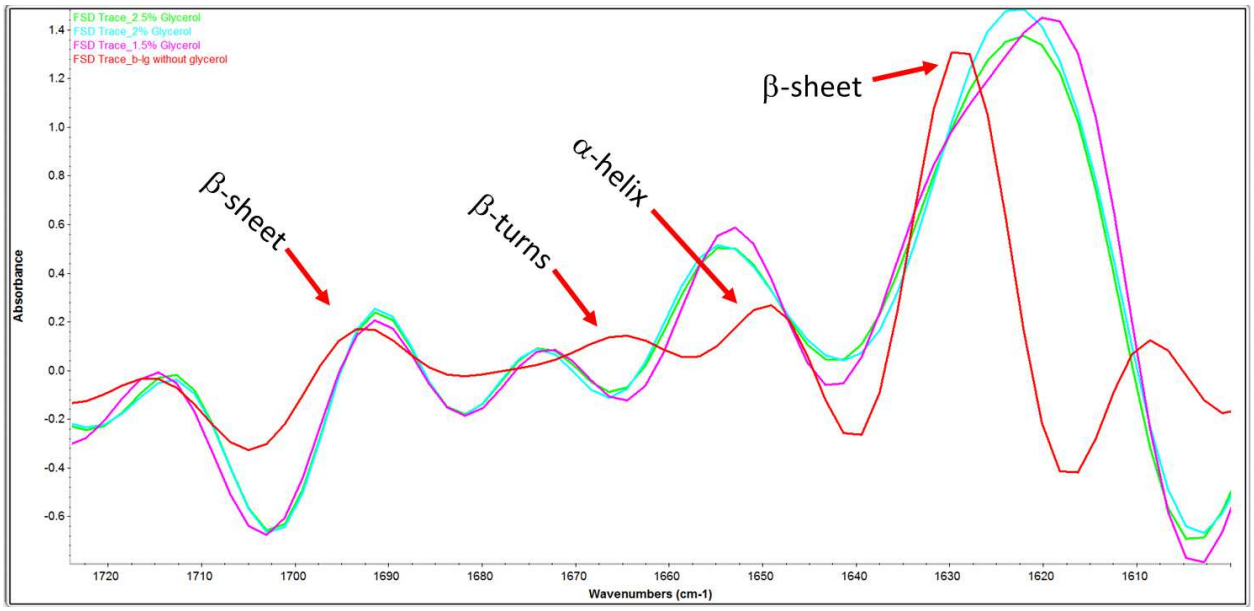

Figure 5.4. Fourier self-deconvoluted amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg films prepared with the use of different glycerol concentrations (1.5%, 2.0% and 2.5%). The red spectrum is that of powdered β-lg from Chapter 3, which is overlaid to allow for direct comparison.

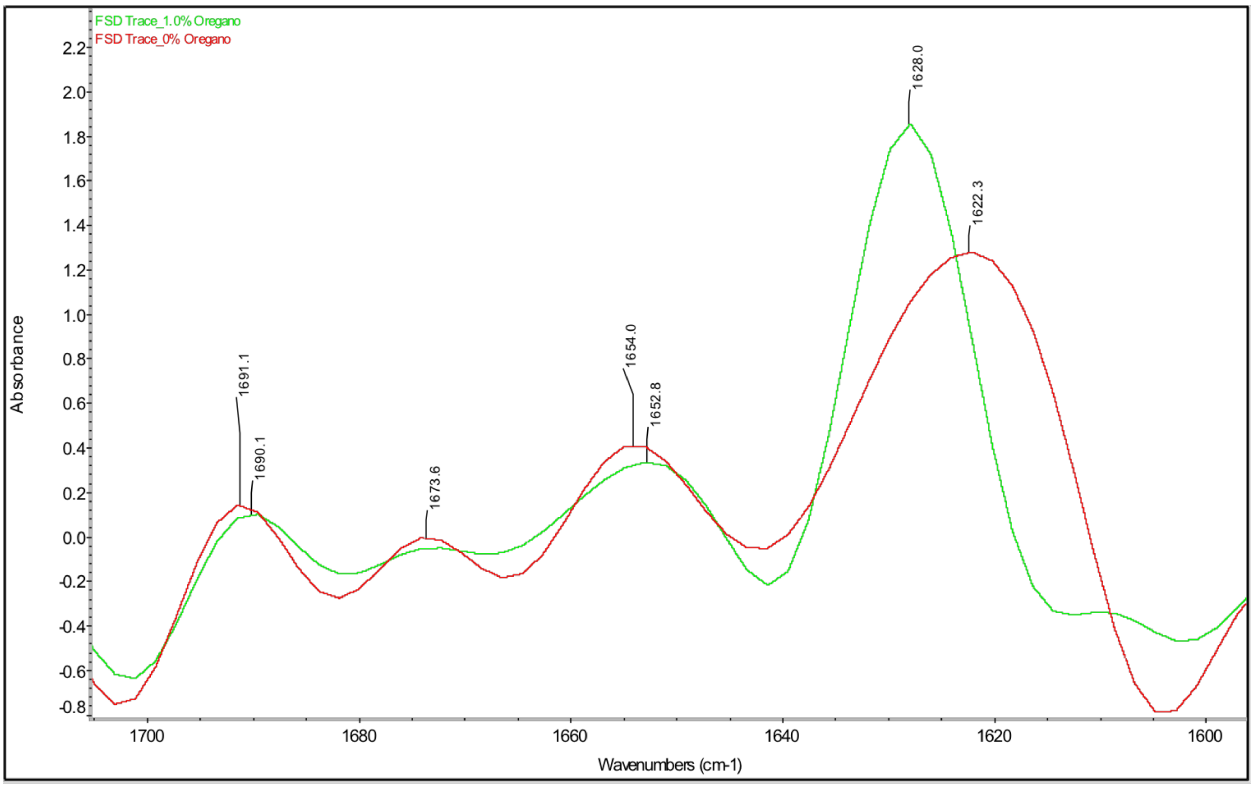

Figure 5.5. Fourier self-deconvoluted amide I region (1700-1600 cm^{-1}) of averaged IR spectra of β-lg films prepared with and without addition of oregano essential oil. The glycerol level in the initial protein solution was 2.5% in both cases.

Comparison of the spectra of the β-lg edible films prepared with the use of different glycerol concentrations with the spectrum of β-lg powder in Figure 5.4 shows the shifting of the bands associated with β-turns and α-helical structure as well as substantial broadening and shifting of the lower-frequency β-sheet band is observed. The broadening of the latter band is indicative of a mixed population of β-sheet structures and possibly the formation of intermolecular β-sheets, given that the formation of intermolecular β-sheets during thermal denaturation of β-lg is characterized by the appearance of an amide I band component at 1618 cm^{-1} (Boye *et al.*, 1996). Previous studies in the literature have reported that glycerol may affect protein secondary structure not only by interacting directly with the protein but also by changing the structure of water around the protein molecules (Huang *et al.*, 1995). The glycerol-induced changes in the amide I band profile of β-lg observed in Figure 5.4 are largely independent of the

glycerol concentration, except that the shift of the lower-frequency β-sheet band is larger at the lowest glycerol concentration.

The two spectra shown in Figure 5.5 were obtained from edible films prepared from β-lg solutions with the same initial glycerol level (2.5%) but one of them was prepared with addition of oregano essential oil at the 1.0% level. It is apparent that the addition of oregano essential oil counteracted the effect of glycerol on the β-sheet structures, as evidenced by the narrowing and shift of the low-frequency β-sheet band back to its position in the spectrum of β-lg powder (1628 cm^{-1}). On the other hand, the bands associated with β-turns and α-helical structure remain shifted from their positions in the spectrum of β-lg powder, although the band associated with α-helical is slightly shifted from its position in the presence of glycerol and absence of oregano essential oil. These observations suggested that the interactions between components of oregano essential oil and β-lg were primarily associated with the β-sheet structure and were sufficiently strong to interfere with the interactions of glycerol with the β-sheet structure of β-lg. Chapter 3 of this thesis demonstrated significant interactions between oregano essential oil and β-lg in freeze-dried microcapsules.

5.5 Conclusion

This study has investigated the incorporation of essential oils in β-lg edible films. Films were cast from film-forming solutions containing different levels of essential oils and glycerol (as plasticizer). It was observed that seasonal effects played an important role in determining the optimum composition of the film-forming solution. Specifically, when the relative humidity of the surrounding environment became lower, the elevated water evaporation rates would result in significantly higher plasticizer requirements. Another finding was that the incorporation of essential oils in the film-forming solution tended to have disruptive effects on edible film formation, as manifested by a cracked appearance. However, it was observed that β-lg films could be readily cast from solutions containing 1.0% (w/v) oregano essential oil. FTIR analysis of these films was unable to detect the principal IR band of oregano essential oil, which may be due to a decrease in the concentration of the essential oil through evaporation during the film drying process. However, FTIR analysis did provide evidence of significant interactions between

oregano essential oil and β-lg, by revealing that the changes in the β-sheet structure of β-lg induced by the presence of glycerol were counteracted by the addition of oregano essential oil.

For future studies, the investigation on incorporating other essential oil species in whey protein or other protein based edible films will be valuable, and testing the compatibility of a variety of plasticizers with the matrix material and essential oil will help to determine the optimum composition of the film-forming solution. On the other hand, due to the volatile nature of essential oils, exploring essential oil related spice powders will be another form of producing active packaging materials. Furthermore, developing means to improve the film preparation process by controlling the water evaporation rate will be of great value. Last but not least, the binding interactions between essential oils and protein matrices probed by FTIR spectroscopy can be further confirmed by other techniques such as circular dichroism and fluorescence spectroscopy.

5.6 References

Arrieta, M.P., Peltzer, M.A., Lopez, J., Garrigos, M.C., Valente, A.J.M., & Jimenez, A. (2014). Functional properties of sodium and calcium caseinate antimicrobial active films containing carvacrol. *Journal of Food Engineering, 121*(4), 94-101.

Avila-Sosa, R., Hernandez-Zamoran, E., Lopez-Mendoza, I., Palou, E., MT, J.M., Nevarez-Moorillon, G.V., & Lopez-Malo, A. (2010). Fungal inactivation by Mexican oregano (*Lippia berlandieri* Schauer) essential oil added to amaranth, chitosan, or starch edible films. *Journal of Food Science, 75*(3), M127-133.

Bahram, S., Rezaei, M., Soltani, M., Kamali, A., Ojagh, S.M., & Abdollahi, M. (2014). Whey protein concentrate edible film activated with cinnamon essential oil. *Journal of Food Processing and Preservation, 38*(3), 1251-1258.

Boye, J. I., Ismail, A. A., & Alli, I. (1996). Effects of physicochemical factors on the secondary structure of β-lactoglobulin. *Journal of Dairy Research*, *63*(1), 97-109.

Cagri, A., Ustunol, Z., & Ryser, E.T. (2001). Antimicrobial, mechanical, and moisture barrier properties of low pH whey protein-based edible films containing p-aminobenzoic or sorbic acids. *Journal of Food Science, 66*(6), 865-870.

Cai, Y.Z., & Corke, H. (2000). Production and properties of spray-dried *Amaranthus betacyanin* pigments. *Journal of Food Science, 65*(7), 1248-1252.

Cho, S.Y., Lee, S.Y., & Rhee, C. (2010). Edible oxygen barrier bilayer film pouches from corn zein and soy protein isolate for olive oil packaging. *LWT-Food Science and Technology, 43*(8), 1234-1239.

Han, J.H., Hwang, H.M., & Krochta, J.M. (2008). Coating of peanuts with edible whey protein film containing α-tocopherol and ascorbyl palmitate. *Journal of Food Science, 73*(8), E349-E355.

Huang, P., Dong, A., & Caughey, W.S. (1995). Effects of dimethyl sulfoxide, glycerol, and ethylene glycol on secondary structures of cytochrome c and lysozyme as observed by infrared spectroscopy. *Journal of Pharmaceutical Sciences*, *84*(4), 387-392.

Jangchud, A., & Chinnan, M.S. (1999). Properties of peanut protein film: sorption isotherm and plasticizer effect. *LWT-Food Science and Technology, 32*(2), 89-94.

Janjarasskul, T., Tananuwong, K., & Krochta, J.M. (2011). Whey protein film with oxygen scvenging function by incorporation of ascorbic acid. *Journal of Food Science, 76*(9), E561-E568.

Jouki, M., Yazdi, F.T., Mortazavi, S.A., Koocheki, A., & Khazaei, N. (2014). Effect of quince seed mucilage edible films incorporated with oregano or thyme essential oil on shelf life extension of refrigerated rainbow trout fillets. *International Journal of Food Microbiology, 174*, 88-97.

Ket-On, A., Pongmongkol, N., Somwangthanaroj, A., Janjarasskul, T., & Tananuwong, K. (2016). Properties and storage stability of whey protein edible film with spice powders. *Journal of Food Science and Technology, 53*(7), 2933-2942.

Kim, S.J., & Ustunol, Z. (2001). Sensory attributes of whey protein isolate and candelilla wax emulsion edible films. *Journal of Food Science, 66*(6), 909-911.

Lent, L.E., Vanasupa, L.S., & Tong, P.S. (1998). Whey protein edible film structures determined by atomic force microscope. *Journal of Food Science, 63*(5), 824-827.

Lin, S-J., & Pascall, M.A. (2014). Incorporation of vitamin E into chitosan and its effect on the film forming solution (viscosity and drying rate) and the solubility and thermal properties of the dried film. *Food hydrocolloids, 35*, 78-84.

Lopez, D., Marquez, A., Gutierrez-Cutino, M., Venegas-Yazigi, D., Bustos, R., & Matiacevich, S. (2017). Edible film with antioxidant capacity based on salmon gelatin and boldine. *LWT-Food Science and Technology, 77*, 160-169.

Ma, Q., Hu, D., Wang, H., & Wang, L. (2016). Tara gum edible film incorporated with oleic acid. *Food Hydrocolloids, 56*, 127-133.

McHugh, T.H., Aujard, J.F., & Krochta, J.M. (1994). Plasticized whey protein edible films: water vapor permeability properties. *Journal of Food Science, 59*(2), 416-419.

Meenatchisundaram, S., Chandrasekar, C.M., Udayasoorian, L.P., Kavindapadi Rajasekaran, R., Muthusamy, S., Kesavan, R.K., & Srinivasan, B. (2016). *Journal of the Science of Food and Agriculture,* 4268-4275.

Moradi, M., Tajik, H., Rohani, S.M.R., & Mahmoudian, A. (2016). Antioxidant and antimicrobial effects of zein edible film impregnated with *Zataria multiflora* Boiss. essential oil and monolaurin. *LWT-Food Science and Technology, 72*, 37-43.

Mu, C., Guo, J., Li, X., Lin, W., & Li, D. (2012). Preparation and properties of dialdehyde carboxymethyl cellulose crosslinked gelatin edible films. *Food Hydrocolloids, 27*(1), 22-29.

Nandane, A.S., & Jain, R. (2015). Study of mechanical properties of soy protein based edible film as affected by its composition and process parameters by using RSM. *Journal of Food Science and Technology, 52*(6), 3645-3650.

Randazzo, W., Jimenez-Belenguer, A., Settanni, L., Perdones, A., Moschetti, M., Palazzolo, E., Guarrasi, V., Vargas, M., Germana, M.A., & Moschetti, G. (2016). Antilisterial effect of citrus essential oils and their performance in edible film formulations. *Food Control, 59*, 750-758.

Seydim, A.C., & Sarikus, G. (2006). Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. *Food Research International, 39*(5), 639-644.

Sivarooban, T., Hettiarachchy, N.S., & Johnson, M.G. (2008). Physical and antimicrobial properties of grape seed extract, nisin, and EDTA incorporated soy protein edible films. *Food Research International, 41*(8), 781-785.

Sohail, S.S., Wang, B., Biswas, M.A.S., & Oh, J-H. (2006). Physical, morphological, and barrier properties of edible casein films with wax applications. *Journal of Food Science, 71*(4), C255-C259.

Soni, A., Kandeepan, G., Mendiratta, S.K., Shukla, V., & Kumar, A. (2016). Development and characterization of essential oils incorporated carrageenan based edible films for packaging of chicken patties. *Nutrition and Food Science, 46*(1), 82-95.

Sun, Q., Sun, C., & Xiong, L. (2013). Mechanical, barrier and morphological properties of pea starch and peanut protein isolate blend films. *Carbohydrate Polymers, 98*(1), 630-637.

Thakur, R., Saberi, B., Pristijono, P., Golding, J., Stathopoulos, C., Scarlett, C., Bowyer, M., & Vuong, Q. (2016). Characterization of rice-ι-carrageenan biodegradable edible film. Effect of stearic acid on the film properties. *International Journal of Biological Macromolecules, 93*, 952-960.

Zsila, F. (2003). A new ligand for an old lipocalin: induced circular dichroism spectra reveal binding of bilirubin to bovine beta-lactoglobulin. *FEBS Letters, 539*(1), 85-90.

CHAPTER 6

GENERAL CONCLUSION

In this study, the microencapsulation of commercial oregano, rosemary and sage essential oils in β-lg by a freeze-drying process was investigated. Using the same technology, the potential of inulin as the wall material for encapsulating oregano essential oil was also investigated. Finally, a preliminary study on incorporation of essential oils in β-lg based edible films was aimed at examining the impact of essential oils on film quality.

Freeze-dried microparticles produced from emulsions having a higher content of the wall material (β-lg or inulin) tended to have higher bulk tapped density and particle density, revealing higher compressibility. The dried β-lg microcapsules containing oregano essential oil exhibited lower hygroscopicity compared to those containing rosemary or sage essential oil, indicating higher powder quality in terms of water sorption.

The encapsulation efficiency obtained by using β-lg as the wall material was slightly higher than that obtained with inulin, but both results were consistent with previous literature. For future studies, employing a mixture of these two compounds, β-lg as the primary and inulin as the secondary coating material, will be of interest to test if there is a synergistic effect on the encapsulation efficiency.

The antioxidant activity of β-lg microparticles containing oregano, rosemary or sage essential oils was found to be significant based on β-carotene assay results.

The *in vitro* release of rosemary and sage essential oils from β-lg microcapsules into phosphate buffer solution was similar whereas that of oregano essential oil was substantially lower. This difference may be attributed to the lower vapor pressure of oregano essential oil or to more extensive interaction between β-lg and components of oregano essential. Since inorganic solvent was tested in this study, further research on the targeted release of essential oils in *in*

vitro (organic solvent or artificial digestive tract) or *in vivo* (real animal model) environments will also be valuable.

The preliminary study of the incorporation of essential oils in β-lg edible films revealed that the addition of essential oils to film-forming solutions reduced the film stability and led to a higher level of brittleness. Among the three essential oils tested in this study, only oregano essential oil in a narrow concentration range yielded satisfactory films. These films tended to possess better water barrier properties than the films prepared without essential oil addition. For future studies, the compatibility of other essential oil species with β-lg and the efficacy of various types of plasticizers should be examined.

Finally, FTIR analysis was conducted to evaluate whether interactions between essential oils and β-lg resulted in protein conformational changes. Based on the IR spectra of freeze-dried microparticles, it was found that the presence of oregano essential oil caused apparent band shifts and intensity changes in the α-helix and β-sheet components of the amide I band. In addition, the spectra of edible films showed that the presence of oregano essential reversed the glycerol-induced changes in the low-frequency β-sheet components of the amide I band. It could be concluded that the interactions between components of oregano essential oil and the protein matrix were significant. The potential binding sites were proposed to be on the surface of the calyx that connects the α-helix and β-barrel.