ANTIMICROBIAL ACTIVITY OF VARIOUS ESSENTIAL OILS AND THEIR APPLICATION IN ACTIVE PACKAGING OF FOOD PRODUCTS

by

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ABSTRACT

Essential oils (EOs) have potential utility as clean-label food preservatives due to their antimicrobial and antioxidant properties. Several attempts have been made to develop active-packaging systems incorporating EOs into polymeric materials that can slowly release the volatile active compounds onto the food surface to inhibit the growth of pathogenic microorganisms. *Listeria monocytogenes* is the primary microorganism of concern in ensuring the safety of refrigerated ready-to-eat foods and also frozen vegetable products, as the latter may be thawed by consumers. In our research, the efficacy of EOs in inhibiting the growth of *L. grayi,* selected as a less pathogenic surrogate for *L. monocytogenes,* was studied *in vitro* and in storage tests with thawed frozen vegetable products. In addition, conditions for the preparation of soy protein films as carriers for EOs and their antimicrobial properties in active-packaging applications were investigated. Finally, the rate of release of active compounds from these and other carrier matrices was monitored by gas-phase Fourier transform infrared (FTIR) spectroscopy, and kinetic parameters were evaluated by fitting a Weibull distribution model to the FTIR data by non-linear regression.

The research began by screening 25 locally sourced EOs for antimicrobial activities against *L. grayi* as well as *E. coli* K12 in disk diffusion and vapor diffusion tests. A cinnamon EO (CIN-03) and an oregano EO (ORE-03) as well as pure carvacrol, a major active compound in oregano EOs, proved to be effective in inhibiting the growth of *L. grayi* and *E. coli* K12 both in direct contact with the microorganisms and in the vapor phase. In additional tests, these EOs also inhibited growth of *L. monocytogenes* as well as Shiga toxin-producing *E. coli* (STEC) strains belonging to serotypes O157:H7 and O26:H11. The slightly lower susceptibility of *L. grayi* in these tests as compared with *L. monocytogenes* makes it a suitable non-pathogenic surrogate for *L. monocytogenes* in this research. In storage tests in which fresh-cut green peppers inoculated with *L. grayi* were packaged with cellulose stickers impregnated with cinnamon EO, the listeria count was reduced to 10 CFU/g after 2 days of storage as compared to 7.54 log CFU/g for the controls. Sensory evaluation of broccoli and french fries that had been exposed to oregano and cinnamon EOs in the headspace of the packaging revealed that cinnamon EO at the higher test level was less acceptable to panelists than oregano EO for both products. A method of delivery of aerosolized oregano EO onto broccoli in a sealed container was found to be effective in inhibiting the growth of *L. grayi*

during subsequent storage at 4 ºC, resulting in a reduction of the listeria count by almost 2 log CFU/g in comparison with controls after 12 days.

As carriers of EOs, soy protein films were prepared and investigated in the following chapter. The film preparation conditions were optimized based on examination of the mechanical properties of films cast from 5% (w/v) solutions of soy protein isolate (SPI) as a function of the concentration of glycerol added to the film-forming solution to serve as a plasticizer, the pH of the solution, and the heating temperature/time applied. These experiments resulted in the selection of the following conditions for film preparation: 2% glycerol, pH 10 for the film-forming solution and heating of this solution at 85 ºC for 10 minutes. These conditions were used to prepare soy protein films incorporating carvacrol, oregano EO and cinnamon EO. The type of EO, the concentrations of glycerol and EO in the film-forming solution, and the emulsification treatment employed in preparing the films influenced their mechanical properties, water vapor permeability, water solubility and transparency. FTIR spectroscopic characterization of the films indicated that interactions between soy proteins and the EOs incorporated in the films resulted in some transformation of β-sheet to α-helical structure, consistent with a plasticizing effect of the EOs. Furthermore, the soy protein films prepared with ≥1.5% oregano EO or ≥2% carvacrol in the film-forming solution exhibited strong antimicrobial activities in vapor diffusion tests, demonstrating that the films released essential oil active compounds into the vapor phase at inhibitory concentrations under the conditions of these tests. The study of the kinetics of carvacrol release from soy protein films differing in glycerol content by the FTIR methodology elaborated in this research indicated that increasing the amount of glycerol promoted the release of carvacrol from the films. In addition, release of carvacrol from the cellulose stickers employed in the storage tests produced a higher equilibrium concentration of carvacrol vapor in a sealed infrared gas cell than evaporation of liquid carvacrol. These results were obtained by fitting a Weibull model to the FTIR data, yielding a high correlation coefficient (>0.995) and low RMSE (≤0.002). Further application of this FTIR methodology to examine the release of carvacrol from common matrix materials showed that the rate of release was strongly influenced by the microstructure of the matrix and decreased in the order silica > cotton > clay > chitosan.

RESUME

Les huiles essentielles (HE) ont une utilité potentielle en tant que conservateurs alimentaires naturels en raison de leurs propriétés antimicrobiennes et antioxydantes. Plusieurs tentatives ont été faites pour développer des emballages actifs incorporant des HE dans des matériaux polymères qui peuvent lentement libérer les composés actifs volatils sur la surface des aliments pour inhiber la croissance des micro-organismes pathogènes. *Listeria monocytogenes* est le principal micro-organisme préoccupant pour assurer la sécurité des aliments réfrigérés prêts à consommer et des produits végétaux surgelés, car ceux-ci peuvent être décongelés par les consommateurs. Dans nos recherches, l'efficacité des huiles essentielles dans l'inhibition de la croissance de *L. grayi*, sélectionnée comme substitut moins pathogène de *L. monocytogenes*, a été étudiée *in vitro* et dans des tests de stockage avec des produits végétaux surgelés décongelés. De plus, les conditions de préparation de films de protéines de soja en tant que supports pour les HE et ses propriétés antimicrobiennes dans les applications d'emballage actif ont été étudiées. Finalement, le taux de libération des composés actifs d'HE à partir de différentes matrices a été surveillé par spectroscopie infrarouge à transformée de Fourier (FTIR) en phase gazeuse et des paramètres cinétiques ont été évalués en ajustant un modèle de distribution de Weibull aux données FTIR par régression non linéaire.

La recherche a commencé par le criblage des activités antimicrobiennes d'environ vingt-cinq huiles essentielles par des tests de diffusion en milieu gélosé et en phase vapeur. Les huiles essentielles de cannelle (CIN-03) et d'origan (ORE-03) et le carvacrol se sont révélés efficaces à la fois en contact direct et en phase vapeur pour inhiber la croissance de *L. grayi* et de *E. coli* K12 ainsi que de *Listeria monocytogenes* et de souches *E. coli* producteurs de Shiga-toxines (STEC) appartenant aux sérotypes O157:H7 et O26:H11. La sensibilité légèrement inférieure de *L. grayi* par rapport à *L. monocytogenes* en fait un substitut non pathogène approprié dans nos études. L'emballage de poivrons verts fraîchement coupés avec des autocollants en cellulose imprégnés d'HE de cannelle a réduit le nombre de listeria à 10 CFU/g après 2 jours de stockage, contre 7.54 log CFU/g pour les témoins. L'évaluation sensorielle du brocoli congelé et des frites congelées qui avaient été exposés à l'HE d'origan et de cannelle dans l'espace de tête de l'emballage a révélé que l'HE de cannelle à la concentration la plus élevée était moins acceptable pour les panélistes que l'HE d'origan. Une méthode de livraison directe d'origan HE en aérosol sur le brocoli dans un récipient scellé

s'est avérée efficace pour inhiber la croissance de *L. grayi* lors d'un stockage ultérieur à 4 ºC, entraînant une réduction du nombre de listeria de près de 2 log UFC/g par rapport aux témoins après 12 jours.

En tant que porteurs d'HE, des films de protéines de soja ont été préparés et étudiés dans le chapitre suivant. Les conditions de préparation des films ont été optimisées sur la base de l'examen des propriétés mécaniques des films en fonction de la concentration de glycérol ajouté à la solution filmogène comme plastifiant, du pH de la solution et de la température / temps de chauffage appliqué. Ces expériences ont abouti à la sélection des conditions suivantes pour la préparation du film: 2% de glycérol, pH 10 pour la solution filmogène et chauffage de cette solution à 85 ºC pendant 10 minutes. Tous ces paramètres étudiés précédemment ont été utilisés pour préparer les films de soja incorporés aux huiles essentielles de carvacrol, d'origan et de cannelle. Le type d'HE, les concentrations de glycérol et d'HE dans la solution filmogène et le traitement d'émulsification utilisé pour préparer les films ont influencé leurs propriétés mécaniques, la perméabilité à la vapeur d'eau, la solubilité dans l'eau et les opacités. La caractérisation spectroscopique FTIR des films a indiqué que les interactions entre les protéines de soja et les HE incorporés dans les films ont entraîné une certaine transformation de la feuille β en une structure hélicoïdale α, compatible avec un effet plastifiant des HE. De plus, le film de protéine de soja incorporé avec plus de 1,5% d'origan ou 2% de carvacrol a démontré de fortes capacités antimicrobiennes en phase vapeur *in vitro*. L'étude de la cinétique de la libération de carvacrol à partir de films de protéines de soja différant en teneur en glycérol par la méthodologie FTIR élaborée dans cette recherche a indiqué que l'augmentation de la quantité de glycérol a favorisé la libération de carvacrol. De plus, la libération de carvacrol des autocollants cellulosiques utilisés dans les tests de stockage a produit une concentration plus élevée de vapeur de carvacrol dans une cellule à gaz infrarouge scellée par rapport à l'évaporation de carvacrol liquide. Ces résultats ont été obtenus en ajustant un modèle de Weibull aux données FTIR, produisant un coefficient de corrélation élevé (>0.995) et un RMSE faible (≤0.002). Une application supplémentaire de cette méthodologie FTIR pour examiner la libération de carvacrol à partir de matériaux matriciels courants a montré que le taux de libération était fortement influencé par la microstructure de la matrice et suivait l'ordre suivant de la silice > coton > argile > chitosane.

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CONTRIBUTIONS OF AUTHORS

This thesis consists of six chapters as follows: Chapter I is a general introduction which includes a brief overview of the recent related work in the literature and states the objectives of the research presented in the thesis. Chapter II is a detailed review of the literature pertaining to essential oils and their antimicrobial activities, the application of biopolymers in active packaging, and the incorporation of essential oils in protein films and sachets. Chapter III presents the investigation of the antimicrobial activities of various essential oils and their potential applicability in active packaging of ready-to-eat and frozen vegetable products. Chapter IV reports on the preparation of soy protein films incorporating essential oils, the characterization of the physical properties of these biopolymer films, and *in vitro* study of their antimicrobial activities. Chapter V examines the release kinetics of essential oil active compounds from various packaging matrices, including those studied previously in Chapters III and IV, by gas-phase FTIR spectroscopy and the application of the Weibull distribution to model the FTIR data. Finally, Chapter VI provides a general discussion of the results obtained in this research together with suggestions for further work.

The author was responsible for designing and performing experimental work, analysis of data and results, and preparation of the thesis. Dr. Ashraf Ismail was the thesis supervisor and guided experimental work and provided advice throughout this research. Dr. Jacqueline Sedman provided guidance in analysis of the FTIR spectra of soy protein films and reviewed and edited this thesis for submission.

Results reported in this thesis were or will be presented at the following conferences:

1. Poster presentation at IFT 19 Annual Meeting & Food Expo-Institute of Food Technologists (New Orleans, LA, USA)

   Antimicrobial Activity of Various Essential Oils and Their Application in Active Packaging of Frozen and Ready-to-Eat Vegetable Products.
   (Authors: Ran Tao, Ashraf Ismail, Jacqueline Sedman)

2. Oral presentation at the 2020 AOCS Annual Meeting & Food Expo (Montreal, QC, Canada)

   Characterization of Biopolymer Films Incorporating Essential Oils Exhibiting Antilisterial Activity as Active-Packaging for Ready-to-Eat Foods.
   (Authors: Ran Tao, Ashraf Ismail, Jacqueline Sedman)

CONTRIBUTIONS TO KNOWLEDGE

1. The susceptibility of *Listeria grayi* to various essential oils was investigated for the first time and compared with that of *L. monocytogenes*. The slightly lower susceptibility of *L. grayi* made it a suitable non-pathogenic surrogate for *L. monocytogenes* in our study.

2. In *in vitro* tests, certain cinnamon and oregano essential oils as well as pure carvacrol, a major active compound in oregano essential oil, proved to be effective in the vapor phase in inhibiting the growth of *L. grayi* and *L. monocytogenes* as well as *E. coli* K12, *E. coli* O157:H7 and *E. coli* O26:H11.

3. Release of essential oil active compounds from cellulose stickers impregnated with oregano and cinnamon essential oils into the headspace of packaging was found to be effective in inhibiting the growth of *L. grayi* on vegetables during storage.

4. Treatment of vegetables with aerosolized essential oil prior to storage was shown for the first time to be effective in inhibiting the growth of *Listeria* on vegetables stored at 4°C.

5. Conditions for the preparation of antimicrobial soy protein films by incorporation of carvacrol or oregano essential oil were established. Soy protein films incorporating cinnamon essential oil were also prepared but lacked antimicrobial activity.

6. The mechanical properties, water vapor permeabilities, and optical characteristics of soy protein films incorporating essential oils were shown to be influenced by the type of essential oil, the concentrations of glycerol and essential oil in the film-forming solution, and the emulsification treatment applied to the film-forming solution.

7. Incorporation of carvacrol or oregano essential oil into soy protein films resulted in changes in the secondary structure of the soy proteins. Some transformation of β-sheet to α-helical structure was observed by FTIR spectroscopy, consistent with a plasticizing effect of carvacrol and oregano essential oil.

8. Gas-phase FTIR spectroscopy in conjunction with fitting of the FTIR data to a Weibull distribution model was used for the first time to study the release kinetics of essential oil active compounds from different carrier matrices. This convenient FTIR methodology aids the selection of components for controlled-release active-packaging systems.

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LIST OF ABBREVIATIONS

AITC	Allyl isothiocyanate
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ATR	Attenuated total reflectance
BHIA	Brain heart infusion agar
CAR	Carvacrol
CED	Cedar
CFIA	Canadian Food Inspection Agency
CHI	Chili seed
CIN	Cinnamon
CINN	Cinnamaldehyde
CIT	Citron
CLO	Clove
CNB	Cranberry
CUM	Cumin seed (*Cuminum cyminum*)
CYP	Cypress
D_{cal}	Calculated diffusion coefficient
EAB	Elongation at break
EHEC	Enterohemorrhagic *Escherichia coli*
EM	Elastic modulus
EO(s)	Essential oil(s)
EUG	Eugenol
FDA	U.S. Food and Drug Administration
FEMA	Flavor and Extract Manufacturer's Association
FPA	Focal-plane array
FTIR	Fourier transform infrared
GAR	Garlic
GRAS	Generally recognized as safe
LDPE	Low-density polyethylene
LSPQ	Laboratoire de Santé Publique du Québec

LSA	*Listeria* selective agar
MAP	Modified atmosphere packaging
MBC	Minimum bactericidal concentration
MEL	Lemon balm
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
MID	Minimum inhibitory dose
OD	Optical density
OIG	Concentrated onion
OPP/PE	Oriented polypropylene/polyethylene
ORE	Oregano
PIM	Pimento
PIN	White pine needle
R^2	Coefficient of determination
RH	Relative humidity
RMSE	Root mean square error
ROS	Rosemary
S(%)	Solubility percentage
SAG	Sage
SMPS	Silica mesoporous supports
SPI	Soy protein isolate
STEC	Shiga toxin-producing *E. coli*
THY	Thyme
TS	Tensile strength
TSB	Tryptic soya broth
TTO	Tea tree oil
USDA	U.S. Department of Agriculture’
UV	Ultraviolet
Vis	Visible light
WS	Water solubility
WPI	Whey protein isolate
WVP	Water vapor permeability
WVTR	Water vapor transmission rate

CHAPTER 1 GENERAL INTRODUCTION

Over the past few decades, consumers' attitudes about food safety and additives have changed a lot. An increasing number of consumers prefer natural foods with no synthetic food additives in them. There is a high demand for 'clean label products' in the market (Axel *et al.*, 2016). Because of this trend of 'green' consumerism (Tuley de Silva, 1996; Smid and Gorris, 1999), new food preservatives that are green and natural are required to extend food shelf life. There are several bio-preservations that are extracted from plants or composed of compounds found in nature, such as carvacrol, benzaldehyde, cymene, cinnamaldehyde, eugenol, limonene, salicylaldehyde, menthol, thymol, terpineol and vanillin (Walsh *et al.,* 2003; Dobre *et al.,* 2011). Natural extracts and their active components are listed as flavoring agents by the European Union and are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (Passarinho *et al.,* 2014).

Essential oils (EOs) are volatile and aromatic oily liquids extracted from some herbs and spices, such as flowers, leaves, seeds, roots and fruits, and have potential utility as clean-label food preservatives due to their antimicrobial and antioxidant properties (Burt, 2004). Among EOs, cinnamon (*Cinnamonumverum*) essential oil and oregano (*Origanum vulgare*) essential oil are reported to exhibit highly effective antimicrobial properties, largely attributed to the active compounds carvacrol and thymol in oregano EO and cinnamaldehyde in cinnamon EO. Recently, these and other EOs have received much attention from researchers, and several attempts have been made to develop active-packaging systems incorporating EOs into polymeric materials that can slowly release the volatile active compounds onto the food surface.

Active packaging is an innovative concept in packaging technology that aims at providing additional functionality beyond the conventional roles of packaging. Active packaging systems actively interact with the inner environment of the package and thus can provide better protection to the food products (Yam *et al.,* 2005). It is commonly known that food spoilage is mostly caused by microbial contamination. To solve this problem, an antimicrobial packaging system is a version of active packaging with an emphasis on controlling growth of microorganisms (Floros *et al.,* 1997). The strong antimicrobial properties of certain EOs make them a promising choice of antimicrobial agent for active packaging systems based on natural extracts (López *et al.,* 2007). Two approaches can be used to introduce EOs into packages: one is by addition of EO to in-package components (sachets and absorbent pads) and the other is by incorporation in biopolymer films/coatings or encapsulation (Suppakul *et al.,* 2002). In antimicrobial packages, EOs can be released by

diffusion through direct contact and by evaporation. Several studies also reported antibacterial or antifungal activity of EOs in active packaging by incorporating them in biopolymer films or in other ways (Seydim *et al.,* 2006; Arancibia *et al.,* 2014; Emiroglu *et al.,* 2010; Tongnuanchan *et al.,* 2012).

Frozen fruits and vegetables are intended to be kept frozen at low temperature (-18 ^{o}C) to maintain their initial quality and nutritive value unchanged. It is undoubtedly the most satisfactory means of preserving food for long-term storage, and the consumption of frozen fruits and vegetables has seen constant growth in many places (Allende *et al.,* 2002). To ensure the quality of frozen foods, many factors need to be taken into account, such as initial quality, pre-freezing treatments, and freezing conditions. Generally, the freezing of vegetables and fruits is often undertaken immediately after postharvest treatment and a blanching process (Sun, 2011; Danyluk *et al.,* 2015). However, although many organisms can be destroyed during freezing, some may still survive. Microbial contamination of frozen food can occur from slicers, choppers or cutters, conveyor belts, lifts, hoppers, and fillers (Splittstoesser, 1973). In frozen vegetables, the common microorganisms mainly come from the soil, including lactic acid bacteria (LAB), enterococci, and coliforms (Mundt *et al.,* 1967; Splittstoesser, 1983; Borgstrom, 1955). Spoilage of frozen food can happen upon thawing due to the growth of psychrotrophic microorganisms, especially *Listeria,* which can survive at low temperature and may be able to slowly initiate growth under thawing conditions (Michener, *et al.,* 1968; Brown, 1991; Golden *et al.,* 1988). Infection with *L. monocytogenes* can cause several illnesses in humans and animals, including septicemia, meningitis, and encephalitis, as well as spontaneous abortion or miscarriage and has a mortality rate of 20-25%. In the 1980s, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service and the U.S. Food and Drug Administration (FDA) established a “zero-tolerance” policy for *L. monocytogenes* in ready-to-eat foods (Kataoka *et al.,* 2017). Federal regulations in Canada permit no greater than 100 CFU/g of *L. monocytogenes* in ready-to-eat vegetables, for which the refrigerated shelf life should be less than 5 days; meanwhile, in frozen vegetable products without cooking instructions, *L. monocytogenes* should not increase in number by 0.5 log CFU/g during the stated shelf life under reasonable condition of storage, distribution and usage. As frozen vegetable products do not support the growth of *L. monocytogenes* throughout their shelf life, they receive low priority for regulatory oversight and industry verification (CFIA, 2016).

For the food industry, detecting and inhibiting the growth of *L. monocytogenes* in food products during storage is critically important and has been the focus of much research. Other *Listeria* species such as *L. grayi* have largely been considered as apathogenic bacteria causing relatively minor injuries to animals and humans and have served as surrogates for *L. monocytogenes* in some studies. However, *L. grayi* needs more attention from researchers for three reasons. (i) In previous studies, *Listeria* spp. have been frequently isolated from vegetables, meat, seafood, fish, and soil. Some studies have shown higher prevalence of *L. grayi* than of *L. monocytogenes* in *Listeria*-contaminated food, and *L. grayi* was found to be the most prevalent species of *Listeria* in raw and ready-to-eat foods (Soriano *et al.,* 2001; Chen *et al.,* 2009; Miyebi *et al.,* 2018). Furthermore, *L. grayi* was found most frequently in meat and vegetable products, while *L. monocytogenes* was found in a large variety of foods. (ii) As *L. grayi* has been regarded as non-pathogenic, its antibiotic susceptibilities have only been reported in some studies comparing various *Listeria* species. The limited studies of *L. grayi* found it to be naturally resistant to all antifolates and some antimicrobial agents, including cephalothin, clindamycin, erythromycin, gentamycin, methicillin, oxacillin, and penicillin, by comparison with *L. monocytogenes* (Troxler *et al.,* 2000; Pesavento *et al.,* 2010). Some MIC/MBC tests of antibiotics against *L. grayi* also demonstrated its resistance to the first-line antibiotics ampicillin and penicillin G, but it was more susceptible to plant-based triterpenes (Penduka *et al.,* 2014). (iii) Although, *L. grayi* is not generally a pathogenic bacterium and infections caused by *Listeria* species other than *L. monocytogenes* are rare (Reda *et al.,* 2016), some cases of *L. grayi* infection have been reported in the past 20 years, including eye infection, isolation of *L. grayi* from joint cavity effusion, and diarrhea in a five-year-old child caused by *L. grayi* (Ao Birong, 2001; Lin Jiuling *et al.,* 2001; Grif *et al.,* 2003) and, more recently, a sepsis infection in a heart transplant recipient and a bacteremia in a stem cell transplant recipient (Rapose *et al.,* 2008; Salimnia *et al.,* 2010). These infections in humans showed the potential pathogenicity of *L. grayi* strains. Therefore, the prevalence of *L. grayi* in *Listeria*-contaminated food, its high resistance to antibiotics and its potential pathogenicity are worrisome and have been brought to the attention of researchers and the public. Unfortunately, beyond its role as an indicator bacterium for *Listeria* spp., *L. grayi* is seldom studied, and there is limited knowledge about its inactivation mechanisms and its susceptibility to various antimicrobial agents such as essential oils.

Thus, four hypotheses proposed in this thesis are: (i) Essential oils with highest antilisterial activity can be incorporated in packaging material to inhibit the growth of *Listeria* on

vegetables (ii) Essential oils incorporated into soy protein isolate (SPI) film can influence the film physical and functional properties, as well as enhance their antimicrobial efficacy. (iii) Fourier transform infrared (FTIR) spectroscopy can be employed to monitor release kinetics of active compounds in essential oils from packaging materials. (iv) FTIR spectroscopy can be employed to determine lot-to-lot variability of essential oil products. The research presented in this thesis addresses some of these knowledge gaps by targeting inhibition of *L. grayi* by essential oils in relation to the development of active packaging for vegetable products.

Objectives of the Research

The overall objective of the research was to lay the scientific foundation for development of food active packaging incorporating essential oils to maintain food quality and inhibit the growth of *Listeria* on vegetables. The specific objectives of this research were as follows:

1. To evaluate the antimicrobial activities of a panel of essential oils from various commercial sources against *L. grayi*.
2. To select essential oils exhibiting the highest antilisterial activity and compare the susceptibilities of *L. grayi* and *L. monocytogenes* to these essential oils.
3. To study the growth of *L. grayi* on vegetable products in the presence of selected essential oils to provide a basis for future development of active-packaging systems.
4. To investigate the effects of incorporated essential oils on physical and functional properties of soy protein isolate (SPI) films and assess the antimicrobial abilities of these films *in vitro*.
5. To study vapor-phase release of active compounds from essential oils by Fourier transform infrared (FTIR) spectroscopy and develop a vapor-phase FTIR methodology to monitor their release kinetics from packaging materials.

CHAPTER 2 LITERATURE REVIEW

2.1 **Essential oils**

Essential oils (EOs) are volatile and aromatic oily liquids which are extracted from some herbs and spices, such as flowers, leaves, seeds, roots and fruits (Burt, 2004). They are known as the secondary metabolites of some plants and are involved in the plants' chemical defense systems (Bassolé & Juliani, 2012). It is estimated that over 3,000 essential oils are known, of which about 300 are commercially important and used by the flavor and fragrance industries (van de Braak & Leijten, 1999). Among these essential oils, there are three major classes of active compounds according to their chemical structure, which are phenylpropenes, terpenes, and terpenoids (Wink, 1999). Because of the antimicrobial activities of active compounds in essential oils, essential oils can be used in the food industry as natural preservatives (Teissedre & Waterhouse, 2000). There are many methods to obtain the essential oil from different parts of the aromatic plants, including expression under pressure, solvent extraction, fermentation and steam distillation.

2.1.1 **The history of essential oils**

As early as 2000 years ago, people in Egypt, India and Persia started to use distillation as a method to produce EOs (Guenther, 1950). A Greek historian, Herodotus (484-425 B.C.), as well as the Roman historian and his contemporary Dioscorides, also mentioned oil of turpentine and partial information about how to produce it in their books. However, in the early Middle Ages, EOs were regarded as undesirable by-products of distilled rose water rather than a desirable product. As the extensive trade of odoriferous oils and ointments took place in ancient Greece and Rome, odoriferous oils were widely used. However, odoriferous oils were still not essential oils since the method of producing them was different. The first authentic description of the distillation of real essential oil was by the Catalan physician Arnald de Villanova, who wanted to introduce distillation into recognized European therapy (Guenther, 2013). By the 13th century, EOs started to be used as a medicine and their pharmacological effects were documented in pharmacopoeias (Bauer *et al.,* 2001). The term "essential oil" was first used by the Swiss reformer of medicine Paracelsus von Hohenheim (1493-1541), who named the effective component of a drug "quinta essentia" (Guenther, 1950; Burt, 2004; Bassolé and Juliani, 2012).

In 1556, a published book by Walter Reiff, *New Gross Destillirbuch*, mentioned clove, mace, nutmeg, spike and cinnamon as sources of essential oils, and there was a French essential oil industry that sold their oil of spike or lavender at high price at that time (Guenther, 2013).

During this period, the most important publication about essential oils was written by a scientific writer, the Neapolitan, whose name was Giobanni Battista della Porta (1537-1615). "In his 'De Destillatione libri IX,' written in 1563, he not only differentiated distinctly between expressed fatty and distilled essential oils, but described their preparation, the ways of separating the volatile oils from water and the equipment used for this purpose" (Guenther, 2013). In 1546, three essential oils were first listed in the official pharmacopoeia *Dispensatorium Pharmacopolarum*, which was published by the Imperial city of Nuremburg. They were oil of turpentine, spike (lavender) and juniper berries oils. The pharmacopoeia also recommended oils imported from France and named Narbonne as the seat of the industry. By the end of the 15th century, the production and use of essential oils became general. Furthermore, in the second official Nuremburg edition of the pharmacopoeia in 1592, no less than 61 distilled essential oils were listed (Winkler, 1934). This fact illustrated the rapid development of essential oils and their official acceptance as well.

In modern times, the first important investigation of the essential oils involved submitting the well-known oil of turpentine to elemental analysis. From this investigation, J.J. Houton de la Billardière found that the ratio of carbon to hydrogen in the oil was 5:8 (*Journal de pharmacie*, 4[1818],245). Later, this ratio was established for all polyterpenes, sesquiterpenes, terpenes and hemiterpenes. Then, in the latter half of the 19th century, the research of O. Wallach (1847-1931) on terpenes and their derivatives caused a wide expansion of the use of essential oils. Gradually, essential oils were not only used in medicinal drugs but also in the production of beverages, food and perfumes (Guenther, 2013).

2.1.2 **Functions of common essential oils**

More and more people have begun to take interest in the use of natural products as bio-preservatives against spoilage microorganisms and foodborne pathogens. These include compounds that can be extracted from plants or essential oils, such as carvacrol, benzaldehyde, cymene, cinnamaldehyde, eugenol, limonene, salicylaldehyde, menthol, thymol, terpineol and vanillin (Walsh *et al.,* 2003; Dobre *et al.,* 2011). Natural extracts and their active components are listed as flavoring agents by the European Union and are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (Passarinho *et al.,* 2014). Among plant compounds, carvacrol, thymol, cinnamon essential oil, and oregano (*Origanum vulgare*) essential oil are reported to exhibit highly effective antimicrobial properties.

2.1.2.1 **Carvacrol and Thymol**

Carvacrol, the major component (50–86%) of thyme and oregano essential oils (*Thymus* and *Origanum spp.*), is a phenolic compound which can be used on many food products as a preservative (Guarda *et al.,* 2011). Its antimicrobial activity has been demonstrated against bacteria, molds and yeasts, and it has a high potential to extend the shelf life and ensure safety of perishable foods (Holley and Patel, 2005; Martínez-Romero *et al.,* 2007). Thymol is an isomer of carvacrol and the two compounds have very similar structures (shown in **Fig. 2.1**), with their hydroxyl groups at different locations on the phenolic ring. The mechanisms of action of thymol and carvacrol are also similar (Lambert *et al.,* 2001): "These two EOs can disintegrate the outer membrane of Gram-negative bacteria, leading to increase the permeability of the cytoplasmic membrane to ATP and release lipopolysaccharides (LPS)" (Burt, 2004). In a study with *Bacillus cereus*, carvacrol was shown to interact with the cell membrane, dissolving in the phospholipid bilayer and aligning between fatty acid chains (Ultee *et al.,* 2000). Thus, the distortion of physical structure may result in expansion and destabilization of the cell membrane and increase membrane fluidity and permeability (Ultee *et al.,* 2002). In addition, magnesium chloride had no effect on this action, indicating a mechanism other than chelation of cations in the outer membrane (Helander *et al.,* 1998).

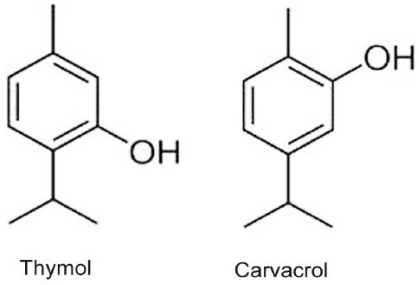


Figure 2.1 Chemical structure of thymol and carvacrol.

Recently, carvacrol and thymol have received much attention from researchers. Several attempts have been made to develop active-packaging systems incorporating EO into polymeric materials that can slowly release the volatile active compounds onto the food surface. Guarda *et al.* (2011) developed plastic films with microcapsules containing carvacrol and thymol. The carvacrol and thymol showed significant antimicrobial activity against a broad spectrum of microorganisms, including *Aspergillus niger, Staphylococcus aureus*, *Escherichia coli* O157:H7, *Listeria innocua,* and *Saccharomyces cerevisiae*, with minimum

inhibitory concentrations (MIC) of 125-250 ppm for thymol and 75-375 ppm for carvacrol. Furthermore, a synergistic effect of the combination of carvacrol and thymol was observed, with the highest effect being achieved with a 50:50 mixture. Du *et al.* (2015) evaluated the efficacy of thymol and carvacrol against pathogenic *Escherichia coli*, *Salmonella* strains, *Clostridium perfringens*, and *Lactobacillus* strains on broiler chicks. The MIC values determined by an *in vitro* assay showed strong antibacterial activity against all the tested bacteria except *Lactobacillus* strains. Arfa *et al.* (2007) prepared antimicrobial papers by coating paper with a soy protein isolate (SPI) solution containing carvacrol. Their study found that aggregation of SPI was induced by carvacrol at 25 ºC and that the loss of carvacrol during coating and drying was low, which was attributed to entrapment of carvacrol by the SPI aggregates. The rates of release of carvacrol from the antimicrobial papers were also low, especially in the first three days after preparation (0.04 g/m^2 per day for coating at 25 °C and 0.31 g/m^2 per day for coating at 90 °C). Irrespective of the heat treatment applied to the SPI solutions, the concentration of residual carvacrol in the SPI-coated papers after 50 days ranged between 0.6 and 0.7 g/m^2 (Arfa *et al.,* 2007).

2.1.2.2 **Cinnamon or Cinnamaldehyde**

Cinnamon, the dried bark of *Cinnamomum cassia* Blume, is usually used to flavor or season various foods or as a therapeutic agent for various diseases. Cinnamon is rich in tannins and essential oils, both of which can inhibit microbial growth (Mau *et al.,* 2001). Cinnamaldehyde (structure shown in **Fig. 2.2**) is the predominant compound in cinnamon essential oil, ranging from 90% to 62%–73% depending on the extraction method, with higher levels being obtained in the case of steam distillation as compared to Soxhlet extraction (Nabavi *et al.,* 2015). Although cinnamaldehyde (3-phenyl-2-propenal) can inhibit the growth of *S. typhimurium* and *E. coli O157:H7* at similar concentration to thymol and carvacrol, its mechanism of action is different, as it does not disintegrate the outer membrane of the bacterial cells or deplete the intracellular ATP pool (Burt, 2004). Wendakoon *et al.* (1995) demonstrated that the carbonyl group in cinnamaldehyde may bind to proteins, resulting in preventing the action of amino acid decarboxylases in *Enterobacter aerogenes*. In addition, some studies reported that aldehyde groups can cross-link covalently with proteins and DNA through amine groups, leading to interference with their normal function (Hyldgaard *et al.,* 2012).

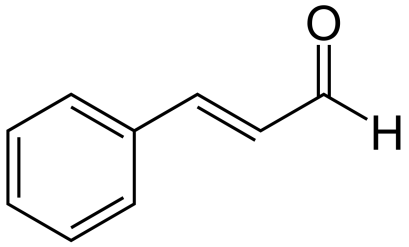


Figure 2.2 Chemical structure of cinnamaldehyde.

In 2007, Muchuweti *et al.* reported that cinnamon contained the highest concentration of polyphenolic compounds. They also showed cinnamon exhibited the highest radical scavenging (92.0%) and antioxidant (61.8%) activities among the investigated herbs and spices. Besides, the components of cinnamon showed antimicrobial activity against Gram-negative and Gram-positive bacteria causing human infection and food spoilage. Due to its antioxidant, anti-diarrheal, anti-flatulent and antibacterial properties, cinnamon can be used in clinical, cosmetic and food applications. A recent investigation showed that cinnamon essential oil controlled the growth of *Listeria monocytogenes* in meat products which were contaminated at 5 ppm and it did not change the products' sensory properties. In addition, cinnamon essential oil inhibited the bacterial growth more effectively in artificially contaminated samples than in untreated controls (Dussault *et al.,* 2014). Similar investigations studied the antibacterial activity of cinnamon against foodborne pathogens in contaminated meat, such as *Salmonella typhimurium, Arcobacter butzeiri, E. coli*, *Staphylococcus aureus*, and *Arcobacter skirrowii*. Cinnamon was also found to be active against *L. monocytogenes*, *Salmonella enterica* and *S. aureus* in a cheese at room temperature (23 °C) (Shan *et al.,* 2011).

2.1.3 **Antimicrobial activity of EOs in food systems**

The antimicrobial activity of EOs has been evaluated in many *in vitro* studies. However, it has generally been found that achieving comparable antimicrobial activity in foods requires a higher concentration of EO than is needed *in vitro* (Smid and Gorris, 1999).

2.1.3.1 **Bakery products**

The shelf life of bakery products without preservative stored in natural atmosphere at room temperature is usually as short as 3–4 days (Gutiérrez *et al.,* 2009). Loss of freshness and spoilage may be attributed to many factors, including microbial spoilage, increasing water activity and oxygen content, and microbial contamination. Molds are known as the common spoilage microorganisms in bread, leading to reduced safety and huge economic losses every year (Passarinho *et al*., 2014).

Most of the active-packaging systems containing EOs, such as oregano, garlic, or cinnamon oil, or cinnamaldehyde applied on food have shown promising results in vivo. Balaguer *et al.* (2013) studied the activity of gliadin films with cinnamaldehyde against food spoilage fungi *in vitro* and on sliced bread. They found that the films with 3% cinnamaldehyde strongly inhibited fungal growth and that growth of *Aspergillus niger* and *Penicillium expansum* was fully retarded after storage for 10 days. Furthermore, the active packaging with the films containing 5% cinnamaldehyde increased the shelf life of bread. Mold could be observed on sliced bread after 27 days of storage at 23ºC, whereas the fungal growth on the control group appeared on the fourth day (shown in **Fig. 2. 3**). Arancibia *et al.* (2014) developed soy protein, lignin and formaldehyde films with incorporated citronella EO. With 3% citronella EO, these films showed good antifungal activity against *Fusarium oxysporum* on bananas. In addition, there was a large reduction in total molds, yeasts and aerobic mesophiles on bananas covered by these films during storage. Passarinho *et al.* (2014) evaluated the antimicrobial activity of sachets containing oregano EO *in vitro* and on sliced bread. These antimicrobial sachets showed effective reduction of the growth of the tested microorganisms, including *Escherichia coli, Salmonella* Enteritidis and *Penicillium sp*., and also inhibited the growth of yeasts and molds on sliced bread. Rodriguez *et al.* (2008) developed a solid wax paraffin with incorporated cinnamon essential oil and used it as a coating on bread. It showed a strong retarding effect on growth of *Rhizopus stolonifer* on bread. However, in a study by Kechinchian *et al.* (2010), the antimicrobial effect of cassava starch films with cinnamon

powders used on sliced bread could not be determined since the physical and chemical properties of the films were changed by the high RH of bread.

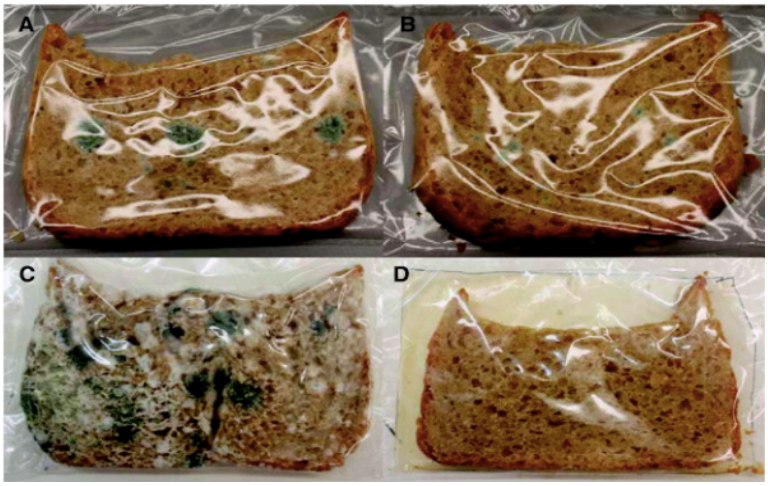


Figure 2.3 Growth of inoculated *Penicillium expansum* and fungi naturally present in inoculated (A) and uninoculated (B) bread in control packaging after 14 days, in uninoculated bread in control packaging after 1 month (C), and in inoculated bread in active packaging after 1 month (D). Adapted from Balaguer *et al.* (2013)

2.1.3.2 **Vegetables and fruits**

Generally, the antimicrobial activity of EOs in vegetables, as in meat products, can be favored by low storage temperature or a decrease in the pH of the food (Skandamis and Nychas, 2000). The low-fat content of vegetables may also increase the exposure of the EO to microorganisms, which would be otherwise limited by its dissolution in the lipid phase (Burt, 2004; Perricone *et al.*, 2015). Some EOs and their components have been tested on vegetables in several research studies. The EOs showed effectiveness against foodborne pathogens and natural spoilage flora at levels of 0.1–10 µl/g in washing water (Wan *et al.,* 1998; Singh *et al.,* 2002). Furthermore, thymol and cinnamaldehyde in hot air at 50 ºC inhibited growth of six *Salmonella* serotypes on alfalfa seeds (Weissinger *et al.,* 2001). However, when the temperature was increased to 70 ºC, the effectiveness of the treatment was reduced because of the volatility of the antibacterial compounds. Skandamis and Nychas (2000) inoculated eggplant salad with *Escherichia coli* O157:H7 and showed that addition of oregano EO at concentrations in the range of 7-12 µl/g was effective in reducing the *E. coli* O157:H7 counts and also reduced the final total bacterial count on the salad. Ayala-Zavala *et al.* (2010) found that garlic EO encapsulated in β-cyclodextrin capsules in cellulose sachets could inhibit microbial growth. The results showed that only 1 g of garlic EO was enough for 100 g of tomato slices to be preserved. Rosemary EO encapsulated in β-cyclodextrin was

found to be thermally stable at high temperatures and preserved its antimicrobial properties after a tomato juice pasteurization process (Garcia-Sotelo *et al.*, 2019). Cinnamon essential oils from both leaf and bark were reported to effect log reduction of *L. monocytogenes* and *Salmonella typhimurium* on celery after storage for 7 days at 4 ºC (Brnawi *et al.,* 2019). In a study of oriented polypropylene/polyethylene (OPP/PE) film packages coated with soy protein isolate containing cinnamaldehyde, the microbial counts on broccoli, radish, and alfalfa sprouts in these active-packaging systems were significantly reduced (Gamage *et al.,* 2009).

In relation to antimicrobial activity of active packaging applied on fruit, Dos Santos *et al.* (2012) evaluated the efficacy of the combination of *Origanum vulgare L.* EO and chitosan in reducing the growth of *Aspergillus niger* URM 5842 and *Rhizopus stolonifer* URM3728 on grapes during storage (12 days at 25 ºC and 24 days at 12 ºC). The results of their study showed that the EO and chitosan inhibited spore germination and even caused changes in the morphology of fungal spores and mycelia. In addition, the growth of the fungal strains was retarded in both artificially infected and autochthonous mycoflora of grapes at room and cold temperature. The authors demonstrated the potential application of EO against post-harvest pathogenic fungi in fruits. Besides, the active compounds carvacrol and cinnamaldehyde were very effective in reducing the viable counts of the natural microbiota on kiwifruit at a concentration of 0.15 µl/g in dipping solution. However, they were less effective on honeydew melon (Burt, 2004). This difference may be attributed to the difference in the pH of the fruits, where the pH of the melon (5.4 – 5.5) was higher than that of kiwi (3.2 – 3.6) (Roller and Seedhar, 2002). Thus, the lower the pH, the more effective the EOs were in reducing microbial growth on fruits. Medeiros developed antifungal sachets with oregano and lemongrass EOs to preserve mangoes. They found that both EO sachets could inhibit fungal growth during storage. The lemongrass EO sachet had higher antimicrobial activity and even decreased the total counts of aerobic mesophilic bacteria (TAM), molds and yeasts by 2 log cycles relative to the control. Both lemongrass and oregano EOs had limited effect on skin color, soluble solids, firmness and titratable acidity of the fruit (Otoni *et al.,* 2016). Likewise, similar results were observed by Espitia *et al.* (2012) with papaya. They reported that sachets with oregano, cinnamon and lemongrass EOs inhibited the growth of yeast, molds and mesophilic aerobic bacteria, and cinnamon EO reduced the total counts by 1.6 log cycles. As in the study with mangoes, EO sachets had limited effects on physicochemical properties of papaya.

2.2 **Other antimicrobial agents**

Antimicrobials in food are used as preservatives and can enhance the quality and safety of processed food by controlling the growth of bacteria, although it has been emphasized that they must not be used as a substitute for good sanitation practices (Brody *et al.,* 2001; Cooksey, 2005). The ability of these preservatives to be used to protect food from deterioration is described in the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA, FSIS) directives. Antimicrobial agents are categorized into three types: processing aids, secondary direct food additives, and direct food additives. The use of an antimicrobial during manufacturing depends on the desired effect, effects on food, and legal limits (Crozier *et al.,* 2004.). Furthermore, these antimicrobials can also be classified into synthetic and natural antimicrobial agents (Kuorwel *et al.,* 2011). Common antimicrobials that can be used in food, such as cheese, bread, fish, and cereal, include benzoic acid, sorbate, nitrate, sulfur dioxide, ethanol, CO_2, UV irradiation, reduced iron complex, nisin, etc. **Table 2.1** shows applications of antimicrobial food packaging and packaging material.

2.2.1 **Synthetic and inorganic antimicrobials**

Common synthetic antimicrobials are organic acids and their salts, sulfites, nitrites, and antibiotics (Jideani & Vogt, 2016). **Table 2.2** shows typical synthetic antimicrobial agents used in food systems. The antimicrobials used in packaging can control the population of the microorganism by reducing the growth rate, resulting in extending the lag phase, or inactivating the microorganisms (Quintavalla and Vicini, 2002). Various types of antimicrobials have been researched to evaluate their suitability and function in food packaging, including organic acids, fungicides, inorganic gases and metals (Scannell *et al.,* 2000).

Table 2.1 Applications of antimicrobial food packaging

Antimicrobial agent	Packaging material	Food or substrate
Organic acids		
Potassium sorbate	LDPE	Cheese
Calcium sorbate	CMC/paper	Bread
Propionic acid	Chitosan	Water
Acetic acid	Chitosan	Water
Benzoic acid	PE-co-MA	Culture media
Sodium benzoate	MC/chitosan	Culture media
Sorbic acid anhydride	PE	Culture media
Benzoic acid anhydride	PE	Fish fillet
Fungicides/bacteriocins		
Benomyl	Ionomer	Culture media
Imazalil	LDPE	Cheese
Nisin (peptide)	Silicon coating SPI	Culture media
Peptides/proteins/enzymes		
Lysozyme	PVOH, nylon	Culture media
Glucose oxidase	Alginate	Fish
Alcohol		
Ethanol	Silica gel sachet	Culture media
Oxygen absorber/antioxidant		
Reduced iron complex	Sachet (Ageless)	Bread
BHT	HDPE	Cereal
Gases		
CO_2	$Ca(OH)_2$ sachet	Coffee
SO_2	Sodium metabisulfite	Grape
Other		
UV irradiation	Nylon	Culture media
Silver zeolite	LDPE	Culture media
Grapefruit seed extract	LDPE	Soya sprouts

LDPE, low-density polyethylene; MC, methyl cellulose (MC); CMC, carboxyl MC; PE, polyethylene; MA, methacrylic acid; SPI, soy protein isolate; PVOH, polyvinyl alcohol; HDPE, high-density PE; BHT, butylated hydroxytoluene.
Adapted from Han *et al.* (2000) and Jideani & Vogt (2016).

Table 2.2 Examples of typical antimicrobial agents used in food packaging

Class of antimicrobial agents	Examples
Organic acids	Propionic, benzoic, sorbic, acetic, lactic, malic, succinic, tartaric
Mineral acids	Phosphoric acid
Inorganics	Sulfites, sulfur dioxide
Parabens	Methyl- and propylparaben
Antibiotics	Natamycin
Metals	Silver, copper
Chelating agents	Ethylenediaminetetraacetate, pyrophosphate, citrates
Bacteriocins	Nisin, pediocins
Fungicides	Benomyl, Imazalil

Adapted from Hotchkiss (1997) and Jideani & Vogt (2016)

Organic acids and their salts, such as sodium benzoate, propionic acid, sorbic acid and potassium sorbate, are the most widely used preservatives in the world and can be used in bakery products to extend their shelf life (Jideani & Vogt, 2016; Axel *et al.,* 2016). Most large commercial bakeries use one of them or a combination of two of them to control the growth of microorganisms. Organic acids, such as acetic acid, benzoic acid, and propionic acid, and sulfite (Neal *et al.,* 1965; Krebs *et al.,* 1983; Stratford, 1983) are referred to as weak acid preservatives and the mechanism of action of these acids against yeast is described by 'weak acid preservatives' theory (Stratford and Anslow, 1997). Generally, at low pH, weak acids in aqueous solution partially dissociate, resulting in a dynamic equilibrium between charged species and molecular acids. The molecular acids predominate in solution and can easily penetrate the plasma membrane into the cytoplasm by simple diffusion on account of their lipid solubility. At the neutral pH of the cytoplasm, these acids dissociate and release protons, causing cytoplasmic acidification. Consequently, cellular metabolism is inhibited (Krebs *et al.,* 1983 and Pearce *et al.,* 2001; Axel *et al.,* 2016). Sorbic acid is different from other preservatives, acting as a membrane-active antimicrobial compound, primarily by inhibiting the plasma membrane H^+- ATPase proton pump (Stratford *et al.,* 2013a & 2009).

Suhr *et al.* (2004) reported that propionate at 0.3% concentration generally had a strong inhibitory effect on all tested fungi on wheat agar at pH 4.5 and a_w 0.95 but it had only a slight effect on mold inhibition when the agar was at pH 6. Membre *et al.* (2001) showed that propionic acid and sorbic acid (applied at 0.2% concentration) have the same effect on inhibiting the growth of *P. brevicompactum* at a_w 0.90, but benzoate was found to be less effective (at 0.05% concentration) (Suhr *et al.,* 2004). In a study reported by Guynot *et al.*

(2005), 0.3% potassium sorbate (pH 4.5) was more effective at preventing fungal spoilage of bakery products than calcium propionate. Although sorbates are the most effective preservative in bakery products, their antimicrobial activities may inhibit yeast fermentation in bakery products as well. Thus, these agents have to be used as a spray application after baking. In the U.S., potassium sorbate or sodium sorbate can be added at less than 0.3% (w/w), while propionate added in bread cannot exceed 0.25% of total flour (FDA, 2016). Within the Europe Union, the use of these chemicals in bakery products as preservatives is limited. Sorbate can be added to a maximum concentration of 0.2% (w/w) and propionate is allowed up to 0.3% (w/w), which pertains only to prepacked sliced bread and rye bread (EEC, 2008). This means that neither the addition of sorbate nor propionate is permitted in unpacked bread and some wheat bread only made from wheat flour, water, yeast and salt (Axel *et al.*, 2016).

Overall, applying a high concentration of sorbate or propionate in bakery products is desirable for inhibition of fungi, but this may alter the physical or sensory properties of the product. In addition, prolonged usage of the same preservatives may result in the development of fungal resistance to those chemicals (Levinskaite, 2012; Stratford *et al.,* 2013b; Suhr and Nielsen, 2004). With the increase in consumer awareness of natural food and food safety, concerns have arisen about the use of chemical additives or preservatives in food products (Azaz *et al.,* 2005). Hence, new food preservatives that are green and natural need to be used as antimicrobial additives to extend the shelf life of food.

2.2.2 **Natural antimicrobial agents**

Natural antimicrobial compounds can be extracted or hydrolyzed from spices, herbs, vegetables, and fruits. Many of these compounds are widely used in the food industry as flavoring agents. Recently, these natural compounds are being used as antimicrobial agents in food. They include bacteriocins (nisin and lacticin), grapeseed extracts, essential oils, lemon extracts, enzymes, pepper, orange extracts, honey and propolis extract (Conte *et al.,* 2007 a & b); Fernandez-Lopez *et al.,* 2005; Choi *et al.,* 2006; Jideani &Vogt., 2016).

2.2.2.1 **Allyl isothiocyanate (AITC)**

Allyl isothiocyanate (AITC) is a volatile compound found in plants that belong to the Cruciferae family. AITC is an aliphatic substance and is the major antimicrobial component in black (*Brassica nigra*) and brown mustards *(Brassica juncea*) (Otoni *et al,* 2016; Nielsen & Rios, 2000). AITC exists in a precursor form and the precursor of AITC is found in many

plants, including mustard, broccoli, cauliflower, cabbage, kale, horseradish and turnips. Previous studies have shown that AITC has high antimicrobial activity in the vapor phase against many food pathogenic microorganisms, such as *Salmonella*, *E. coli* O157:H7*, Staphylococcus aureus, L. monocytogenes, Penicillium expansum,* and *Aspergillus flavus* (Luciano *et al.,* 2008; Mari *et al.,* 2002; Isshiki *et al.,* 1992). Various hypotheses about the antimicrobial mechanisms of AITC have been reported so far. Lin *et al.* (2000) showed that AITC may cause cell leakage and then lead to cell death. Troncoso-Rojas *et al.* (2005) showed that 2-phenylethyl isothiocyanate can react with enzymes present in the plasma membrane resulting in inhibition of cell growth and cell death. Furthermore, Kojima *et al.* (1971) and Banks *et al.* (1985) proposed that AITC can react nonspecifically and irreversibly react with the sulfhydryl groups, disulfide bonds, and amino groups of proteins and amino acid residues (Otoni *et al.,* 2016).

Table 2.3 Food preservation by sachets containing allyl isothiocyanate

Carrier/sachet material	Target microorganism(s)	Food matrix	References
Porous HDPE resin	Yeasts and molds	Cottage cheese	Gonçalves *et al.* (2009)
Nonwoven tissue	*Aspergillus flavus*	Peanuts	Otoni *et al.* (2014)
Porous HDPE resin	Yeasts and molds, *Staphylococcus* spp., psychrotrophic bacteria	Mozzarella cheese	Pires *et al.* (2009)
LDPE film/allyl isothiocyanate beads	*E. coli* O157:H7, molds and yeasts	Fresh spinach	Seo *et al.* (2012)

Adapted from Otoni *et al*., 2016, with slight modifications.

AITC is commonly used as a food preservative in Japan and has obtained GRAS status in the United States (Kim *et al.,* 2002, Seo *et al.,* 2012; U.S. FDA, 2011). However, the United States has not y*et al*lowed its use as a food additive, but it can be found in food with added condiments like mustard, wasabi, and horseradish (Delaquis & Sholberg, 1997; Luciano *et al.,* 2008). Research on the potential use of AITC in food packaging has shown that such packaging can be used in the preservation of cottage and mozzarella cheese, fresh spinach and so on (Otoni *et al.,* 2016) (**Table 2.3**). Nadarajah *et al.* (2005) reported that AITC can substantially reduce the amount of E. *coli* O157:H7 in fresh beef patties during refrigerated or frozen storage (4 ºC and -18 ºC) for at least 21 days, while the number of mesophilic aerobic bacteria in ground beef was not affected by AITC. In another study, AITC was applied on

pears to inhibit growth of the mold *Penicillium expansum*. Among treated pears inoculated with the mold at an inoculum density of $1x10^3$ conidia ml^{-1}, the percent of wounds infected was less than 20%. In comparison, more than 98% of wounds were infected in untreated samples. In addition, AITC treatment was effective more than 24 h after inoculation and it also controlled a thiabendazole-resistant strain of *Penicillium expansum* (Mari *et al.,* 2002). Gonçalves *et al.* (2009) developed sachet with 0.5% or 1% concentration of AITC for cottage cheese preservation against yeast and mold growth. Quiles *et al.* (2015) reported that AITC in a sachet or on a filter paper at 10 µl/l reduced the growth of *Aspergillus parasiticus* in fresh pizza crust after 30 days and completely inhibited the formation of aflatoxins (AFs). Similarly, AITC sachets were also applied in the storage of peanuts to control post-harvest sporulation by *Aspergillus flavus.* Otoni *et al.* (2014) reported that a 10-fold reduction in *A. flavus* survival was observed after 7 days and its survival was reduced by 4.81 log cycles after 60 days at 25 ºC. The volatile AITC in the headspace of packaging was decreased to 7.6% after 15 days and it could not be detected after 30 days (**Fig. 2.4**). The study of Seo *et al.* (2012) showed that AITC encapsulated in a sachet inhibited growth of *E. coli* O157:H7 on spinach leaves, on which the number of the *E. coli* O157:H7 was decreased by approximately 2.1-5.7 log CFU/leaf at 25 ºC and 1.6-2.6 log CFU/leaf at 4 ºC within 5 days. These results indicate that AITC sachets retard fungal and bacterial growth and can be used as an antimicrobial agent to ensure the safety of food products (Otoni *et al.,* 2014; Otoni *et al.,* 2016).

Figure 2.4 AITC-containing sachet in peanut packaging.
Adapted from Otoni *et al.,* 2016.

2.2.2.2 **Ethanol and ClO_2**

Ethanol is very common in our daily lives. It is the alcohol in alcoholic beverages such as beer, red wine, tequila and vodka. Ethanol is also widely used medically as a sterilant in venipuncture, hypodermic injections and finger pricks (Russell *et al.,* 2003). Furthermore, ethanol is a volatile antimicrobial agent used as a food preservative, having been widely used for more than 100 years in the preservation of fruit against fungi. In bakery, a patent by Kuchen (1963) described the use of ethanol post-baking to prolong the shelf life of bakery products, especially bread. After the cooling step, the baked bread can be dipped in or sprayed with ethanol solution before it is packaged. According to the patent, the treated bread can be stored for 2 years (Russell *et al.,* 2003). Subsequently, the first reference which included the use of low levels of ethanol as a food preservative was published in the Federal Register in 1974, in which up to 2% ethanol is permitted to be applied on part-baked pizza bases by spraying and its use was affirmed by the U.S. Food and Drug Administration (Anon 1974; Plenons *et al.,* 1976). Later, Toyama *et al.* (1987 & 1994) and some scientists used ethanol in noodle production and did not observe any microbial growth on noodles for almost 14 days at 30 ^{o}C because of the ethanol remaining on them. Suenaga *et al.* (1995) used ethanol generated by ethanol-resistant yeasts through consumption of oxygen to preserve food against the growth of *E. coli* and *Aspergillus oryzae* (Russell *et al.,* 2003).

With wrapping machine and modified atmosphere packaging (MAP) of food gaining wide acceptance, ethanol is now used in sachets and films as a preservative in food packaging. However, the use of ethanol in films is not widespread due to the problem of controlling the release of ethanol from films into the headspace of the package. To hold and release the ethanol in a controlled manner, ethanol films usually require additional layers, resulting in increasing cost of this system (Ozdemir & Floros, 2004). In the case of sachets, ethanol is either absorbed to or encapsulated in a carrier material inside the sachet, which generates or releases the alcohol vapor (Otoni *et al.,* 2016). Ethanol-containing sachets are also known as ethanol emitters, and the first 'Ethicap' sachet contained food-grade ethanol absorbed on a fine silicon dioxide powder to gain at least 55% of alcohol by weight (Russell *et al.,* 2003; Freund Industrial, 1980 & 1981). Vanilla or other flavors need to be added into sachets as well to mask the undesirable odor of the high concentration of ethanol (Kerry and Butler, 2008). Because of the antimicrobial activities of ethanol, much research has been done to apply it in food products, especially bakery products (**Table 2.4**). Daifas *et al.* (2000) tested the antibacterial activity of ethanol on English-style crumpets. According to their research,

sachets containing 2 g of ethanol retarded toxin production by *Clostridium botulinum* for 10 days and those containing 4 g and 6 g of ethanol completely inhibited it. Franke *et al.* (2002) developed an ethanol-containing sachet to prolong the shelf life of pre-baked buns for at least 7 days and also resist spoilage by bacteria and molds (Russell *et al.,* 2003). Latou *et al.* (2010) combined an ethanol sachet and oxygen absorbers to produce a synergistic antimicrobial effect on sliced wheat bread. According to this research, counts for yeast and mold were reduced from 5.1to 2.0 log CFU/g and for *Bacillus cereus* counts were decreased to 2.0 log CFU/g after 30 days' storage (Otoni *et al.,* 2016). In addition to their antimicrobial activities, ethanol-emitting sachets can delay staling of bakery products. In addition, ethanol-emitting sachets may reduce the need for other preservatives, such as sorbates and propionate, for inhibition of microorganisms (Ozdemir & Floros, 2004).

Table 2.4 Application of ethanol-containing sachets alone or in combination with other preservatives on bakery products.

Preservative agent(s)	Food matrix	Reference
Oxygen absorbers, ethanol	Rye bread	Salminen *et al.* (1996)
CO_2, N_2, ethanol or oxygen absorbers	Baked pita bread	Black *et al.* (1993)
CO_2- or ethanol-generating oxygen absorbers	Sponge cake	Naito *et al.* (1991)
Antimold 102	Madeira cake	Pafumi and Durham (1987)
Ethanol	English-style crumpets	Daifas *et al.* (2000)
Ethanol	Pre-baked buns	Franke *et al.* (2002)
Ethanol and oxygen absorbers	Sliced wheat bread	Latou *et al.* (2010)

Chlorine dioxide (ClO_2) is one of the few compounds that exists almost entirely as monomeric free radicals (WHO, 2000). It was first used in water disinfection in Belgium at the beginning of the 20th century (Tzanavaras *et al.,* 2007). It is commonly used in the paper industry to bleach pulp and in public water treatment facilities to make drinking water safer (Han *et al.,* 2003). Due to the explosive properties of ClO_2, it is impossible to store it under pressure or transport it in gas form. Therefore, ClO_2 has to be generated on-site. In food applications, ClO_2 is generally generated in the packaging system, by mixing two precursors in individual sachets together (Lee *et al.,* 2004; Sy *et al.,* 2005). The antimicrobial effect of ClO_2 is attributed to non-specific oxidative damage to the outer membrane, resulting in the

destruction of the trans-membrane ionic gradient. Similarly, Yong *et al.* (2003) reported that ClO_2 can also cause the death of spores of *Bacillus subtilis* since ClO_2-treated spores cannot undergo the further process of spore germination (Gómez-López *et al.,* 2009). Therefore, ClO_2 can be used as a bio-preservative in food for decontamination.

Previous studies have shown antimicrobial activities of ClO_2 against many kinds of microorganisms, including viruses, *Cryptosporidium parvum* oocysts, *E. coli* O157:H7*, B. cereus, Salmonella, Listeria monocytogenes,* yeasts and molds (Gómez-López *et al.,* 2009; Sy *et al.,* 2005). Chen *et al.* (1990) demonstrated that ClO_2 can inhibit rotaviruses under alkaline conditions. A study by Peeters *et al.* (1989) showed that ClO_2 can also decrease the viability of *Cryptosporidium parvum* oocysts to some degree. Han *et al.* (2001) tested the activity of ClO_2 gas in inhibiting the growth of *E. coli* O157:H7 on green peppers. In the response surface modeling study conducted by these authors, the gas concentration (in the range of 0.1-0.5 mg/l) was the most important factor for inactivation of *E. coli* O157:H7. Beuchat *et al.* (2005) reported that ClO_2 at a concentration of 200 mg/ml can kill spores of *B. cereus* and achieved a reduction of more than 6.4 log CFU/ml within 5 min. In addition, ClO_2 (5 mg/l) was also used to treat strawberries, resulting in approximately 4.3-4.7 log CFU reduction in *E. coli*, *L. monocytogenes* and *S. enterica* per strawberry. Furthermore, the ClO_2 did not change the color of strawberries and prolonged their shelf life from 8 days to 16 days (Sy *et al.,* 2005). Other studies on the potential of ClO_2 to ensure microbiological safety of fruits and vegetables including apples, peaches, tomatoes, onions, lettuce, cabbage and carrots are listed in **Table 2.5**.

Table 2.5 Application of ClO_2 on fruits and vegetables.

Produce	Targeted microorganism(s)	Reference
Green bell peppers	*Escherichia coli* O157:H7	Han, Sherman, *et al.* (2000)
Green bell pepper Baby carrot	*Listeria monocytogenes*	Han, Linton, *et al.* (2001)
Blueberry	*Salmonella*	
Strawberry	*Salmonella*	Sy, McWatter, *et al.* (2005)
Raspberry	*Salmonella*	
Apple	*Allicyclobacillus acidoterrestris*	Lee *et al.* (2006)
	L. monocytogenes	
Blueberry	*Salmonella*	Popa *et al.* (2007)
	E. coli O157:H7	
	E. coli O157:H7	
Strawberry	*L. monocytogenes*	Mahmoud *et al.* (2007)
	Salmonella enterica	
Lettuce	*E. coli* O157:H7 *S. enterica*	Mahmoud and Linton (2008)

Adapted from Gómez-López *et al*., 2009.

2.2.2.3 **Plant-derived compounds**

The growing interest in the application of natural compounds in food as preservatives has led to extensive research on plant extracts. Some plant-derived compounds, such as raisin extract, cherry laurel leaf extract, and the water-soluble extract from *Amaranthus* spp. seeds, are used as preservatives to control spoilage fungi *in vitro* (Axel *et al.,* 2016). Wei *et al.* (2009) investigated the antifungal activity of different raisin extracts in conventional bread. The results showed that the breads with raisin water extract (7.5%) and raisin paste had the best mold inhibition properties when compared to the control group (no addition), but there was no significant different to the bread with 0.24% propionate. The authors indicated that the raisin extract could be a potential chemical preservative in food in the future. Another natural compound is cherry laurel leaf extract (*Prunus laurocerasus L.*), a novel bio-preservative, which was reported to retard a range of bread spoilage fungi at very low MIC (μg/ml) (Sahan, 2011). However, there has been no further research about it. Rizzello *et al.* (2009) reported that the water-soluble extract from *Amaranthus* spp. seeds showed good inhibition of spoilage fungal on gluten-free wheat bread and that antifungal peptides, agglutinin sequences, were active compounds in the extract. In 2014, Gänzle reported that the amaranth water-soluble extract had more pronounced reduction of fungal growth than sourdough in breads.

With the addition of this extract to breads, the appearance of fungal mycelia was delayed to at least 7 days and it also improved the taste and volume of gluten-free breads.

2.3 **Active packaging to extend the shelf life of frozen vegetables**

2.3.1 **Frozen vegetables or fruits and their contamination**

Freezing of fruits and vegetables at low temperature (-18 ºC) to maintain their initial quality and nutritive value is undoubtedly the most satisfactory processing method for their long-term preservation, and the consumption of frozen fruits and vegetables has steadily increased in many places, such as the European Union, Canada, the United States, and Japan (Allende *et al.,* 2002). In 2008, the North American frozen food market (Canada, the United States, and Mexico) was still the largest in the world with 166,100 tons of frozen fruits valued at $1030.7 million and 9,862,200 tons of frozen vegetables valued at $47,182 million.

To ensure the quality of frozen food, many factors need to be taken into account, such as initial quality, pre-freezing treatments, and freezing conditions. The factors known as P-P-P (Product-Processing-Packaging) and T-T-T (Time-Temperature-Tolerance) are important critical control points (Sun, 2011). Generally, vegetable and fruits often undergo freezing immediately after postharvest and blanching processes (Sun, 2011; Danyluk *et al*., 2015). The total count of bacteria on frozen vegetables tends to be lower than on non-frozen food. However, the freezing process does not normally kill microorganisms, because it is not a lethal process. Thus, although many organisms can be destroyed during freezing, some may still survive. Microbial contamination of frozen food can occur from slicers, choppers or cutters, conveyor belts, lifts, hoppers, and fillers (Splittstoesser, 1973). Bacterial numbers may decrease during storage under frozen conditions, but this depends on many factors, including time, food matrix, microorganism type, and storage conditions (Van Schothorst *et al.*, 2009). In frozen vegetables, the common microorganisms are lactic acid bacteria (LAB), *Leuconostoc mesenteroides*, micrococci, enterococci, and coliforms, with LAB being the predominant organisms (Mundt *et al.*, 1967; Splittstoesser, 1983). The normal microflora of frozen fruits are fungi (ICMSF, 2005). Because of the low water activity and temperature, microbial growth may not occur in frozen fruits and vegetables, but spoilage can happen upon thawing (Michener *et al.*, 1968). In 1999 and 2010, contamination of frozen mamey pulp with Salmonella Typhi caused foodborne outbreaks of typhoid fever, with 19 cases and 9 cases, respectively (CDC, 2010; Katz *et al.*, 2002). In 2005, foodborne outbreaks of

norovirus infection associated with the consumption of frozen raspberries occurred in France and it was suggested that the contamination might have occurred before the raspberries were frozen, although this could not be confirmed (Cotterelle *et al.*, 2005).

2.3.2 **Active packaging**

Active packaging has been defined as "an intelligent or smart system that involves interactions between package or components in package and food or internal gas atmosphere and complies with consumer demands for high quality, fresh, and safe products" (Floros *et al.*, 1997; Labuza *et al*., 1989; Ozdemir *et al.*, 2004). This packaging can prolong the shelf life of foods and maintain the nutritional quality or safety of food by making it resistant to the growth of pathogenic microorganisms, preventing or indicating the migration of gas and moisture and absorbing food leakage (Hotchkiss, 1995). Active packaging includes many types of systems that provide different functions, including absorbers or scavengers (oxygen, carbon dioxide, ethylene, moisture, flavors or odors, and UV light); emitters and release (CO_2, ethanol, SO_2, flavors, pesticides, antioxidants and antimicrobials); removing components such as lactose and cholesterol; temperature changing (insulating, self- heating or self-cooling, microwave susceptors and modifiers, and temperature-sensitive packaging); microbial and quality control (UV and surface treated) (Kerry *et al.,* 2006). These functions are outlined in **Fig. 2.5**.

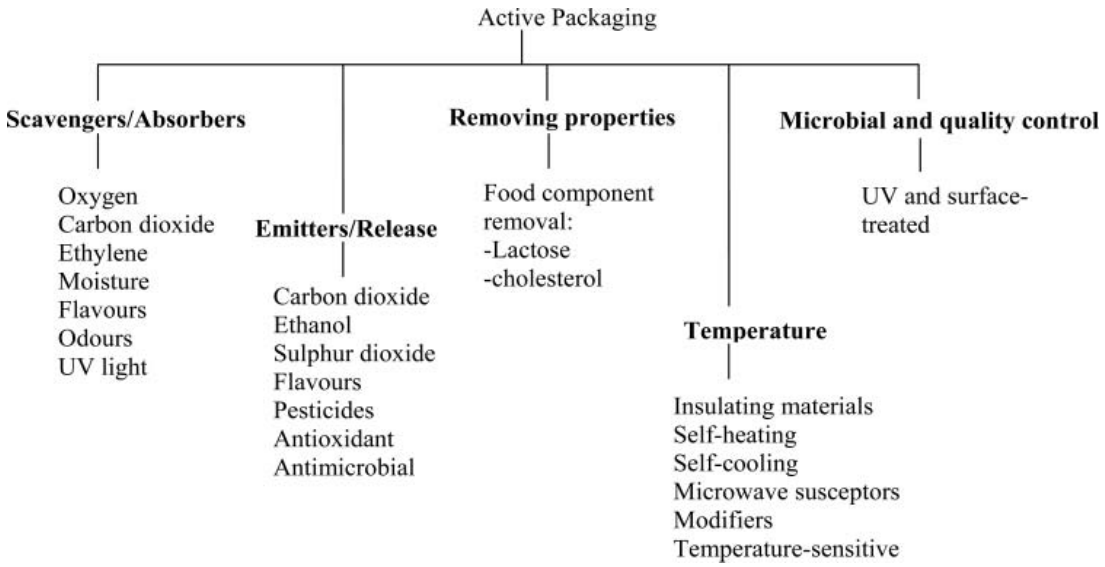


Figure 2.5 Active-packaging systems.
Reproduced from Kerry *et al*., 2006.

Among these types, the most important and promising active-packaging systems are those that emit/release and those that scavenge/absorb. They can be responsible for preventing rancidity (Rooney, 1982), protecting the food from spoilage by molds, yeasts, and, especially, aerobic bacteria (Vermeiren *et al.,* 1999), controlling insect infestation in cereal products, extending shelf life of foodstuffs and eliminating the use of chemical preservatives (Nakamura and Hoshino, 1983; Vermeiren *et al.,* 1999). Scavengers/absorbers, especially oxygen scavengers, appear to be the earliest and most widely used type of active packaging. The use of cans made of tinplate is the evidence of this. The tin in the tinplate is sacrificially corroded to protect the iron-based can and concurrently ensure the protection the food from iron contamination. The oxidant state of iron can be the autoxidation agent catalyst when there is residual oxygen in the can. The tin can be a reducing agent for food components such as pigments and is responsible for the traditional flavor of canned orange juice (Rooney, 1995). Similarly, the next development, in a patent, was also targeted at canned food. Tallgren (1938) reported that iron, manganese or zinc powder can be used to remove oxygen from the headspace of cans. This invention may be the precedent for the subsequent development of the iron-based oxygen scavengers in use today (Rooney, 1995).

Oxygen scavenger systems can provide an alternative environment to vacuum or gas flushing. The mechanism of oxygen scavenging is oxidation, including iron powder oxidation, ascorbic acid oxidation, enzymatic oxidation (glucose or alcohol oxidase), photosensitive dye oxidation, and oxidation of unsaturated fatty acids (oleic or linolenic acid) (Vermeiren *et al.,* 1999). In the case of oxygen-absorbing systems, the amount and type of absorbent, permeability of the packaging material, initial oxygen level, amount of oxygen present in the food, and water activity should be taken into account. These systems are commonly developed in sachet or multi-layer form. Since the sachet form is not appropriate for liquids, multi-layer scavenging systems have been developed to address this limitation and have been widely used recently. The typical structure of this type of system is shown in **Fig. 2.6**. From left to right of the multi-layer system are the outer layer, barrier layer, oxygen-absorbing layer, and inner layer, respectively. The barrier layer limits the penetration of oxygen from the outside environment due to its impermeability to oxygen. The oxygen-absorbing layer is used to absorb O_2 present in the headspace of the package. This layer is highly permeable to oxygen, and oxygen absorbing compounds, such as powdered iron oxide and ascorbic acid, are embedded in it. The inner layer, also called control layer, adjacent to the oxygen-absorbing layer, controls migration of the oxygen-absorbing compounds into the foodstuff

(Ozdemir *et al*., 2004). Typical and common oxygen scavenger systems are iron-based oxygen-absorbing systems, and these systems can be used in many foods, including high- or low-moisture foods and lipid foods (Hoshino, 1995). In addition, they can also work in frozen and refrigerated foods and can even be effective oxygen scavengers in microwaveable food. However, the iron powder in oxygen scavengers may impart undesirable flavor to the food and can also be accidentally ingested. Recently, Cryovac Corporation developed new polymer films in which the scavenging compound is co-extruded as part of a layer of the package and is invisible to the consumers (Ozdemir *et al*., 2004).

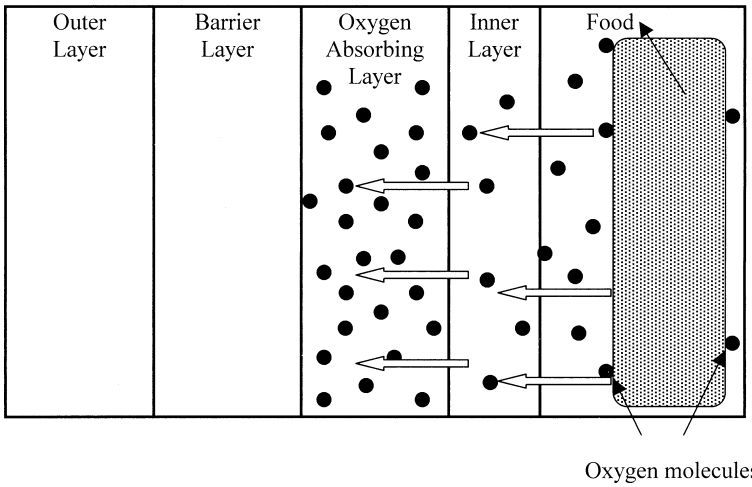


Figure 2.6 Structure of a typical oxygen-absorbing multi-layer active film. Reproduced from Ozdemir *et al.,* 2004.

Other important scavenger systems in food packaging are moisture scavenger systems. According to Rooney (1995), "respiration of fresh produce, the drip of tissue fluid from cut meat and produce, or temperature fluctuations in high equilibrium relative humidity packages are the main reasons for causing development of the excess moisture". The control of excess water in packaging can inhibit microbial growth, prevent fogging, and extend the shelf life of food. Effective moisture scavenger components in food packaging include silica gel, calcium oxide or chloride, modified starch, natural clay, and molecular sieves. Among these, silica gel is the most widely used moisture scavenger due to its non-corrosive and non-toxic properties. Moisture-absorbing packaging is usually used to keep low levels of relative humidity in packages of dried food, such as chips, spices, nuts, biscuits, milk powder, crackers, and instant coffee. The most common moisture-absorbing systems used to absorb or control

moisture for water-sensitive packaged foods are various brands of sachets and the moisture-absorbing label Desimax (Multisorb Technologies, USA) (Ozdemir *et al.,* 2004).

2.3.3 **Edible films and coatings (spray)**

Plastic, the most common packaging material, is increasingly produced; however, only 5% of plastic packaging is recycled, causing an enormous accumulation of plastic in the environment. Environmental pollution caused by synthetic packaging material has triggered the development of novel eco-friendly materials. Edible films and coatings are thereby studied as an alternative because of their biodegradability (Espitia *et al.,* 2014). Edible films are traditionally used to improve food appearance and prolong shelf life. Edible films such as wax for fruits and lipids for meat products have been widely used in the food industry for years (Ramos, 2012). Some records even indicated that citrus fruits were already waxed in the 12th century in China and lard or fats were used to extend the shelf life of meat products in England. In the early to mid- twentieth century, coatings started to be used on fruits and vegetables to prevent moisture loss and add shine to them (Salgado *et al.,* 2015). These are some simple and early attempts at coating of foods.

Now films or coatings are mainly produced from edible biopolymers and food-grade additives. Since they are part of a food and can be eaten, they are called edible films or coatings. Films are thin layers that are self-supporting and are used to wrap food products, while coatings can be either applied to or formed directly on foods by spaying or dipping into solution. Films and coatings are generally located on the food surface or between different food components (Guilbert *et al.,* 2005; Salgado *et al.,* 2015). All of these edible materials should be safe in accordance with their intended use and processed under good manufacturing practices. In the United States, materials employed to produce edible films and coatings should have FDA approval or acquire GRAS status at least (Sothornvit *et al.,* 2005). These edible films and coatings are food packaging materials and can produce foods from physical, chemical and biological deterioration in order to enhance food quality and prolong shelf life. **Fig. 2.7** shows some main functions of edible films and coatings. They have good barrier properties against gas, moisture, oils and solutes. Furthermore, their mechanical properties can provide protection against mechanical damage during transportation. In addition, special components in the films can prevent food from oxidation or microbial growth and carry active compounds such as antimicrobials, nutraceuticals, and flavors (Han and Scanlon, 2014).

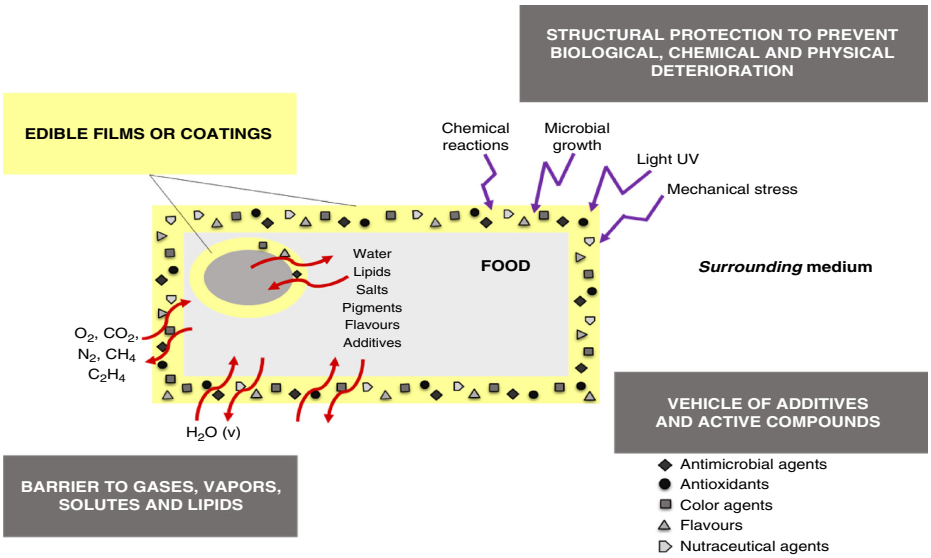


Figure 2.7 Functions of edible films.
Reproduced from Salgado *et al*., 2015.

2.3.3.1 **Components of edible films**

Edible films are basically manufactured from proteins, lipids, and polysaccharides as well as mixtures of two or more of these components (Khwaldia *et al*., 2004). Plasticizers and other additives may be employed to modify the physical properties or functions of the film-forming materials. The composition of edible films contributes substantially to their properties. Both protein- and polysaccharide-based edible films have good barrier properties against gases. Furthermore, they have moderate mechanical properties and excellent organoleptic functions. However, the hydrophilic nature of these films, which results in high permeability to water vapor, limits their performance. Although lipid-based films provide a better moisture barrier, they are associated with undesirable sensory properties such as off-flavors and less effective mechanical properties. Generally, protein-based edible films have superior mechanical and barrier properties. Furthermore, the nutritional value of proteins as well as their various functional properties make them extensively explored in research in this area (Fabra *et al.*, 2008).

2.3.3.1.1 Proteins: SPI

Films and coatings are made from proteins of both plant and animal origin, including casein, whey protein, gelatin, corn, soybean, wheat, rice, and peanut (Mellinas *et al*., 2016). They are macromolecules with specific molecular structure and amino acid sequences. Compared to other film-forming materials, proteins are more complex in structure, three-dimensional

shape and other properties. The final properties of the films can be affected by the film components and processing factors (Panyam and Kilara, 1996). In the case of protein-based edible films, relevant intrinsic properties of proteins include hydrophobicity or hydrophilicity, amino acid composition, pI, surface charge, and molecular size. The cysteine residues in proteins such as β-lactoglobulin and glycinin are responsible for potential disulfide bridge formation, and some proteins such as α-zein (corn) that have a high content of leucine and alanine residues have hydrophobic zones (Embuscado and Huber, 2009). Processing factors include temperature, ionic strength, drying conditions, pH and relative humidity during storage (Damodaran, 1996).

Proteins, plasticizer and solvent are the three main components of protein-based films and coatings. The solvent is either water or ethanol or a mixture of them (Kester and Fennema, 1986). Protein films can be generally formed and peeled off after solvent or carrier evaporation. Generally, proteins can be denatured by acid or base and heat to unfold their chains and expose some hidden active groups. Once unfolded, protein chains can associate through hydrogen, hydrophobic, ionic and covalent bonding and this interaction can be affected by the degree of chain extension and sequence of amino acid residues (Bourtoom, 2008). The numbers of polar, hydrophobic, or thiol groups along the polymer chain can affect chain-to-chain interactions. If these interactions are extensive, films are stronger but less flexible and less permeable to gases and liquids (Kester and Fennema, 1986). Hydrogen and ionic bonding between different protein groups result in films that have excellent oxygen barrier properties but are susceptible to moisture. Thus, protein films have good oxygen barrier properties at low relative humidity (Bourtoom, 2008).

Soybean protein is one of the most widely available plant proteins and has been used in food products for a long time, especially in Asian countries. It has been cultivated in China for more than 3000 years and can be utilized in many forms such as tofu, natto, and miso (Nishinari, 2014). Soybeans contain approximately 20% oil and 40% protein on a dry matter basis. Soy protein isolate (SPI) is obtained from soybeans by removing the oil at low temperature. SPI is a mixture of proteins, which can be classified into four types according to their sedimentation coefficients 2S, 7S, 11S and 15S (Nishinari, 2014). Among them, the two major components of SPI are glycinin (11S) and β-conglycinin (7S), which together account for approximately 70-80% of total soybean protein; the ratio of 7S to 11S is in the range of 0.5-1.3 (Saio *et al*., 1969). Glycinin (11S) is made of six subunits and its weight is around 340-375 kDa. Each of the six subunits is made up of an acidic subunit A and basic subunit B

linked by a disulfide bond. β-Conglycinin (7S) consists of three subunits (α, α', andβ), and its weight is about 140-170 kDa. Studies have shown that soy proteins have excellent film-forming ability and that their films provide a better oxygen barrier (Miller and Krochta. 1997; Cho *et al*., 2007) and are low cost and biodegradable. However, edible films made only of soy protein are brittle and exhibit poor moisture resistance. Incorporation of other materials, such as polysaccharides, lipids or essential oils, can improve the mechanical or physical properties of soy protein-based films. Sivarooban *et al*. (2008) reported that the incorporation of grapeseed extract (GSE), nisin and EDTA significantly increased the thickness and puncture and tensile strengths of SPI films. Furthermore, SPI films containing GSE (1%), nisin (10,000 IU/g) and EDTA (0.16%) exhibited the greatest inhibitory activities against *Listeria monocytogenes, Escherichia coli* O157:H7 and *Salmonella typhimurium*. In the study of Cao *et al.* (2007), a high concentration of gelatin in SPI-based films increased the elongation to break, tensile strength (TS), elastic modulus (EM) and swelling properties of composite films. In addition, the films became more transparent and were easy to handle. Friesen *et al.* (2015) investigated the mechanical, optical and water vapor barrier properties of SPI-based edible films with added rutin and epicatechin as cross-linking agents. The results showed that the addition of rutin significantly increased puncture strength and TS by comparison with control films. In addition, the water vapor permeability of films with epicatechin was higher than that of films with rutin. These findings indicated that rutin and epicatechin can be used to improve specific properties of SPI films. In the case of edible films made of the subunits of SPI, 11S-based films had greater tensile strength than 7S-based films due to the high concentration of cysteine groups in glycinin (Kunte *et al.,* 1997).

2.3.3.1.2 Polysaccharides and lipids

Many materials belong to the category of polysaccharide film-forming materials, including cellulose, chitosan, starch, fibers, and gums. Compared to protein molecules, which are made up of 20 common amino acids, polysaccharides are made up of simpler monomers. However, because of the higher molecular weight of polysaccharides by comparison with proteins, the conformation of their structures is more complicated and unpredictable. Usually, most carbohydrates are neutral, while some gums are negatively charge and very exceptional cases are positively charged. Due to the large numbers of hydrophilic moieties or hydroxyl groups in the structure of neutral carbohydrates, hydrogen bonds play the most significant role in film formation and in determining film characteristics (Han, 2014).

Lipids can also be used as film-forming materials, but they are, of course, not polymers. They are biodegradable, edible, and cohesive biomaterials, which are used as a type of modified additive to improve the properties of films or coatings. Most lipids and edible resins used in films are soft solids at room temperature and have characteristic phase transition temperatures. Due to their hydrophobic nature, films or coatings made from lipids have very low surface energy and high-water resistance. In addition, lipids can be combined with other film-forming materials, including proteins or polysaccharides, to form emulsion films or multilayer coatings in order to decrease the water vapor permeability (Han *et al*., 2006; Mehyar *et al*., 2012; Pérez-Gago and Krochta, 2002).

In addition, biopolymer composites can improve film properties and create desirable film structures for specific applications. Similar to multilayered plastic films, biopolymer films can be created as layer-by-layer multiple films, for example, lipid layers on polysaccharide-protein films or protein coatings (or film layers) on polysaccharide films. This multilayered structure optimizes the final characteristics of the film. Composite films can also be produced by mixing two or more composites together to make one homogeneous film layer (Debeaufort *et al*., 1998; Were *et al*., 1999). Various biopolymer composites can be mixed together to form a smart packaging film with unique properties and combine desirable attributes of each component (Wu *et al*., 2002).

2.3.3.1.3 Plasticizers and other additives

Plasticizers are incorporated into polymeric film materials to increase the thermoplasticity of the polymers (Han, 2014). They are small molecules that can fit between large polymer molecules, such as proteins or polysaccharides, and interfere with their interactions to improve the flexibility of the polymeric films (Krochta, 2002). They can not only change the mechanical properties of films, especially the elastic modulus, but also affect the moisture and gas permeabilities of edible films and coatings (Sothornvit and Krochta, 2000, 2001). Possible food-grade plasticizers include glycerol, sorbitol, mannitol, propylene glycol, and sucrose. Most plasticizers can attract water molecules and form a larger plasticizer complex due to its hydrophilic and hygroscopic properties. Plasticizers disrupt the hydrogen bonds between protein or polysaccharide molecules, thereby increasing the distance between them, and thus reduce the proportion of crystalline to amorphous regions to lower the glass transition temperature (Krochta, 2002). Water is also a good plasticizer, but it can easily be lost during dehydration. So, there are two types of plasticizers: one is used to form hydrogen

bonds with polymer molecules and increase the distance between polymer chains; the other is used to attract more water molecules in order to maintain higher moisture content in the films (Sothornvit and Krochta, 2000, 2001). Size, shape and compatibility with the matrix are three factors that may affect the effectiveness of a plasticizer. Solid plasticizers may bring an anti-plasticizing effect to films, resulting in decreasing their flexibility but improving permeability (Dangaran and Krochta, 2007). Chinma *et al*. (2012) reported that addition of 20% or more glycerol can enhance the mechanical and barrier properties of cassava starch-SPI edible films. Similarly, Sothornvit and Krochta (2001) reported that glycerol is a very effective plasticizer in a whey film matrix and that the properties of the protein would also affect the plasticizer-protein interaction.

Additives are the active agents in edible films and coatings, such as antioxidants, antimicrobials, flavors and emulsifiers, and can enhance food quality and safety or change mechanical properties of the films (Baldwin *et al*., 1995; Han, 2014). Emulsifiers are surface-active agents and are employed to control the adhesion and wettability of the films by reducing the surface tension (Krochta, 2002). Essential oils and lipids are hard to dissolve in aqueous solution, and thus incorporating emulsifiers into the film-forming solution can make it easier to produce uniform lipid emulsion films. Antioxidant and antimicrobial additives in the edible film and coating system can protect food from oxidation and microbial spoilage, leading to improved quality and safety (Kang *et al*., 2013). Other additives in films are flavors and colorants that have an impact on the organoleptic properties of the food (Han, 2014). Due to the various characteristics of these active additives, film composition can be modified to keep a homogeneous film structure and obtain suitable physical properties (Debeaufort *et al*., 1998).

2.3.3.2 **Formation of films and coatings**

2.3.3.2.1 Ways of forming films and coatings

There are many ways to form edible films and coatings from proteins and all of these can affect the properties of the films or coatings. Typical film-forming methods include solvent casting, extrusion, and spinning. The solvent casting method is one of the most favorable methods for research purposes. There are various types of equipment available for casting films, from casting plates to more continuous lab coaters. The use of casting plates is the most common method for forming protein films as it is effective and low cost (Embuscado and Huber, 2009). In this method, a dilute protein solution (5-10% solids) and plasticizer are

spread into Petri dishes or plates which are then left to dry in room-temperature or ambient conditions with controlled humidity. Kozempel and Tomasula (2004) reported that they developed a continuous process for casting casein-based films and described some parameters that need to be determined in producing such films. Thus, more sophisticated equipment produces larger films by mechanically controlling the thickness of the spreading solution. Extrusion is an alternative to solvent casting, which produce a cohesive film matrix by using high temperature and shear. Compared to solvent casting, extrusion is faster and uses less energy because a high concentration solution can be used. Obuz *et al*. (2001) found that wheat gluten can be extruded with different concentrations of MCEBC or LDPE and that the TS of films containing more than 25% of wheat gluten were similar to those of the pure synthetic materials. However, the elevated temperature and higher concentration of biopolymer can result in detrimental effects on color, elongation, and tensile strength (Embuscado and Huber, 2009).

The processes and mechanisms of forming edible coatings are the same as those for edible films. A dilute film-forming solution is applied to the surface of food and the coating forms after solvent evaporation. The methods for forming a coating include spraying, dipping, panning, and fluidized-bed processing. Spray coating is a more controlled method than other methods of applying coatings and is used to apply a uniform coating on a food surface. It is best suited for large surface area items. Furthermore, the bottom surface of the food may be exposed and coated after initial coating and drying during the coating process (Embuscado and Huber, 2009). The dipping method, which is another method of forming a coating on the surface of a food product, is preferred for irregularly shaped food products. With this coating method, multiple dippings may be required to ensure full coverage of the food surface. For this reason, coatings formed by the dipping method are less uniform than other coatings (Krochta *et al*., 1994). Finally, panning and fluidized-bed processing are methods used by the confectionery and the pharmaceutical industry to coat chocolate or tablets.

2.3.3.2.2 Drying temperature

A drying process is required to obtain protein edible films, and the drying conditions are critical. Drying conditions may affect the final functions and properties of edible films.

The drying temperature employed in forming edible films is determined by the characteristics of the protein material such as the presence of a preexisting gel phase or thermally induced gelation. Several phenomena may take place during drying, such as crystallization, phase

separation due to thermodynamic incompatibility, and conversion from a rubbery state to a vitreous state (Denavi, 2009). Temperature can denature proteins, although the thermal stability depends on amino acid content. When the protein is denatured, the interactions between protein chains are easier and stronger especially by disulfide bonds. Throughout drying, as water is gradually eliminated, protein structure changes, while the extent of protein unfolding determines the proportion and type of both covalent (S-S) and non- covalent (hydrogen bonds and hydrophobic interactions) interactions that can be formed between protein chains.

Moreover, as temperature increases, hydrophobic interactions become more active, while hydrogen bonds and electrostatic interactions decrease. This leads to increase adhesion between edible films and substrates. In addition, heating to high temperature, ranging from 70 to 100°C, results in the formation of a rigid protein structure because of denaturation. Thus, excessive thermal treatment as well as excessive solvent drying rate may contribute to non-adhesive films.

2.3.3.2.3 Relative humidity

In order to account for modifications of the physical characteristics of protein-based edible films caused by changes in water content, understanding their interaction with water becomes a priority. It is generally assumed that the binding of water molecules to hydrophilic sites such as amino residues and peptide groups is essentially related to the absorption of water. When relative humidity is high, multi-molecular adsorption can occur by swelling or conformational changes in protein structure.

Relative humidity (RH) affects permeability of edible films. According to Gontard (1996), at low relative humidity, edible films exhibited low oxygen as well as low carbon dioxide permeability. When RH increased (60%), both oxygen and carbon dioxide permeability increased exponentially. Moreover, an effect of relative humidity on tensile strength was also observed. As relative humidity increased, tensile strength decreased (Gennadios, 1993). The influence of relative humidity on mechanical and barrier properties of protein-based films has been explained in terms of plastification phenomena. The plasticizing effect of water on protein films results in the decrease of the glass transition temperature.

2.3.3.2.4 Concentration

Protein films are complex in their structure. During aggregation, protein-protein interactions can be an important factor that leads to different categories of cohesiveness. The mobility of proteins as well as their ability to form films can be affected in this way. The concentration of the protein solution changes the self-adhesion and the speed of matrix formation during film preparation. Moreover, the concentration of protein in the film solution can also affect the formation of a protein matrix. When protein concentration is lower, protein-protein interactions may decrease; conversely, at a higher protein concentration, self-diffusion is increased, contributing to inferior properties. Therefore, the concentration should be selected appropriately (Wittaya, 2012).

2.3.3.3 **Effect of incorporated EOs on physical properties of edible films**

2.3.3.3.1 Tensile properties

The tensile properties or mechanical properties of films are usually determined by tensile tests (ASTM D883, 2001), which involve extending the film at a given rate until it breaks and recording the force versus time or distance. The elastic modulus (EM) represents the film strength and can be related to the parameter of rigidity, tensile strength (TS). The percentage elongation at break (E %) represents the film stretchability upon breakage. All three parameters are important for measurement of packaging materials (Krochta *et al*., 1997). The tensile properties of edible protein-based films are determined by several factors, such as the microstructure of the film and the film constituents and their relative concentrations (Krochta *et al*., 1997; Cuq *et al*., 1995). Thus, the effect of addition of EO in films on tensile properties may be influenced by the characteristics of the EO and its interaction with the protein matrix (Pires *et al*., 2011).

A lot of studies have been published in this area, but the conclusions of these studies are quite different. Many works reported a negative effect of EO addition on TS of protein films. Tongnuanchan *et al*. (2012) found that addition of citrus oil caused a decrease in TS of fish skin gelatin films, which was attributed to the reduced interaction between gelatin molecules. However, this reduced interaction might lead to an increased extension of films. This result indicated that addition of EO into protein films weakens the polymer interaction (Limpisophon, 2010). Similarly, a reduction in TS was also observed to result from addition of *Origanum vulgare* EO in fish gelatin-chitosan films (Hosseini *et al*., 2015). The TS

decrease can be affected by the concentration of EO in the films. Zinoviadou *et al*. (2009) investigated elongation of whey protein films containing different concentrations of oregano EO. The results showed that the film's elongation properties increased at a concentration of EO up to 1%. On the other hand, several studies reported an increase of the TS of films due to the addition of EO. Atarés *et al*. (2010a) found this behavior for soy protein isolate films with incorporated cinnamon EO. The formation of intermolecular covalent bonds between gliadins gave the explanation for the increase of TS and EM in gliadins films with cinnamaldehyde (Chiralt *et al*., 2018). The same effect of cinnamon EO was also observed by Ojagh *et al*. (2010) in chitosan films. This phenomenon may be caused by the rearrangement of the polymer network due to the presence of EO. In some cases, some compounds in EOs can interact with the polymer chain, leading to improved tensile properties (Tongnuanchan *et al*., 2012). Phenolic compounds, one of the main components in oregano and thyme EO, can associate with more than one protein site, resulting in protein cross-links (Haslam, 1989). In addition, cinnamon EO can interact strongly with the chitosan film matrix, leading to an increase in TS (Ojagh *et al*., 2010). Pires *et al*. (2013) studied the interaction of EO and protein in hake protein films. In this research, the high resistance to break of films with citronella oil was attributed to the cross-linking promoted by β-citronellal. However, several studies showed no significant effect of addition of EO on TS of films. For example, Atarés *et al*. (2010b) observed no significant difference in TS of sodium caseinate films with cinnamon EO.

Unlike pure components, EOs have a complex composition, and this complicates prediction of their effects on the tensile properties of films. According to Atarés and Chiralt (2016), the presence of EOs in films typically causes some weakening of the film due to the heterogeneous biphasic structure produced by addition of EO. However, the effects of EO addition on tensile properties of films are quite variable and depend on the specific interactions between compounds in the EO and the food matrix.

2.3.3.3.2 Barrier properties

The ability to retard moisture loss from food is an important characteristic of films to ensure product quality (Pranoto *et al*., 2005). Thus, when films and coating materials are used on food, their water barrier properties need to be taken into account. The water vapor permeability (WVP) of films and coatings can be measured by a gravimetric method (ASTM E96-95). The ratio of hydrophilic and hydrophobic groups in films can affect the water vapor

permeation processes through films (McHugh *et al*., 1994). Pires *et al*. (2013) observed that the addition of thyme EO could improve the water vapor barrier properties of hake protein films. Similar trends have also been reported in many studies on polysaccharide films. However, owing to the complex composition of EOs, various effects of EO on WVP have been reported for different EOs and polymer matrices. Oregano EO may not affect the WVP of whey protein films (Zinoviadou *et al*., 2009). In the case of soy protein films, Pruneda *et al*. (2008) found an increase in WVP after addition of oregano EO in soy-based films. Atarés *et al*. (2010a) reported that the presence of a small proportion of ginger EO and cinnamon EO led to a decrease in the water vapor barrier properties of soy films. This result might be due to the low concentration of EO such that the lipid discontinuities were not sufficient to increase the tortuosity factor for transfer of water molecules, which is responsible for the reduction of WVP (Perez-Gago and Krochta, 2001a). All these studies demonstrated that the reduction of WVP in edible films cannot simply be achieved by adding a hydrophobic component, since the amount added can impact the microstructure of the emulsified films, which is a decisive factor in WVP. In turn, the microstructure of films can also be affected by the distribution of EO in the matrix and by its physical state (Atarés and Chiralt, 2016). With regard to the effect of lipid distribution in the protein matrix, smaller oil droplets are more homogeneously distributed in the film matrix, resulting in lower WVP (Pérez-Gago and Krochta, 2001a). Atarés *et al*. (2010a) found that cinnamon EO was more effectively integrated than ginger EO in the soy protein film network at the same ratio of oil to protein.

The oxygen permeability of films is another characteristic of edible films, but it is measured more rarely than WVP. Oxygen permeability increases with increases in temperature and relative humidity since the diffusion of oxygen in the film is dependent on molecular mobility, which increases when the film moisture content or temperature increases (Miller *et al*., 1997). Oxygen permeability of films is important for the packaging which aims to reduce lipid oxidation.

2.3.3.3.3 Transparency

Transparency is one of the optical properties (color, transparency and gloss) of films and one of the factors affecting the food product's appearance. It can be measured by different methods. Tongnuanchan *et al*. (2012) measured the transparency of gelatin films at 200-800 nm, and Han and Floros (1997) calculated a transparency value by dividing the absorbance at 600 nm by the thickness of the film. This transparency value was also called opacity index,

and the greater the opacity index or transparency value, the lower the transparency of films (Gomez-Estaca *et al*., 2009b). The addition of EO to films resulted in a reduction in film transparency. The EO in films caused a decrease of light transmission, and this may be due to light scatter at the interface of EO droplets in the matrix. Many researchers observed this trend using the same method (Atarés and Chiralt, 2016). Another method of calculating opacity (%) uses reflectance measurements of each sample with a black and with a white backing: Opacity (%) $= \frac{Y_{\text{black backing}}}{Y_{\text{white backing}}} \times 100\%$, where Y is the tristimulus value Y. Pires *et al*. (2011) studied the opacity (%) of hake protein film with thyme EO. They found that as the EO proportion increased, the transparency value increased, but the opacity (%) did not change. This may have been caused by variable film thicknesses, as the transparency value will be affected by thickness. Ortiz *et al*. (2018) reported that incorporation of clove EO in SPI films resulted in an increase of the opacity value, In a study in which oregano, clove, and garlic EOs were incorporated in fish protein films, the transparency of the films was dependent on the type of EO incorporated (Teixeira *et al*., 2014).

2.3.3.4 **Antimicrobial activity of edible films with incorporated EOs**

Food degradation is mainly caused by spoilage microorganisms and growth of foodborne pathogens. Spoilage microorganisms in food can produce toxin, accelerate oxidation processes, decompose nutrients, and produce changes in the structure and sensory properties of food products (Saggiorato *et al*., 2012) whereas foodborne pathogens can result in illnesses in human beings. Antimicrobial packaging could extend shelf life of food and ensure the quality of food products. Essential oils (EOs) are volatile and aromatic oily liquids extracted from some herbs and spices, such as flowers, leaves, seeds, roots and fruits, and have been recognized as antimicrobial agents (Burt, 2004). The antimicrobial mechanisms of EOs involve various effects on microbial cells, including disrupting enzyme systems, attacking the phospholipid bilayer of the cell membrane, and compromising the genetic material of bacteria (Burt *et al*., 2007). The cell membrane is an important site where many components in EOs can act. The interaction between EOs and bacterial membranes can affect the transport of ions and nutrients, the permeability of the cell, and the membrane potential (Hyldgaard *et al*., 2012). Once cell death occurs, the large molecules in the cytosol will come out, such as ATP and DNA. The antimicrobial activity of EOs can be measured *in vitro* by various methods, including disk diffusion, agar wells, agar dilution and broth dilution (Gomez-Estaca *et al*., 2010).

Many studies reported antibacterial or antifungal activity of EOs in edible films or coatings. Seydim *et al*. (2006) investigated the antimicrobial activity of whey protein films in which garlic, oregano, cinnamon; ginger or rosemary EO had been incorporated. They found that oregano EO films were the most effective against *Staphylococcus aureus, Escherichia coli* O157:H7*, Listeria monocytogenes, Salmonella enteritidis* and *Lactobacillus plantarum* at the 2% level. The WPI films with garlic EO exhibited an inhibitory effect at 3% and 4% levels. Orsuwan & Sothornvit (2018) reported that incorporation of garlic EO in banana flour nanocomposite films could improve the film properties and preserve the quality of roasted peanuts during high-temperature storage (45 ^{o}C). Zein coatings containing compounds present in certain essential oils (eugenol, carvacrol and thymol) were found to be effective in inhibiting the growth of *L. innocua* and *E. coli in vitro* and reduced the counts of these bacteria on inoculated melon surfaces at 10 ^{o}C (Boyacı *et al*., 2019). In addition, application of sodium alginate films incorporating oregano EO on sliced ham inoculated with strains of *L. monocytogenes* combined with the use of high-pressure processing led to a reduction or even absence of the pathogen and improved the aroma as well (Pavli *et al.,* 2019). Emiroglu *et al*. (2010) reported that soy edible films with oregano EO, thyme EO or a mixture of both had higher inhibitory effect against *E. coli, E. coli* O157:H7 and *S. aureus,* but *L. plantarum* and *P. aeruginosa* were more resistant to these antimicrobial agents. However, when these antimicrobial films were applied on ground beef patties, there was no significant effect on total viable counts.

With regard to antifungal activity, Balaguer *et al*. (2013) studied the antifungal activity of gliadin films containing cinnamaldehyde, which is the main component of cinnamon EO, against *P. expansum* and *A. niger* on bread. After 30 days, fungal growth remained absent on bread slices packed with gliadin films treated with 5% cinnamaldehyde. Otoni *et al*. (2014) developed a nanoemulsion of oregano and clove bud EOs in edible films; in their *in vitro* tests, the essential oil at 20 mg/ml inhibited spore germination of *Penicillium spp.* and *A. niger*. Arancibia *et al*. (2014) developed soy protein, lignin and formaldehyde films with citronella EO. Films cast with 3% (w/w) citronella EO in the film-forming solution showed good antifungal activity against *Fusarium oxysporum* in bananas. In addition, there was a large reduction in total molds, yeast and aerobic mesophiles on bananas covered by the films during storage.

2.3.4 **Antimicrobial Sachets**

The placement of sachets alongside a food inside an enclosed packaging system is a type of active packaging, employed to extend shelf life or ensure food safety. Typical sachets used in food packaging include oxygen scavengers, antimicrobial emitting sachets, and moisture scavengers.

2.3.4.1 **Types of emitting sachets**

There are two types of emitting sachets: one generates the antimicrobial compound in situ and releases it (G sachet) and the other type carries and releases antimicrobials (C sachet). The production of G sachets is very simple: materials that can generate antimicrobial compounds are placed inside the sachet and the sachet is then sealed. Ma (2012) developed a G sachet that uses an enzyme to generate AITC vapor by the sinigrin-myrosinase reaction and releases it in the sachet (Otoni *et al*., 2016). Similarly, ClO_2 can also be used in sachets as an antimicrobial. Due to its instability and risk of explosion, ClO_2 can only be generated in situ. In the study of Keskine and Annous (2010), sodium chlorite and an acid were mixed together in sachets to generate ClO_2 ($4H^+ + 5NaClO_2 \rightarrow 4ClO_2 + NaCl + 4Na^+ + 2H_2O$). Sachets of the C sachet type are produced in three steps: the antimicrobial compound is incorporated into a carrier, the carrier is then placed inside the sachet, and the sachet is sealed. The carrier in emitting sachets can control the release of antimicrobial compounds into a closed package, so the sachet material, which is the outer layer of the sachet, is usually permeable to the active compounds (Otoni *et al*., 2016). In addition, the sachet material should withstand transportation and handling to prevent leakage of the carrier. Soares *et al*. (2008) developed an antimicrobial sachet by adding liquid AITC into a porous resin carrier. This carrier was made of a high-density polyethylene resin, placed inside a nonwoven fabric to form the antimicrobial sachet.

2.3.4.2 **Materials employed in sachet packaging (carrier material, sachet and external packaging materials)**

2.3.4.2.1 Carrier materials

Carrier materials often serve as a support material to carry active substances in sachets. Materials commonly employed as carriers include powders, polymers, earths, and gels. For instance, silicon dioxide powder in Ethicap^{TM} sachets is considered a carrier because it releases ethanol vapor into the headspace of the food packaging (Day, 2008). Furthermore,

different sizes and types of carriers can affect the loading of volatile compounds in sachets and their release from the sachets. Seo *et al*. (2012) demonstrated that larger calcium alginate beads can load a larger quantity of AITC.

2.3.4.2.2 Sachet materials

The diffusion of active compounds from sachets into the headspace of the food packaging is controlled by the permeability of the sachet material. It may be either porous or semipermeable to the antimicrobial compounds. Several types of materials can be used as sachet materials, including perforated barrier films, porous nonwoven fabric, semipermeable polymer films, and paper (Ozdemir and Floros, 2004). Studies have shown that polymer type, pore dimensions or thickness and number of layers in perforated barrier films can all affect the release rate of the active compound Ma (2012) and Seo *et al*. (2012) reported that the release of AITC from sachets decreased with increased thickness of the sachet material, which was a low-density polyethylene film.

Additionally, sachet materials are required to be sealable to avoid leakage of the carrier and uncontrolled release. Generally, these materials are heat-sealed. However, if the active compound or sachet material is heat-sensitive, cold-seal coatings may be required (Otoni *et al*., 2016). Last but not least, like all food contact materials, sachet materials must be safe to human health (Directive 89/ 109/EEC) and also have no negative effect on composition and sensory properties of the food in the package. In addition, the plastic or other materials used in fabrication of the sachet, such as paper, ink, and adhesive, must be also covered by EU or food safety regulations (Directive 90/ 128/EEC; De Kruijf *et al*., 2002).

2.3.4.3 **Factors affecting the release of volatile EOs**

The rate of release of the active compound from a sachet is one of the most important factors to evaluate in assessing whether the food packaging system is successful or not. There are several factors affecting the release kinetics. Internal factors include inherent properties of the active compound, the affinity between the active compound and the carrier material, the concentration of the active compound, the permeability of the sachet material, the release time, and the composition and volume of the headspace in the packaging. External conditions like ambient temperature or relative humidity must also be taken into account (Otoni *et al*., 2016). Much research about the influence of various factors on release behavior has been reported in the literature. Ayala-Zavala *et al*. (2010) studied the effect of RH on the release kinetics of garlic EO encapsulated in β-cyclodextrin. They found that high RH could favor

release of garlic EO. The research showed that water weakened the interaction between the EO and β-cyclodextrin, such that the EO was replaced by water and released into the headspace. Han *et al*. (2014) evaluated how water affected the release of rosemary and thyme essential oils from sachets. In this study, the sachets were made of filter paper and microcellular foam starch and were placed into a closed jar with or without water at 23±2 °C for 0.5 h. The results showed that in the absence of added water, EO compounds were not detected in the headspace of the jar, indicating that the volatile compounds were trapped in the starch structure. However, when water was present in this system, the starch repelled the EO and attracted water, and thus EO compounds were detected by gas chromatography in the headspace. Buttery *et al*. (1999) demonstrated that water could promote the release of volatile compounds from microcellular foam starch. Similarly, Seo *et al*. (2012) noted that higher RH conditions could increase the release of active compounds. However, in a study of release of AITC from LDPE sachets, the release at 43% and 85% RH was only significantly different under certain conditions, indicating the importance of other factors (Otoni *et al*., 2016). Temperature is a factor that can have a substantial effect on release kinetics. Thus, in the study referred to above, the rate of release of AITC from sachets at 25 °C was almost twofold higher than that at 4 °C (Seo *et al.,* 2012).

In many cases, it is preferable for the rate of release of active compounds to be low and constant, so that the active compounds will be released into the headspace of the packaging for a long period of time. Slowing the release rate may be achieved by judicious selection of the sachet material and thickness to alter the permeability of the sachet to the active compounds. For instance, in the study of AITC-containing LDPE sachets mentioned above, the rate of AITC release from the sachets decreased with an increase in the thickness of the LDPE film (Seo *et al.,* 2012).

Generally, the release rate of volatile antimicrobials is maximum at the beginning and then decreases exponentially during the storage time (Jo *et al*., 2013). The concentration gradient between the inner and outer environments of the sachet is the driving force of this release. With the diffusion of the volatile antimicrobials into the headspace of the packaging, the concentration gradient is reduced, leading to a decrease in the rate of release of antimicrobials from the sachet.

2.3.4.4 **Antimicrobial activity of EO-containing sachets**

The effectiveness of antimicrobial sachets in retarding, lessening or inhibiting microbial growth on food has been investigated both *in vitro* and in vivo (Otoni *et al.,* 2016). For *in vitro* assays of their antimicrobial activity, sachets have either been attached to the inner side of the lids of Petri dishes or placed with Petri dishes inside a sealed package to simulate the antimicrobial sachet packaging system (Otoni *et al.,* 2014b). Both methods are shown in **Fig. 2.8**. Some of the results of these studies presented in this section have already been mentioned in other contexts in this literature review.

The EOs commonly used in sachet applications include cinnamon (*Cinnamomum zeylanicum*), garlic (*Allium sativum L.*), oregano (*Origanum vulgare*) and lemongrass (*Cymbopogon zeylanicum*) EOs. Both cinnamon and garlic EOs have also been widely used in food processing because of their flavor and aroma. Thus, when employed as antimicrobials in active packaging, these EOs may not have a negative impact on the food's sensory properties. Many studies have been done on the antimicrobial effects of sachets against foodborne pathogens or on their efficacy in prolonging shelf life. Ayala-Zavala *et al*. (2010) found that garlic EO encapsulated in β-cyclodextrin capsules in a cellulose sachet could inhibit microbial growth. The results showed that only 1 g of garlic EO was enough for 100 g of tomato slices to be preserved. Medeiros *et al*. (2011) developed antifungal sachets with oregano and lemongrass EOs to preserve mangoes. Both EO sachets were found to inhibit fungal growth during storage. The lemongrass EO sachet had higher antimicrobial activity and even decreased the total counts of aerobic mesophilic bacteria (TAM), molds and yeasts by 2 log cycles relative to the control. This study also showed that both lemongrass and oregano EOs had minimal effect on physicochemical properties of mangoes such as skin color, soluble solids, firmness and titratable acidity. Similarly, Espitia *et al*. (2012) reported that sachets containing oregano, cinnamon or lemongrass EO had little effect on the physicochemical properties of papaya and inhibited the growth of yeast, molds and aerobic mesophilic bacteria on papayas, with cinnamon EO having the highest antimicrobial activity, In the study of Han *et al*. (2014), sachets containing rosemary (*Rosmarinus officinalis L.*) and thyme (*Thymus vulgaris L.*) EOs were placed inside packages of shredded mozzarella cheese during cold storage. The results showed that both EOs were able to prevent growth of *L. monocytogenes* and lactic acid bacteria (LAB) and lessen total counts of aerobic bacteria (TAB). For applications in the bakery industry, sachets incorporating oregano EO were reported to control fungal growth on the surface of sliced bread (Passarinho *et al*., 2014). In

another study, sachets containing cinnamon EO were shown to retard mold growth on sliced bread that had been inoculated with molds isolated from moldy bread, subsequently identified as *A. flavus* and *Penicillium* spp., and extended the shelf life of sliced bread by more than 14 days (Cheng, 2018).

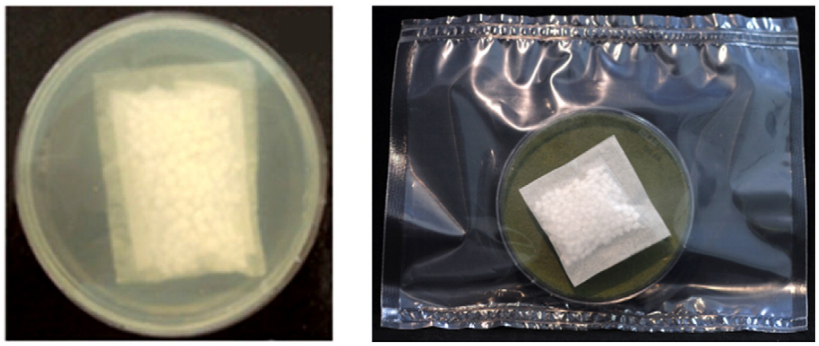


Figure 2.8 Petri dishes with sachets attached to the inner or outer side of the top lid. Reproduced from Otoni *et al*., 2016 and Ayala-Zavala *et al*., 2010.

CONNECTING STATEMENT 1

A thorough literature review in Chapter II presented the antimicrobial and antioxidant properties of essential oils (EOs) as clean-label food preservatives. Several attempts have been made to develop active-packaging systems incorporating EOs into polymeric materials that can slowly release the volatile active compounds onto the food surface to inhibit the growth of pathogenic microorganisms. In the research presented in Chapter III, the efficacy of essential oils in inhibiting the growth of *L. grayi,* selected as a less pathogenic surrogate for *L. monocytogenes, in vitro* and in storage tests with thawed frozen vegetable products was investigated. Sensory evaluation was also conducted to investigate the taste impact of essential oils on vegetables. In addition, the direct delivery of aerosolized oregano EO onto vegetables in a sealed chamber was also established to inhibit the growth of *L. grayi* on the vegetables during subsequent storage at 4ºC.

These results were first presented as a poster presentation at the McGill-CTAQ workshop and subsequently at IFT 19 Annual Meeting & Food Expo of the Institute of Food Technologists and will be submitted for publication in the *Journal of Food Microbiology*.

Tao, R., Sedman, J., & Ismail, A. (2019) Antimicrobial Activity of Various Essential Oils and Their Application in Active Packaging of Frozen and Ready-to-Eat Vegetable Products. IFT19 Annual Meeting & Food Expo, New Orleans, LA, USA, June 2 – June 5.

Tao, R., Sedman, J., & Ismail, A. (2020) Antimicrobial Activity of Various Essential Oils and Their Application in Active Packaging of Frozen and Ready-to-Eat Vegetable Products. *To be submitted*.

CHAPTER 3 EVALUATION OF ANTIMICROBIAL ACTIVITY OF VARIOUS ESSENTIAL OILS *IN VITRO* AND IN STORAGE TESTS WITH VEGETABLE PRODUCTS

Abstract

Essential oils (EOs) have potential utility as clean-label food preservatives due to their antimicrobial and antioxidant properties. Several attempts have been made to develop active-packaging systems incorporating essential oils into polymeric materials that can slowly release the volatile active compounds onto the food surface to inhibit the growth of spoilage and pathogenic microorganisms. In this study, the efficacy of this approach in inhibiting the growth of *Listeria* on thawed frozen vegetable products was investigated. Twenty-five essential oils, including essential oils of the same type from different commercial sources, were screened for their antimicrobial activities against *L. grayi* and *E. coli* K12. A cinnamon EO (designated CIN-03) and an oregano EO (ORE-03) proved to be effective both in direct contact and in the vapor phase in inhibiting the growth of *L. grayi* and *E. coli* K12 as well as *L. monocytogenes* and *E. coli* O157: H7 and O26: H11 strains. The slightly higher susceptibility of *L. monocytogenes* as compared to *L. grayi* makes the latter a suitable non-pathogenic surrogate in this study. The packaging of fresh-cut green peppers with cellulose stickers impregnated with cinnamon EO reduced the listeria count to 10 CFU/g after 2 days of storage as compared to 7.54 log CFU/g for the controls. Sensory evaluation of frozen broccoli and french fried potatoes thawed in packaging with incorporated EOs revealed that cinnamon EO at the higher concentration tested was less acceptable to panelists than oregano EO. Delivery of aerosolized oregano EO onto broccoli in a sealed container was found to be effective in inhibiting the growth of *L. grayi* during subsequent storage at 4^{o}C, resulting in a reduction of the listeria count by almost 2 log CFU/g (99% reduction) in comparison with controls after 12 days.

Keywords: essential oils, carvacrol, *Listeria grayi*, *Listeria monocytogenes*, vegetable products, sensory evaluation.

3.1 **Introduction**

Over the past decades, consumers' concerns about the safety of synthetic food additives have led to high demand for "clean label products" in the market. A number of essential oils have potential utility as green-label preservatives in food because of their antimicrobial and antioxidant properties. The active compounds, such as carvacrol (the major component of thyme and oregano essential oils) and cinnamaldehyde (present in cinnamon bark essential oil), have been demonstrated to have activity against bacteria, molds, and yeasts and high potential to extend the shelf life and ensure safety of perishable foods, including meat, fruits, and vegetables. Several attempts have been made to develop active packaging systems incorporating essential oils into polymeric materials that can slowly release the volatile active compounds onto the food surface to inhibit the growth of spoilage and pathogenic microorganisms.

Generally, the antimicrobial activity of EOs on vegetables, as in meat products, can be favored by low storage temperature or a decrease in the pH of the food (Skandamis and Nychas, 2000). The low-fat content of vegetables may also contribute to the successful results obtained with EOs. Some EOs and their components have been tested on vegetables in several research studies. The EOs showed effectiveness against foodborne pathogens and natural spoilage flora at levels of 0.1–10 µl/g in washing water (Wan *et al*., 1998; Singh *et al*., 2002). Furthermore, thymol and cinnamaldehyde in hot air at 50 ºC inhibited growth of six *Salmonella* serotypes on alfalfa seeds (Weissinger *et al*., 2001). However, when the temperature was increased to 70 ºC, the effectiveness of the treatment was reduced because of the volatility of the antibacterial compounds. Ayala-Zavala *et al*. (2010) found that garlic EO encapsulated in β-cyclodextrin capsules in cellulose sachets could inhibit microbial growth. The results showed that only 1 g of garlic EO was enough for 100 g of tomato slices to be preserved. In relation to antimicrobial activity of active packaging applied on fruit, the active compounds carvacrol and cinnamaldehyde were very effective in reducing the viable counts of the natural microbiota on kiwifruit at a concentration of 0.15 µl/g in dipping solution. Medeiros *et al.* (2011) developed antifungal sachets with oregano and lemongrass EOs to preserve mangoes. The lemongrass EO sachet had higher antimicrobial activity and even decreased the total counts of aerobic mesophilic bacteria (TAM), molds and yeasts by 2 log cycles relative to the control. Both lemongrass and oregano EOs had limited effect on skin color, soluble solids, firmness and titratable acidity of the fruit (Otoni *et al*., 2016). Likewise, similar results were observed by Espitia *et al*. (2012) with papaya. They reported

that sachets with oregano, cinnamon and lemongrass EOs inhibited the growth of yeasts, molds and mesophilic aerobic bacteria, and cinnamon EO reduced the total counts by 1.6 log cycles. Hyun *et al*. (2015) found a strong inhibitory effect of vapor from lemongrass, oregano, and thyme EOs against *E. coli* and *Listeria monocytogenes* and reported that the combination of modified atmosphere packaging (MAP) and vapor of lemongrass EO was highly effective in extending the shelf life of fresh cabbage. The low concentration of EOs in the vapor phase has the benefit of minimizing their sensory impact

Among foodborne pathogens, *Listeria monocytogenes* has presented the food industry with some unique challenges for a number of reasons. *Listeria* spp. are widely distributed in nature, being found in soil, plants, and the environment, and have been isolated from a large variety of foods of animal and plant origin (Troxler *et al*., 2000). Seventeen *Listeria* species are known in the world, including the pathogens *L. monocytogenes* and *L. ivanovii* and apathogenic *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* and eleven *Listeria* species newly described since 2009 (*L. aquatica, L. booriae, L. cornellensis L. fleischmannii, L. floridensis, L. grandensis, L. marthii, L. newyorkensis, L. riparia, L. rocourtiae, and L. weihenstephanensis*) (Orsi & Wiedmann, 2016). Among *Listeria* spp., *L. monocytogenes* is predominantly responsible for infections in humans and animals, termed listeriosis, although occasional human infection cases have also been reported with *L. grayi, L. ivanovii, L. seeligeri*, and *L. innocua* (Cummins *et al*., 1994; Rocourt *et al*., 1986; Perrin *et al*., 2003; Tonk *et al*., 2014). In general, *L. monocytogenes* is not a problem for the person who is immunocompetent, but neonates, young and old people, transplant recipients, cancer and HIV/AIDS patients, and pregnant women are more susceptible to listeriosis (Khan *et al*., 2016). In these susceptible populations, listeriosis can lead to septicemia, meningitis, and encephalitis, as well as spontaneous abortion or miscarriage, and has a mortality rate of 20-30%. These outcomes are mediated by the ability of *L. monocytogenes* to cross three barriers in the body: the intestinal barrier, the blood-brain barrier and the placental barrier. By crossing the intestinal barrier and targeting the mesenteric lymph nodes, the infecting bacteria access the systemic circulation, allowing them to reach and cross the blood-brain barrier and the fetal/placental barrier, leading to meningitis or encephalitis and spontaneous abortion or generalized neonatal infection, respectively (Cossart & Lebreton, 2014). Consumption of food contaminated with *L. monocytogenes* during production or processing is the main route of infection. Furthermore, the psychrotrophic properties of *Listeria* make growth of these bacteria in refrigerated foods possible, leading under certain conditions to substantial

increases in the number of contaminating bacteria in the food (Farber and Peterkin, 1991). In the 1980s, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service and the U.S. Food and Drug Administration (FDA) established a "zero-tolerance" policy for *L. monocytogenes* in ready-to-eat foods to address these public health risks (Kataoka *et al.,* 2017).

L. grayi is one of the subspecies in *Listeria* species, which is apathogenic bacterium. "Cells are small in range from 0.4 to 0.5 by 0.5 to μm, gram-positive, asporogenous, peritrichous rods, and can motile at 28 ºC. Colonies on tryptose agar are small, regular and smooth, whose diameter is 1 to 2 mm after 1 to 2 days incubation at 37 ºC. *L. grayi* can also grow at 4 ºC within 5 days. In addition, its metabolism is facultatively anaerobic, catalase produced, negative for the oxidase test, and reduction of nitrates to nitrites is variable. Acid is produced from glucose, mannitol, and other sugar without gas. The content of guanine and cytosine in the DNA is 41 mol% (Rocourt et al., 1992)." *L. grayi* have been observed to have a high level of similarity with *L. murrayi* according to DNA homology values, isoenzymes patterns, and rRNA genes restriction patterns. Comparing with the other *Listeria* spp., *L. grayi* have a specific clone lgr20-246 with an insert of 722bp. Primers lgr20-246R and lgr20-246 F derived from this clone region, which can amplify a specific band of 420bp from the genomic DNA of *L. grayi*, designed to provide a precise and independent method to distinguish *L. grayi* from other *Listeria* species and common bacteria (Liu *et al.*, 2005).

In addition, isolation of *L. grayi* from *Listeria*-contaminated vegetables has been reported in a number of studies, making it a suitable target organism in our subsequent development of EO-based active-packaging systems for vegetable products. For example, Soriano *et al*. (2001) investigated the occurrence of *Listeria* spp. among 103 samples collected from restaurants, including pork, beef, chicken, fish, lettuce, spinach, and Spanish potato omelette. *L. monocytogenes* was isolated from 2.9% (n = 3) of the samples while *L. grayi* was isolated from 13.6% (n = 14), half of the samples from which *L. grayi* was isolated were raw vegetable (lettuce and spinach) samples. The detection of the *Listeria* species from lettuce sold in markets in Benin City, Nigeria, also revealed the predominance of *L. monocytogenes* and *L. grayi* among all isolated strains (Miyebi *et al*., 2018). In a survey of the Chinese literature that encompassed over 12,000 food samples from 13 provinces in China (Chen *et al*., 2009), only 3.7% (n = 464) were found to be contaminated with *Listeria*; *L. grayi* was isolated from 5.4% of *Listeria*-contaminated vegetable samples in addition to 17.2% of *Listeria*-contaminated milk samples and 11.1% of *Listeria*-contaminated fish samples.

Beyond its roles as test organism in this research, inhibition of *L. grayi* in food is in itself a potentially important target for several reasons. Although infections caused by *Listeria* species other than *L. monocytogenes* are rare (Reda *et al*., 2016), several reports of *L. grayi* infections have appeared in the past two decades. In 2001, there were two cases of *L. grayi* infection in China, including eye infection and the isolation of *L. grayi* from the joint cavity effusion (Ao Birong, 2001; Lin Jiuling *et al*., 2001), and in 2003 it was isolated from a five-year-old child with symptoms of diarrhea as well (Grif *et al*., 2003). A case of sepsis stemming from *L. grayi* infection in a heart transplant recipient was studied in 2008 (Rapose *et al*., 2008), and another case of *L. grayi* bacteremia in a stem cell transplant recipient was reported in 2010 (Salimnia *et al*., 2010). These cases showed the potential pathogenicity of *L. grayi*. Even though the susceptibility of *L. grayi* to antibiotics has been seldom studied owing to its non-pathogenic behavior, information is provided by some studies comparing different *Listeria* species. Troxler *et al*. (2000) reported that *L. grayi* is naturally resistant to all antifolates. Furthermore, compared with *L. monocytogenes*, a higher percentage of *L. grayi* isolated from foods showed resistance to some antimicrobial agents, including cephalothin, clindamycin, erythromycin, gentamycin, methicillin, oxacillin, and penicillin, (Pesavento *et al*., 2010). In research on antilisterial activities of triterpenes, the minimum inhibitory and minimum bactericidal concentrations of ampicillin and penicillin G (first-line antibiotics in treatment of listeriosis) as well as neomycin and gentamicin were reported to be higher for *L. grayi* than for *L. monocytogenes* ATCC-19115 and *L. ivanovii*, while the antilisterial activities of triterpenes were not species-dependent (Penduka *et al*., 2014).

Despite the occurrence of *L. grayi,* like *L. monocytogenes,* in many food products and its potential pathogenicity to humans, there have been few studies on inactivation mechanisms of *L. grayi*, and its susceptibilities to some antimicrobial agents such as essential oils have not been fully examined. Therefore, in the research presented in this chapter the antimicrobial activities of twenty-five essential oils against *L. grayi* were tested and in certain cases compared with their activities against two strains of *L. monocytogenes*, as well as against non-pathogenic and pathogenic *E. coli* strains. Subsequently, the essential oils exhibiting the highest antilisterial activities *in vitro* were selected and their efficacy in active packaging in inhibiting the growth of *L. grayi* on fresh-cut and thawed frozen vegetable products was investigated.

3.2 **Materials and methods**

3.2.1 **Microorganisms and chemicals**

3.2.1.1 **Microorganisms**

The Gram-positive bacterium *Listeria grayi* (ATCC 25401) and Gram-negative bacterium *Escherichia coli* K12 were obtained from the Laboratoire de santé publique du Québec (LSPQ) and maintained on 5% sheep's blood agar at 4 °C. *L. grayi* was subcultured on brain heart infusion agar (BHIA) and *E. coli* K12 was sub-cultured on Mueller Hinton agar (MHA). All microorganisms were isolated twice and incubated at 37 °C for 24 h. Plates were stored at 4 °C to use. Two strains of the pathogenic bacterium *L. monocytogenes* (strains 519 and 523) from the Ottawa Culture Collection of the Canadian Food Inspection Agency, and the pathogenic bacteria *E. coli* O157:H7 and *E. coli* O26:H11 were obtained from Health Canada and the Listeriosis Reference Service, Ottawa. All microorganisms were enriched in tryptic soy broth (TSB) at 37 °C for 24 h to prepare concentrations of bacteria inoculum.

3.2.1.2 **Chemicals**

Most of the essential oils employed in the assays of antibacterial activity were supplied by the following companies: BSA Frutarom (Saint-Leonard, QC, Canada), Novo-taste Corporation Inc. (Dollard-Des Ormeaux, QC, Canada), Nascent Naturals Inc. (Midland, Ontario, Canada), New Directions Aromatics (Mississauga, ON, Canada), and Aliksir (Quebec City, QC, Canada). The essential oils used included cinnamon, oregano, thyme, clove, citron, sage, rosemary, white pine (needle), cedar, chili seed, garlic, pimento, lemon balm, cranberry seed, cumin seed, concentrated onion, and cypress essential oils and were labeled as CIN (01-04), ORE (01-03), THY (02), CLO (04), CIT (01), SAU (01), ROM (01), PIN (01), CED (01), CHI (01), AIL (01-03), PIM (01), MEL (01), CNB (01), CUM (01) OIG (01), and CYP (01), respectively. Carvacrol, eugenol, cinnamaldehyde (CINN), and allyl isothiocyanate (AITC) were labeled as PC-CAR-01, PC-EUG-01, PC-CIN-01 and PC-ISA, respectively. All these pure compounds were obtained from Sigma Aldrich (St. Louis, MO, USA). Sodium chloride, peptone water, trypsin soya broth (TSB), brain heart infusion agar (BHIA), and Mueller Hinton agar (MHA) were purchased from Fisher Scientific Company (Pittsburgh, PS, USA).

3.2.2 **Assays of antibacterial activity of various essential oils**

3.2.2.1 **Standard curve of optical density measurements**

L. grayi and *E. coli* K12 were incubated in 10 ml of TSB for about 20 h at 37ºC. The bacterial suspension was then diluted by the following factors: 1/10, 1/20, 1/40, and 1/80. Each diluted suspension was enumerated by plate count. The optical density (OD) of each diluted bacterial suspension was measured at 660 nm by a spectrophotometer.

3.2.2.2 **Disk diffusion test**

Filter papers were cut into a disk shape with a diameter of 6.5 mm using a metal hole-puncher, and both sides of the disks were sterilized under ultraviolet light for 15 min prior to use. In the disk diffusion test, 5 µl of EO or saline (as control) were pipetted onto sterile filter disks. The disks were then placed on BHIA or MHA plates that had been previously smeared with 100 µl of a standardized inoculum containing 10^6 CFU/ml of *L. grayi* or *E. coli* K12. The plates were incubated at 37°C for 24 -48 h. At the end of that period, the diameter of the clear zone around the disk was measured with a caliper (mm).

3.2.2.3 **Vapor diffusion test**

Disks (6.5 mm diameter) were sterilized by ultraviolet light for 15 min prior to use. BHIA and MHA plates were seeded with 100 µl of standardized inoculum containing 10^6 CFU/ml of *L. grayi* or *E. coli* K12. Then a disk impregnated with 5 µl of EO was laid on the inside surface of the upper lid of the Petri dish, with no direct contact between the disk and the plated bacteria. The plate was then sealed using Parafilm to prevent leakage of essential oil vapor and incubated at 38 ºC for 24-48 h. The essential oils were replaced by an equal amount of saline in the control group. The diameter of the zone free of bacterial growth was measured in millimeters (zone of inhibition).

3.2.2.4 **Estimation of minimum inhibitory concentrations (MIC) of EOs**

The broth microdilution method in National Committee of Clinical Laboratory standards (CLSI, 2006; Jiang *et al*., 2011) was used to evaluate the MIC and MBC of the various EOs. Serial doubling dilutions of various essential oil solutions were prepared in a 96-well plate in the range of 0.313-10 µl/ml. Tween 80 (5%) was used to emulsify the essential oil in the distilled water. Bacterial suspensions (*L. grayi* and *E. coli* K12) were prepared in the same way as described in Section 3.2.2.1. Bacterial suspensions were diluted in brain heart infusion (BHI) or Mueller Hinton (MH) broth to yield an approximate inoculum size of 5×10^5

CFU/ml, of which 100 µl was added to each well to a density of 3×10^5 CFU $well^{-1}$. The essential oil solution, bacterial suspensions and BHI or MH broth alone were added as control. Plates were incubated at 37 °C for 24 h, and optical density values were determined at 600 nm every hour using a Bio-Rad Microplate Reader. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of EOs at which no visible growth of microorganism was observed after 24 h. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of EOs at which inoculated bacteria were completely killed. To determine the MBC, 10 µL of medium taken from microplates was reinoculated on agar plates for 48 h at 37 °C.

3.2.2.5 **Antimicrobial activities of essential oils against pathogenic strains of *Listeria* and *Escherichia coli***

Based on the results of the tests conducted with *L. grayi* and *E. coli* K12, carvacrol, ORE-03, CIN-03 and AIL-03 were chosen to determine the antimicrobial activities against two strains of *L. monocytogenes* (strains 519 and 523), *E. coli* O157:H7 and *E. coli* O26:H11. Disk and vapor diffusion tests were used to evaluate the antimicrobial activities of these essential oils and compare the susceptibilities of the different strains of *Listeria* and *E. coli.* All the procedures were the same as described above, except that three different volumes of the EO (2, 5, and 8 µl) were placed on the disk. The diameter of the inhibition zone was measured after 24 h and 48 h. These experiments were done in the microbiology laboratory at Health Canada in Longueuil, Quebec. The Health Canada reference numbers of *L. monocytogenes* 519 and *L. monocytogenes* 523 were HPB 2989 and HPB 2993, and they had been isolated from naturally contaminated food samples.

3.2.2.6 **Estimation of minimum inhibitory doses (MID) of EOs in the vapor phase**

The MIC vapor diffusion test described by Lopez *et al*. (2005) was used with slight modification. Solidified medium was inoculated with 100 µl of a bacterial suspension containing 10^5 CFU/ml of the microorganism under study. Different volumes of each test substance (carvacrol or essential oil) were added to 90-mm-diameter sterile blank filter paper and placed in the lid of the Petri dish. The Petri dishes were then sealed using sterile adhesive tape (Deltalab, Rubi, Spain). No hermetic sealing was needed because experiments were designed to simulate a worst-case situation, where leaking of the active components into the atmosphere can occur. Blanks were prepared by adding 10 µl of sterile saline to the filter

paper, which was demonstrated to have no effect on the viability of any of the tested bacteria. The plates were incubated at 37 °C for 24-48 h and the analyses were carried out in triplicate.

The concentration of essential oil was expressed as volume of EO per unit volume of headspace (µl/l), dividing the volume of EO added to the filter paper by the volume of the headspace in the Petri dish (µl/l). The tested concentrations varied from 120 to 556 µl/l of headspace of the Petri dish. The effectiveness of the essential oil was calculated by measuring the diameter (mm) of the inhibition zone above the filter paper. Inhibition (%) was expressed as T/C × 100, where T is the diameter of the inhibition zone and C is the diameter of the petri dish (90 mm). The minimum inhibitory doses (MID) was defined as the lowest essential oil concentration resulting in the lack of visible microbial growth.

3.2.3 **Storage of vegetables with EOs**

Fresh potatoes and frozen green peppers were supplied by a frozen vegetables company in Montreal. The fresh-cut potatoes were weighed out in 8.5±0.3 g portions, and then sterilized under ultraviolet light with ethanol for 20 min. An inoculum (60 µl) containing 10^3-10^4 CFU/ml of *L. grayi* was spot applied to 10 random locations on the surface of the potatoes and red peppers. The inoculated vegetables were dried in a laminar flow hood for 10 min before storage. The dry fresh-cut potatoes were sprayed with 1% carvacrol in ethanol and dried for 5 min to evaporate the ethanol before being placed into glass jars. For the storage tests with green peppers, 80-g portions of the frozen samples, which had already been cut into small pieces, were weighed into sterilized stomacher bags while they were still frozen. An inoculum of *L. grayi* was spread over the sample surface with a pipette, and the bags were then shaken for 1 min to make the distribution of the inoculated bacteria uniform. The initial inoculation level was approximately 10^2 -10^3CFU/g. The samples were then randomly separated into control and treatment groups placed in Petri dishes. Frozen broccoli was taken and left under UV light for 20 min to thaw and remove any competing microflora. The samples were dipped into the bacteria suspension (10^2-10^3CFU/ml) of *L. grayi* for 15 s and dried in the hood for 30 min. For samples in the treatment group, a filter paper disk impregnated with carvacrol (242 µl/l of headspace), oregano-03 (360 µl/l of headspace), or cinnamon-03 essential oil (CIN-03; 556 or 702 µl/l of headspace) was placed on the lid of the Petri dish or one side of the 1-L glass jar without touching the sample. Petri dishes and glass jars were sealed with Parafilm to prevent leakage and incubated at 22 °C (room temperature) for up to 1 week (Kwon *et al.*, 2017; Kataoka *et al.*, 2017).

3.2.4 **Microbial analysis of samples during storage**

The total number of tested bacteria was evaluated periodically throughout the storage period. Treated and untreated samples were subjected to microbial analysis during storage at the initial point (day 0) and at days 1, 2, 3, 4 and 5. Under sterile conditions, each sample was diluted in sterilized saline solution (0.9 % NaCl w/v) and shaken for 3 min. Serial dilutions of each suspension were prepared and plated on Petri dishes containing BHI agar or Listeria-selective agar (LSA). The Petri plates were incubated for 48 h at 37 °C (Jovanovic *et al*., 2016). Microbiological counts were converted to log CFU/g.

3.2.5 **Sensory evaluation of vegetables packaged with oregano and cinnamon EOs**

Frozen broccoli and french fried potatoes were provided by a frozen vegetable processor in Quebec and two stickers (filter paper) containing 275 or 556 µL/L of CIN-03 and 90 or 180 µL/L of ORE-03 were attached to the inside of the plastic packaging without making contact with the broccoli or the french fries. Vegetables packaged with empty stickers served as the controls. All the vegetables were stored at 4 °C for 1 day before the sensory evaluation. The broccoli samples were removed from the package and steamed for at least 10 min. The french fried potatoes were warmed in an oven for 30 min.

Two 5-point category difference (No difference, slightly different, moderately different, very different, and extremely different) and hedonic liking test (extremely worse than the control, moderately worse than the control, no difference, moderately better than the control, extremely better than the control) were designed for evaluation of organoleptic properties by 43 panelists, who were McGill University Food Science graduate and undergraduate students Compusense software (Compusense Inc., Guelph, ON, Canada) was used to analyze the sensory attributes. The panelists were considered to be semi-trained and familiarized with the attributes of the overall difference and overall liking prior and during testing.

3.2.6 **Tests of an aerosolization method**

3.2.6.1 ***In vitro*** **assessment of antimicrobial activity of aerosolized essential oils against *L. grayi* and *E. coli* K12**

An ultrasonic aroma diffuser (Spherebrook Laboratory Inc., Canada; specifications: humidification 25 ml/h (+/-5 ml), power 2.4 million vibrations/second, and diffusing capacity up to 350 ft^2) was employed in these experiments to produce aerosolized essential oils. BHI agar and MHA plates were inoculated with *L. grayi* or *E. coli* K12 (10^2 and 10^3 CFU/ml) and placed in a container (28.4 L) connected to the aroma diffuser, which had been filled with

carvacrol, oregano EO, or cinnamon EO. The container was then sealed, and the aroma diffuser was switched on (time point zero). Plates were taken out at 15, 30, 45 and 60 min and then hourly for up to 3 h. The controls were left in a biosafety cabinet for the same amount of time. Following incubation for 24 h at 37 °C, the number of colonies on the treated plates was ratioed against the number of colonies on the control plates and multiplied by 100 to obtain percent viability.

In a subsequent set of experiments, the aerosolized essential oil generated by the aroma diffuser was accumulated in the sealed container for a specified amount of time, following which inoculated agar plates prepared as described above were placed in the container. Plates were removed at different time points (1, 5, 10 and 15 min) and incubated for 24 h at 37 °C. The controls were prepared, and percent viability determined as described above.

3.2.6.2 **Preliminary tests of vegetables inoculated with *L. grayi* exposed to aerosolized oregano essential oil**

Frozen broccoli was supplied by a frozen vegetables company in Montreal. Frozen vegetables were thawed under UV light in a biosafety cabinet for 20 min to remove any competing microflora. The samples were dipped into a suspension of *L. grayi* (10^2 CFU/ml) for 15 s and dried in the biosafety cabinet for 30 min. The inoculated samples were moved into the treatment chamber and exposed to aerosolized oregano EO from the aroma diffuser at 23 °C. Treated vegetables were taken out at 15, 30 and 45 min after a 60-min accumulation of aerosolized EO in the chamber. The controls were left in a biosafety cabinet for the same amount of time.

To investigate the effectiveness of treatment of vegetables with aerosolized EO before storage, the total count of *Listeria* on vegetables was determined immediately after treatment. Vegetable samples exposed to aerosolized EO for different amounts of time were placed in sterilized saline solution (0.9% NaCl w/v) and stomached for 5 min, and the total number of *Listeria* in each diluted suspension was determined by growth on listeria selective agar. The Petri plates were incubated for 48 h at 37 °C. To determine the effectiveness of aerosolized EO on vegetables after storage, samples were prepared and exposed to aerosolized oregano EO in the same way as described previously. The total population of *Listeria* on vegetable samples exposed for different amounts of time was then determined after 8 days storage at 4 ºC and compared with the population on control samples. Microbiological counts were converted to log CFU/g.

3.2.7 **Preliminary storage tests of vegetables inoculated with *L. grayi* and treated with aerosolized oregano EO**

Frozen vegetable samples were prepared and inoculated with *L. grayi* as described previously and exposed to aerosolized oregano-03 EO for 45 min after 60-min accumulation of the aerosolized EO within the sealed container. Following treatment, the samples were randomly moved to several sterilized bags and sealed inside of bags. Then, all treated samples and untreated controls were stored at 4 ºC. Treated and untreated samples were subjected to microbial analysis at the initial point (day 0) and on days 4, 8, 12, 15 and 20 of the storage periods. Under sterile conditions, each sample was diluted in sterilized saline solution (0.9 % NaCl w/v) and shaken for 5 min. Serial dilutions of each suspension were prepared and plated on Petri dishes containing Listeria selective agar. The Petri plates were incubated for 48 h at 37 °C. Microbiological counts were converted to log CFU/g.

3.2.8 **Fourier transform infrared (FTIR) spectroscopy of essential oils and pure compounds**

Fourier transform infrared (FTIR) spectra were recorded in the range between 4000 and 400 cm^{-1} using an Excalibur FTIR spectrometer (Agilent Technologies, Santa Clara, CA), equipped with a single-bounce diamond attenuated total reflectance (ATR) accessory (Specac, Orpington, UK). After obtaining the background of the clean ATR crystal with 128 scans, around 100 µL of the sample essential oil or carvacrol were deposited on the surface of the ATR crystal and their spectra scanned at a resolution of 4 cm^{-1} with co-addition of 64 scans. Spectra were analyzed using Omnic software (Thermo Electron Corp.). Dendrograms were generated by hierarchical cluster analysis using SpectrAnalysis software (CogniSolve Inc., Montreal, QC, Canada).

3.2.9 **Statistical analysis**

All experiments were performed in triplicate. The data were expressed as the mean ± SD. One-way analysis of variance (one-way ANOVA) followed by Tukey's test was used to determine statistical significance of differences ($P < 0.05$) between means. SPSS statistics program (Version 24) was applied for the statistical analysis.

3.3 Results and discussion

3.3.1 Evaluation of antibacterial activities of essential oils

3.3.1.1 Standard curve of optical density measurements

The relationship between the optical density of bacterial suspensions and bacterial counts were shown in **Fig. 3.1**. We found that the total count of *L. grayi* was about 7×10^6 CFU/mL and that of *E. coli* K12 was around 5×10^7 -7×10^7 CFU/mL after 20-22 h incubation, showing that *L. grayi* grew much more slowly than *E. coli* K12 during the same incubation period. By using the linear regression equations on **Fig. 3.1**, we can easily calculate the total count of bacteria in a suspension by measurement of OD.

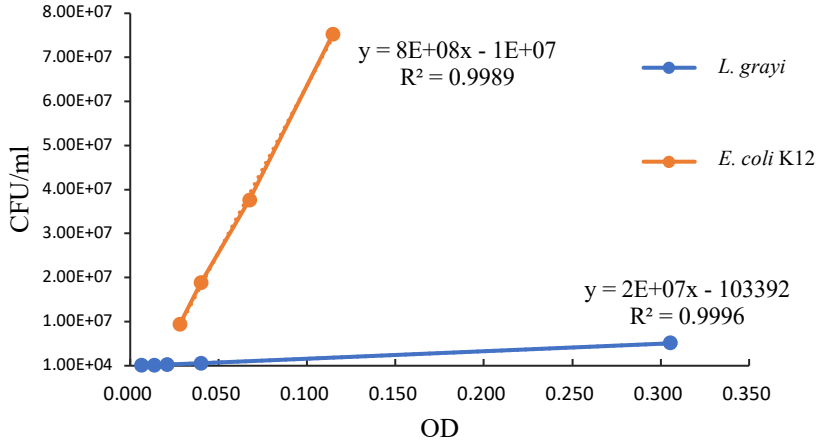
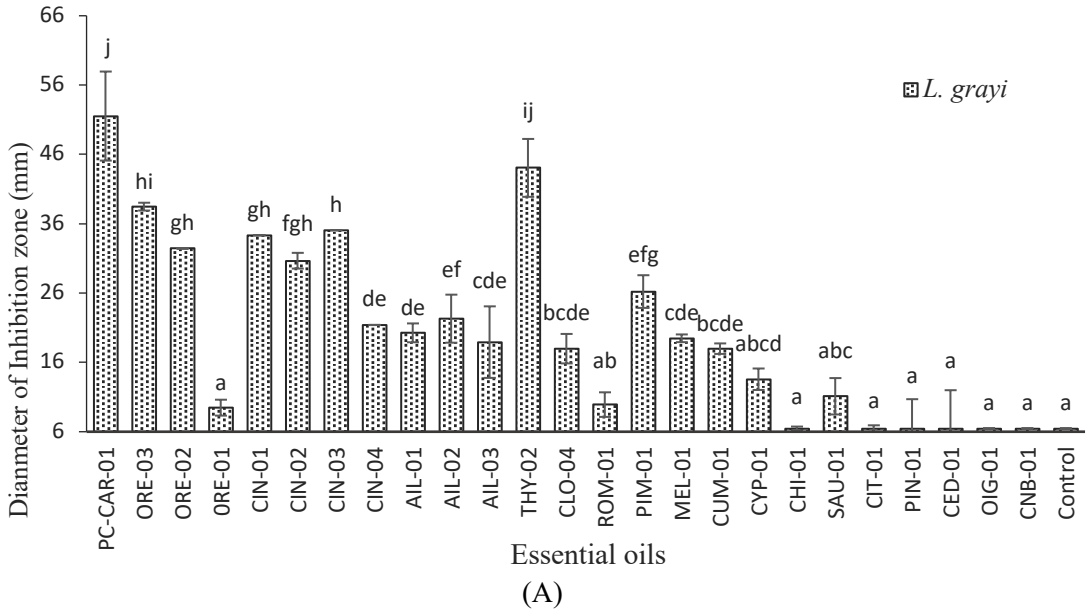


Figure 3.1 Standard curves for *L. grayi* and *E. coli* K12**.**

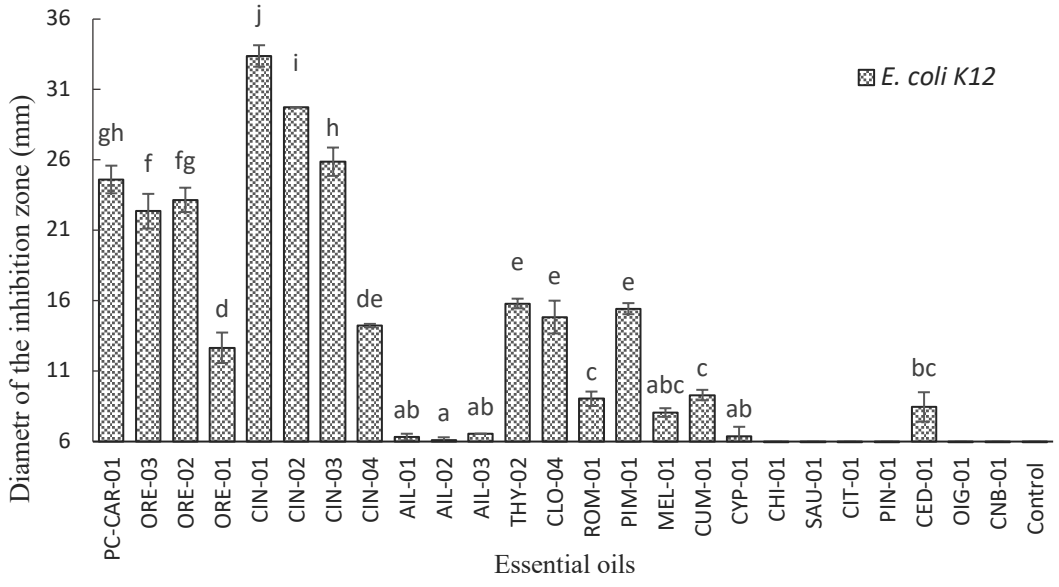
3.3.1.2 Antibacterial activities of essential oils by disk diffusion test

The antimicrobial activities of various EOs and carvacrol (PE-CAR-01) against *L. grayi* and *E. coli* K12 were evaluated by disk diffusion tests. In each test, a disk impregnated with 5 µl of the test substance was placed on an agar plate that had been inoculated with 100 µl of a 10^6 CFU/ml suspension of *L. grayi* or *E. coli* K12. **Fig. 3.2** (A) shows the diameter of the inhibition zone measured after the incubation period in the tests with *L. grayi,* and different essential oils and the pure active compounds exhibited the difference anitimicrobial activities against *L. grayi* by size of inhibition zone. The pure active compound carvacrol gave the highest inhibitory effect against *L. grayi*, while among the essential oils tested thyme EO (THY-02) was the most effective in inhibiting *L. grayi*. In addition, the antimicrobial activities of cinnamon (CIN-01, CIN-02, CIN-03) and oregano (ORE-02, ORE-03) EOs were

significantly higher than those of the other essential oils ($P \leq 0.05$), some of which showed no inhibition zone, including citron, PRA, chili, cedar, cranberry seed, and concentrated onion. In the case of chili EO, we found that there were bacteria or yeast growing besides *L. grayi* on the BHI agar (**Fig. 3.3**). It was confirmed that microbial contamination was present in the chili oil, and accordingly this oil was not employed in the subsequent work.



(A)



(B)

Figure 3.2 Diameter of inhibition zone in disk diffusion tests measuring inhibitory activities of carvacrol (PC-CAR-01) and various EOs against *L. grayi* (A) and *E. coli* K12 (B). (Different letters represent significant differences, $P \leq 0.05$).

In the case of *E. coli* K12 (**Fig. 3.2** (B)), the results of the disk diffusion tests indicated that the antimicrobial activities of two cinnamon EOs (CIN-01, CIN-02) were significantly higher ($P \leq 0.05$) than those of carvacrol and the other EOs. A third cinnamon EO (CIN-03), carvacrol (PC-CAR-01) and two oregano EOs (ORE-02, ORE-03) ranked after CIN-01 and CIN-02 followed by thyme EO (THY-02), clove EO (CLO-04) and piment EO (PIM-01). While sage EO had inhibitory activity against *L. grayi*, it did not inhibit growth of *E. coli* K12. Garlic EOs (AIL-01, AIL-02, AIL-03) also showed quite low inhibitory activity against *E. coli* K12 in comparison with their activity against *L. grayi*. EOs that showed no inhibition against *L. grayi*, such as citron, chili, cedar, cranberry seed, and concentrated onion, also did not inhibit the growth of *E. coli* K12.

Overall, based on comparison of the diameters of the inhibition zone, *L. grayi* was more susceptible to EOs than *E. coli* K12. Farag *et al* (1989) and Smith-Palmer (1998) also found that Gram-positive bacteria were more sensitive to inhibition by plant essential oils than Gram-negative bacteria. Studies aimed at elucidating mechanisms of action revealed that both cinnamon and oregano EOs affected membrane integrity in a Gram-positive bacterium (*L. monocytogenes*) and induced depletion of the intracellular ATP concentration. Furthermore, the intracellular pH was decreased by these two EOs. Taken together, these findings indicated that the toxic action of both EOs involved the cytoplasmic membrane (Oussalah *et al*., 2006). On the other hand, in the case of Gram-negative bacteria, differences in the mechanisms of action of carvacrol and cinnamaldehyde (active compounds in oregano and cinnamon EO, respectively) have been described by others. Carvacrol disintegrated the outer membrane of Gram-negative bacteria, leading to increased permeability of the cytoplasmic membrane to ATP and release of lipopolysaccharides (LPS) (Burt, 2004). At similar concentrations, cinnamaldehyde also inhibited the growth of *S. typhimurium* and *E. coli O157:H7*, but it did not disintegrate the outer membrane of the bacterial cells or deplete the intracellular ATP pool (Burt, 2004). In this context, it may be noted that in the present study cinnamon (CIN-01, CIN-02, CIN-03) and oregano (ORE-02, ORE-03) EOs had comparable activities against *L. grayi*, consistent with a similar mechanism of action, whereas the same cinnamon EOs were significantly more effective than the two oregano EOs in inhibiting the growth of *E. coli* K12, suggesting that the active compounds in these two types of EO differ in their mechanism of action against this Gram-negative bacterium.

Finally, the results presented in **Fig. 3.2** demonstrate substantial variability among EOs of the same type from different companies. For example, all four cinnamon EOs differed in their

antimicrobial activities against *L. grayi* and *E. coli* K12, especially CIN-04, which had low efficacy in inhibiting growth of the tested strains. Similarly, among the three oregano EOs included in this study, ORE-01 showed very low antimicrobial activities against both *L. grayi* and *E. coli* K12 whereas ORE-02 and ORE-03 produced significantly larger inhibition zones. These findings may be attributed to differences in chemical composition among EOs of the same type and could most simply be explained in terms of differences in the concentrations of the major active compounds, i.e., cinnamaldehyde in cinnamon EO and carvacrol and thymol in oregano EO. As described in the following section, these differences were examined by FTIR spectroscopy.

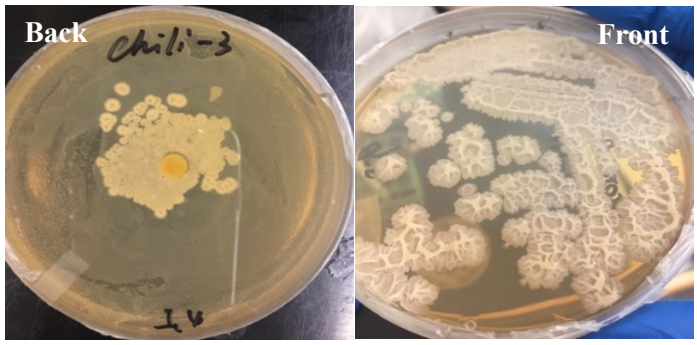
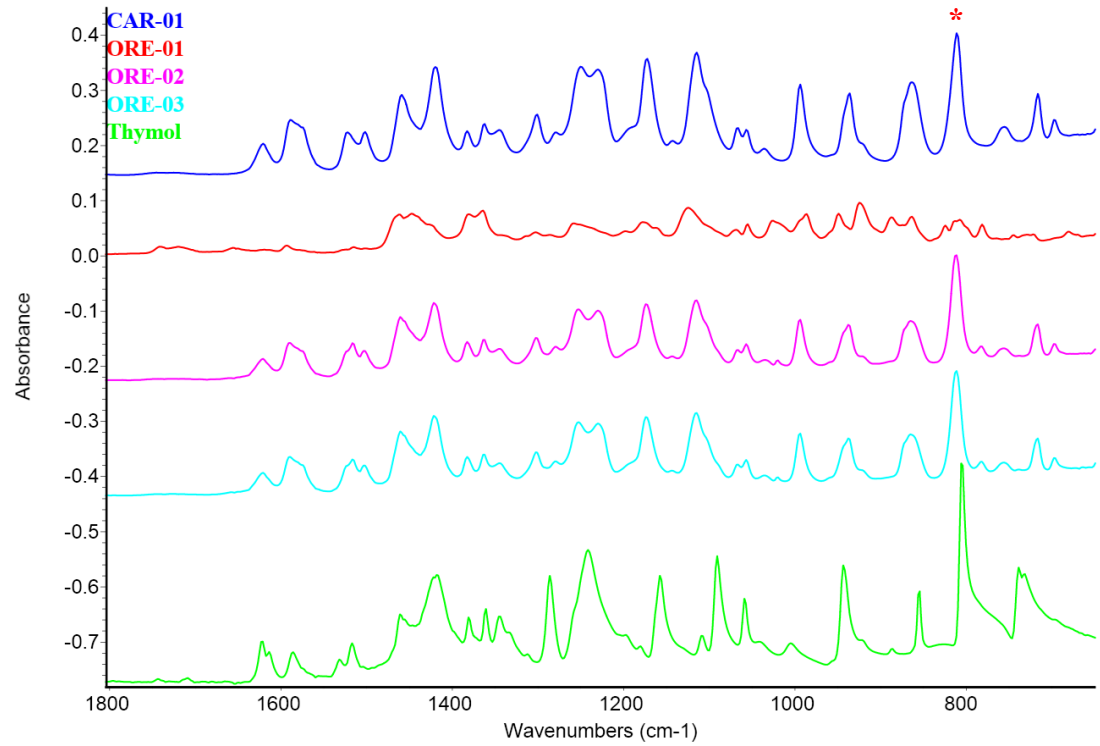


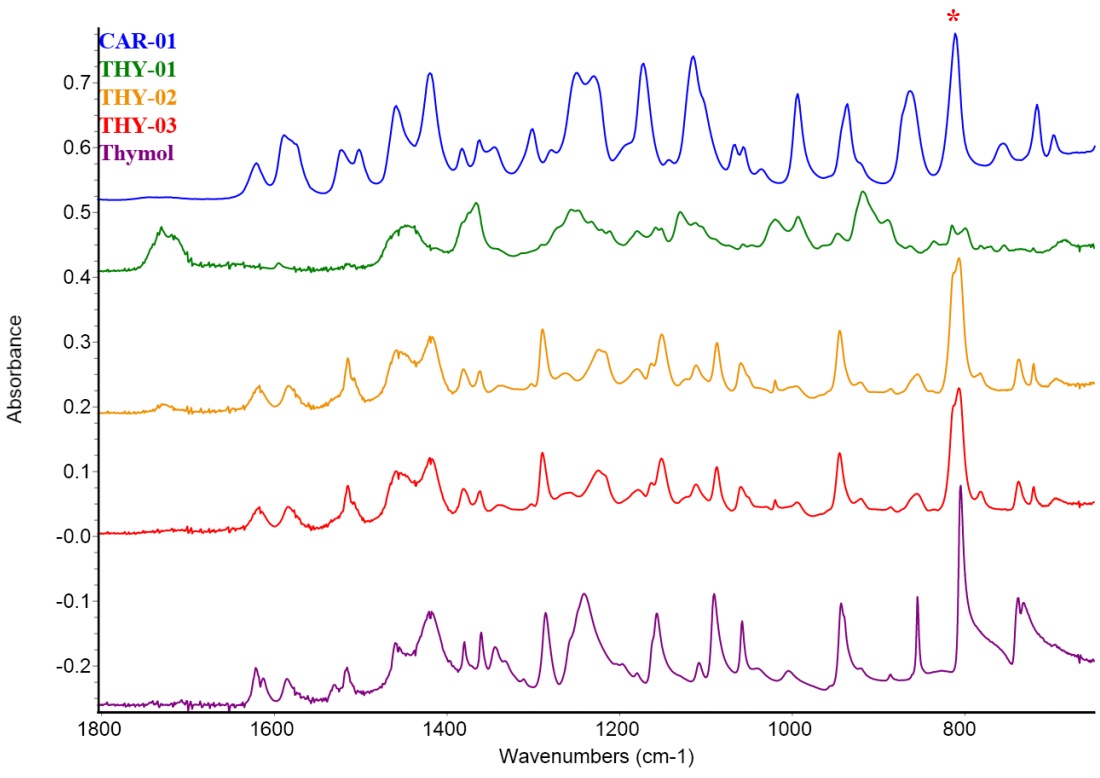
Figure 3.3 The microbe isolated from chili essential oil.

3.3.1.3 **FTIR spectroscopic analysis of essential oils**

From the research of Topala and Tataru (2016), isomeric compounds like thymol and carvacrol showed significant differences in ATR-IR spectra. These authors reported that the most intense bands in the spectra of thymol and carvacrol are observed at 804 cm^{-1} and 811 cm^{-1}, respectively. These bands are assigned to out-of-plane CH wagging vibrations, which are the most significant signals used in distinguishing different types of aromatic ring substitution (Topala and Tataru, 2016). Accordingly, we used the bands at 811 cm^{-1} and 804 cm^{-1} as the characteristic key peaks to identify carvacrol and thymol. The FTIR spectra of three thyme EOs and three oregano EOs together with those of carvacrol and thymol are shown in **Fig. 3.4**. We found that the spectra of carvacrol and thymol had intense peaks in the wavenumber range reported by Topala and Tataru (2016). Two of the oregano EOs (ORE-02, ORE-03) and two of the thyme EOs (THY-02, THY-03) also showed a strong peak in this range, indicating that the content of carvacrol and thymol was high in these EOs. However, no distinct peak was observed in this region in the spectra of ORE-01 and THY-01. These results indicate that the content of carvacrol and thymol in ORE-01`was low, which accounts for the low antimicrobial activity of this EO in **Fig. 3.2**. Russo *et al*. (1998) studied 24 steam-distilled essential oils from Italian oregano spices (*Origanum vulgare* ssp.) and found that the concentrations of carvacrol and thymol varied widely (0.3%-56% and 8-53%, respectively). In the research of Figuérédo *et al*. (2006), representative samples of nine oregano species were analyzed by gas chromatography-mass spectrometry and were found to be composed mainly of carvacrol (55.9-86.1%), the amount of thymol being less than 1%. For *Thymus* species, Ozcan and Chalchat (2004) found thymol (46.2%) to be dominant in thyme from Turkey. In addition, thymol was the main component (19%-54%) in the thyme essential oils analyzed by Hudaib *et al*. (2002) but was present in low concentration (0.24%) in those analyzed by Imelouane *et al*. (2009). In addition to variations among plant species, the amounts of carvacrol and thymol in wild oregano plants were reported to fluctuate from one season to another (thymol: 149.2–1124.4 mg/100 g; carvacrol: 51.6–564.3 mg/100 g), and the seasons in which the plants were collected also affected the qualitative and quantitative composition of the extracted EOs (Jerković *et al*., 2001).



(A)



(B)

Figure 3.4 FTIR spectra of oregano (A) and thyme (B) EOs and their pure active compounds.

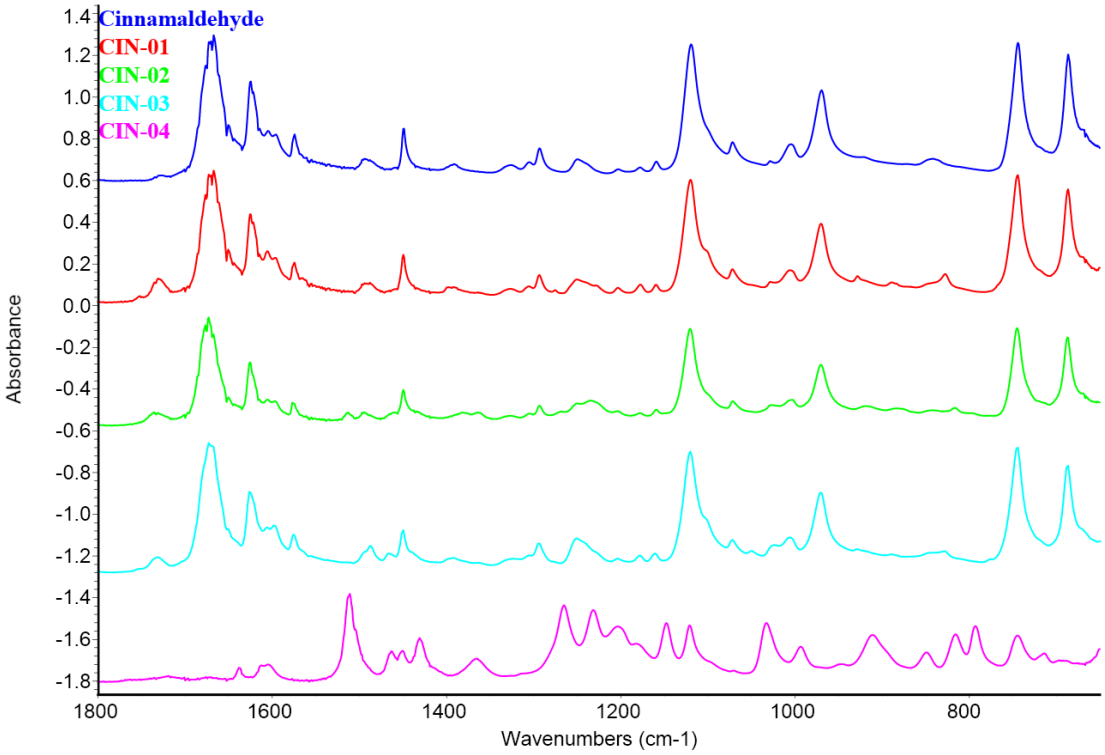


Figure 3.5 FTIR spectra of cinnamon essential oils and cinnamaldehyde.

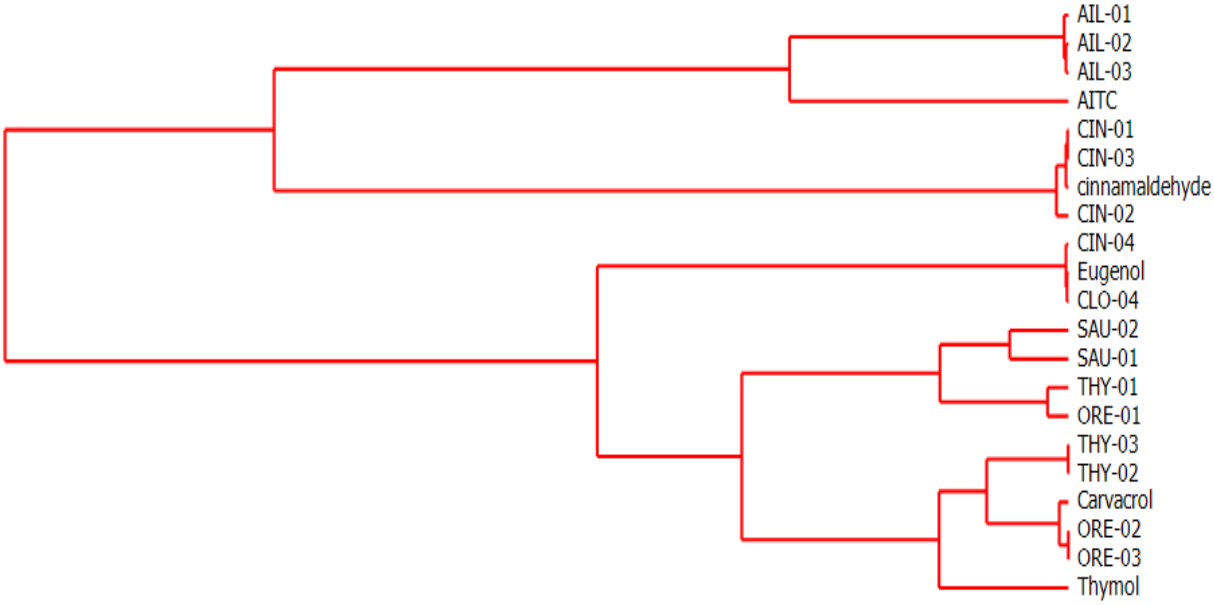


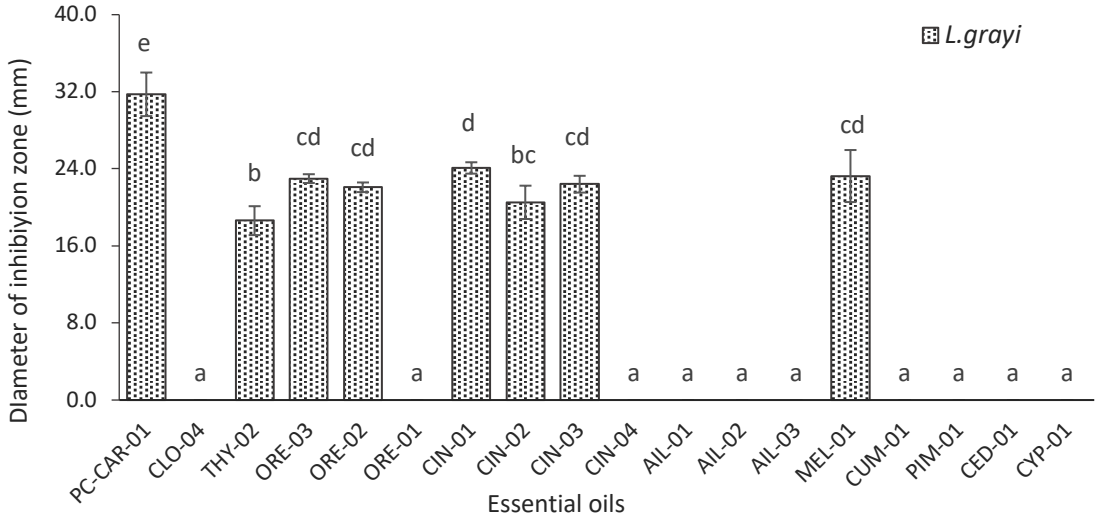
Figure 3.6 Dendrogram obtained by hierarchical cluster analysis of FTIR spectra of 16 essential oils and 5 key components of essential oils.

The FTIR spectra of cinnamon essential oils and cinnamaldehyde were shown in **Fig. 3.5**. Cinnamon EOs are extracted from the bark of *Cinnamomum cassia* Blume, and cinnamaldehyde is the predominant compound in cinnamon EOs, at levels ranging from 90% to 62%–73% (Nabavi *et al*., 2015). The FTIR spectrum of cinnamaldehyde exhibits a peak at 1683 cm^{-1} ascribed to the stretching vibration of the C=O bond (Demitri *et al*., 2016). This characteristic band is not observed in the spectrum of CIN-04. The spectra of the other cinnamon EOs tested (CIN-01, CIN-02, CIN-03) are quite similar to the spectrum of cinnamaldehyde, indicating that the concentration of cinnamaldehyde in these cinnamon EOs is high. The FTIR spectroscopic evidence is consistent with the results of the disk diffusion tests presented in **Fig. 3.2**, which show that the antimicrobial activity of CIN-04 was significantly lower than that of the other three cinnamon EOs.

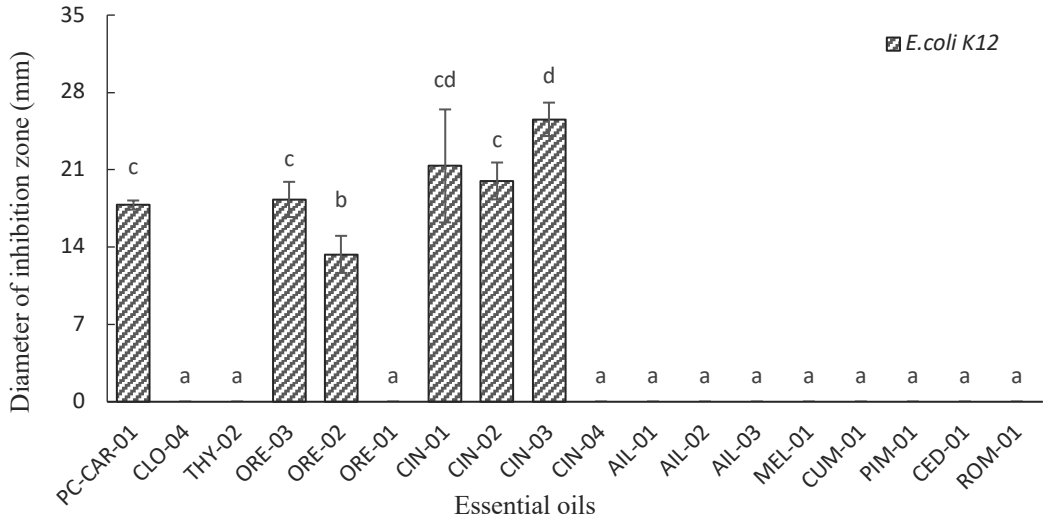
Hierarchical cluster analysis of the FTIR spectral data acquired for the 16 EOs as well as the active compounds carvacrol, thymol, eugenol, cinnamaldehyde and allyl isothiocyanate (AITC), all analyzed in their neat form, was then used to obtain the dendrogram presented in **Fig. 3.6,** showing the grouping of these samples based on overall spectral similarity. From **Fig. 3.6**, it can be seen that two of the oregano EOs (ORE-02, ORE-03) grouped with carvacrol, indicating that they contained a large amount of this compound. Moreover, two thyme EOs (THY-02, THY-03) were close to this cluster, which may indicate that these EOs also contained larger amounts of carvacrol than of thymol, which is in an adjacent arm of the dendrogram. The positions of ORE-01 and THY-01 in the dendrogram indicate that they have a different spectral profile from the other oregano and thyme EOs, as seen in **Fig. 3.4**. Similarly, CIN-04 is completely separated from the other three cinnamon EOs, which cluster together with cinnamaldehyde. CIN-04 clusters with CLO-04 and eugenol, which is the most abundant compound in clove EOs and cinnamon leaf EOs. At the top of the dendrogram, the three garlic essential oils (AIL-01, AIL-02, AIL-03) are seen to form a separate cluster, showing that they are spectrally similar to each other.

3.3.1.4 **Antibacterial activities of essential oils by vapor diffusion test**

The experimental protocol for the vapor diffusion tests differed from that for the disk diffusion tests in only two respects. First, there was no direct contact between the filter paper disk on which the test substance had been applied and the agar plate. Second, the inhibition zone was determined by measuring the diameter of the area on the agar plate that was free of bacterial growth after 48-h incubation, rather than the clear inhibition zone around the disk in the disk diffusion test. From **Fig. 3.7**(A), these EOs showed different antimicrobial effect on growth of *L. grayi*. In addition, pure carvacrol had significantly higher activity against *L. grayi* than the EOs tested ($P \leq 0.05$). The antimicrobial activities of cinnamon (CIN-01, CIN-02, CIN-03), oregano (ORE-02, ORE-03) EOs, and lemon balm (MEL-01) EOs were significantly higher than those of the other essential oils ($P \leq 0.05$), including the EO (THY-02) that had the highest activity in the disk diffusion tests. However, in the case of the lemon balm (MEL-01) EO and garlic (AIL-02, AIL-03) EOs (not shown), the zone of inhibition became smaller or totally disappeared after 48 hours of incubation. The same phenomenon was observed with AITC, which is the main component of mustard essential oil; the zone of inhibition was large after 24 h of incubation, but the bacteria then began to grow, and only a very small zone of inhibition was observed after 48 h of incubation, demonstrating that *L. grayi* survived the exposure to AITC. Among the three oregano and four cinnamon EOs tested, ORE-01 and CIN-04 showed no inhibitory effect on the growth of *L. grayi,* indicating that the concentrations of volatile antimicrobial compounds in these EOs were lower than in the other oregano and cinnamon EOs. Clove (CLO-04), piment (PIM-01), cumin seed (CUM-01) and cypress (CYP-01) EOs also had no antimicrobial activity against *L. grayi* in the vapor diffusion test, even though piment (PIM-01) had worked well in the disk diffusion test.



(A)



(B)

Figure 3.7 Effects of the various EOs in vapor phase on *L. grayi* (A) and *E. coli* K12 (B) after 48 h. (Different letters represent significant differences, $P \leq 0.05$)

In the case of *E. coli* K12, **Fig. 3.7** (B) shows that the antimicrobial activity of one of the cinnamon EOs (CIN-03) was significantly higher than that of carvacrol and the other EOs ($P \leq 0.05$). Two other cinnamon EOs (CIN-01, CIN-02) and one of the oregano EOs (ORE-03) were also very effective in preventing the growth of *E. coli* K12 in the vapor diffusion test, and the results obtained for these three EOs and for pure carvacrol were not statistically different ($P > 0.05$). In contrast, while thyme EO (THY-02) showed antimicrobial activity against *E. coli* K12 in the disk diffusion test, it did not form a clear zone of inhibition in the vapor diffusion test, as there were some colonies of *E. coli* growing in the inhibition zone. Overall, the results of the vapor diffusion and disk diffusion tests followed similar trends,

with the inhibitory effects of EOs in the vapor phase being weaker than those induced by direct contact. The antimicrobial effects in the disk diffusion test include contributions from the activities of the hydrophilic and less volatile components, whereas the antimicrobial effects in the vapor diffusion test are due to an equilibrium concentration of both hydrophilic and hydrophobic volatile substances released into the headspace of the sealed Petri dish and partially adsorbed on the agar surface. Since the active compounds are easily lost by evaporation in the disk diffusion test and their apolar properties retard their diffusion through the media, the disk diffusion test is considered to be less suitable than the vapor diffusion test in evaluating the antimicrobial activity of essential oils (Kalemba & Kunicka, 2003; Kubo *et al*., 1995; Goni *et al*., 2009).

3.3.1.5 **Antimicrobial activities of pure active compounds against *L. grayi* in disk diffusion and vapor diffusion tests**

Disk diffusion and vapor diffusion tests were also performed with four pure compounds that are present in essential oils, including oregano, thyme, cinnamon, clove, and mustard EOs. Two phenolic compounds (carvacrol and eugenol), cinnamaldehyde, and allyl isothiocyanate (AITC), an aliphatic substance present in high concentrations in essential oils from black (*Brassica nigra*) and brown mustards *(Brassica juncea*) (Otoni *et al*, 2016; Nielsen & Rios, 2000), were tested. As shown in **Fig. 3.8**, in the disk diffusion test with *L. grayi*, carvacrol exhibited the highest antimicrobial activity among the compounds tested, followed by cinnamaldehyde and eugenol. However, in the vapor diffusion test, eugenol did not produce a zone of inhibition, which is consistent with the results obtained for clove EO (in which eugenol is the predominant active compound) in the disk diffusion and vapor diffusion tests shown in **Figs. 3.3** & **3.7**. In the case of AITC, no growth of *L. grayi* in the disk diffusion test was discernible after 24 hours of incubation, but the bacteria began to grow in the following 24 hours and the inhibition zone had almost disappeared after 48 hours of incubation. In the vapor diffusion test, AITC was not effective in inhibiting the growth of *L. grayi*. It has been reported in the literature that Gram-positive bacteria are more resistant to AITC vapor than Gram-negative bacteria (Isshiki *et al*., 1992). Lin *et al*. (2000) found that *E. coli* O157:H7 was more susceptible to AITC than *L. monocytogenes*. Rhee *et al*. (2003) reported that *L. monocytogenes* survived longer than *E. coli* O157:H7 under treatment with mustard and acetic acid at 22 ^{o}C, and a small population of *L. monocytogenes* was even recovered after 12 h.

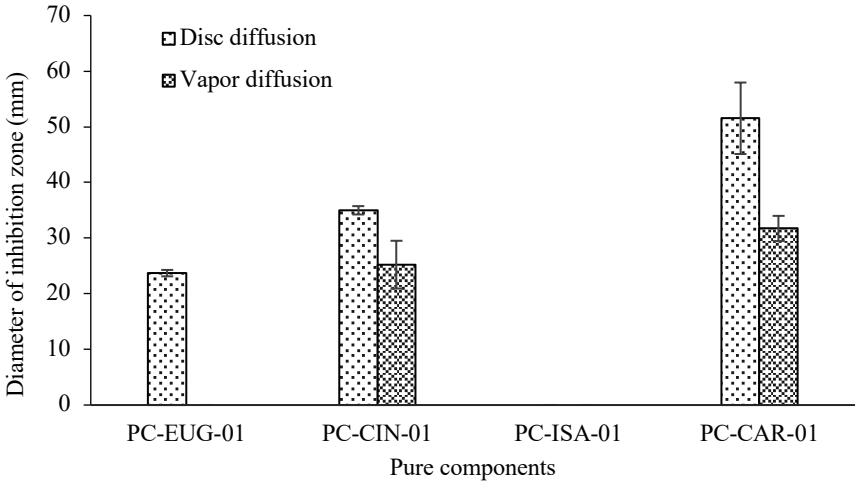


Figure 3.8 Effects of four pure active compounds in direct contact and in the vapor phase on the growth of *L. grayi*. The compounds tested are (from left to right) eugenol, cinnamaldehyde, allyl isothiocyanate, and carvacrol.

3.3.1.6 **Minimum inhibitory concentrations (MIC) of essential oils**

The minimum inhibitory concentration (MIC) is used by many researchers as a measure of the antimicrobial performance of EOs. The definitions of MIC are different between publications. In our experiments, the definition of MIC we used is the lowest concentration that inhibits the visible growth of the test organism (Delaquis *et al*., 2002). The MICs and MBCs of various EOs and carvacrol against *L. grayi* and *E. coli* K12 were determined by the broth microdilution method (**Table 3.1**) in 96-well plates. The lowest MIC of CIN-01, CIN-03 and AIL-03 (*L. grayi* only) against *L. grayi* and *E. coli* K12 were observed as 0.625 µl/ml in EOs, which indicated the best effect of CIN-03 on inhibiting the growth of *L. grayi* and *E. coli* K12. As the higher antimicrobial activities of ORE-03, CIN-02, carvacrol, AIL-02 and AIL-03 in disk and vapor diffusion tests, 1.250 µl/ml of MIC of these EOs against growth of *L. grayi* and *E. coli* K12 were ranked following the top three EOs with exception to AIL-02& 03 to *E. coli* K12. However, the MIC of carvacrol was slightly higher than some cinnamon essential oils, indicating the slight decrease of antimicrobial ability of carvacrol over time, corresponding to the behavior of carvacrol in the disk and vapor diffusion test against different *Listeria* species. Besides, garlic (AIL) EOs showed the same high antilisterial effect after 24 h as the total inhibition on *Listeria* happened in previous test, while the minimum bactericidal concentration (BMC) were quite higher than other EOs and *L. grayi* could be recovered from almost all wells, thereby, antilisterial effective duration of bacteriostatic garlic EOs were only 24 h.

Table 3.1 Estimated minimum inhibitory concentrations (MICs) of 24 EOs and carvacrol against *L. grayi* and *E. coli* K12 in broth microdilution method.

EOs	*L. grayi*		*E. coli* K12	
	MIC (µl·ml^{-1})	BMC (µl·ml^{-1})	MIC (µl·ml^{-1})	BMC (µl·ml^{-1})
CIN-03	0.625	1.250	0.625	1.250
CIN-01	0.625	1.250	0.625	1.250
AIL-03	0.625	>10.000	>10.000	>10.000
ORE-03	1.250	1.250	1.250	5.000
PC-CAR-01	1.250	2.500	1.250	5.000
CIN-02	1.250	2.500	1.250	2.500
AIL-01	1.250	>10.000	>10.000	>10.000
AIL-02	1.250	>10.000	10.000	>10.000
MEL-01	5.000	5.000	>10.000	>10.000
CIN-04	5.000	10.000	5.000	10.000
PIM-01	5.000	10.000	5.000	10.000
CLO-04	5.000	10.000	5.000	5.000
THY-02	5.000	10.000	10.000	>10.000
ORE-02	5.000	10.000	2.500	10.000
CUM-01	10.000	>10.000	>10.000	>10.000
ORE-01	10.000	>10.000	10.000	>10.000
SAG-01	10.000	>10.000	>10.000	>10.000
ROM-01	>10.000	>10.000	>10.000	>10.000
CED-01	>10.000	>10.000	>10.000	>10.000
CYP-01	>10.000	>10.000	>10.000	>10.000
CIT-01	>10.000	>10.000	>10.000	>10.000
CHI-01	>10.000	>10.000	>10.000	>10.000
PIN-01	>10.000	>10.000	>10.000	>10.000
OIG-01	>10.000	>10.000	>10.000	>10.000
CNB-01	>10.000	>10.000	>10.000	>10.000

The MBC of most of EOs were twice of their MIC, which MBC meant that no bacteria growth was shown on the agar plates inoculated by inoculum from each well from MIC, 2 x MIC or 3 x MIC well. MIC of CIN-04, PIM-01, CLO-04, THY-02, ORE-02 and MEL-01 were 5.000 µl/ml, all EOs exhibited the lower antibacterial effect on *L. grayi* as their behaviors in previous tests, with exception of THY-02 and ORE-02. They showed high antimicrobial abilities against *L. grayi* and *E. coli* K12 in previous tests due to its major

active compounds, including carvacrol or thymol etc., while degradation or gradually weak effect of some components led to its effect decreasing during the storage time. Some alterations of EOs during storage were mentioned in other research. Temperature, light and oxygen in the air can cause conversion reactions in essential oils, such as oxidation, dehydrogenation, isomerization, polymerization and thermal rearrangement (Turek & Stintzing, 2013). Terpenoids as one of the major classes of compounds in EOs were volatile and tended to be thermolabile and be easily hydrolyzed or oxidized due to their structure (Scott, 2005). Some degradation of monoterpenes was observed in rosemary oil, and 10% reduction of α-terpinene occurred within 3 weeks of storage (Turek & Stintzing, 2012). Some thyme EOs were even shown to undergo color alteration with naked eye at elevated temperature (Turek & Stintzing, 2011). However, phenols such as carvacrol and thymol can disrupt and rather retard autoxidative reactions (Treibs, 1960). Thus, efficiency of some oregano and carvacrol were slightly decreased comparing to their fresh stage. At last but not least, the MIC of rest of EOs, such as CED-01, ROM-01, CIT-01, CHI-01, were over 10.000 µl/ml, corresponding to our previous results for these ineffective EOs against *L. grayi* and *E. coli* K12.

Other researchers have used the same method as a way to estimate the minimum inhibitory concentration of EOs. From the research of Kim *et al*. (1995a), Cosentino *et al*. (1999) and Ghrairi & Hani (2015), MIC values of carvacrol were 0.225-5 µl/ml for *E. coli* and 0.375-5 µl/ml for *L. monocytogenes*, and the MIC values of thyme were 0.45 – 2.2 µl/ml for *E. coli* and 0.156-0.45 or 3.6 µl/ml for *L. monocytogenes*. In addition, Farag *et al*. (1989) and Smith-Palmer (1998) studied the antimicrobial properties of plant essential oils and essences against five important foodborne pathogens. MIC values of clove EO were 0.4 - 2.5 µl/ml for *E. coli* and 0.3 µl/ml for *L. monocytogenes,* and those of cinnamon EO were 0.1% for *E. coli* 8007 and 0.075% for *L. monocytogenes*. The results we obtained are similar to the literature data, although they cannot be directly compared due to the different species of test bacteria and differences in the total number of bacteria in the inoculum. Therefore, according to all the MIC results, CIN-03, CIN-01, ORE-03 and Carvacrol were the best choice for the later research if taken the gradually changing of EO by time into consideration.

3.3.1.7 **Antimicrobial activities of essential oils against pathogenic *Listeria* and *E. coli* strains**

3.3.1.7.1 *Listeria* strains

3.3.1.7.1.1 Results of disk diffusion test

Disk diffusion tests were conducted to compare antimicrobial activities of carvacrol (CAR) and cinnamon (CIN-03), oregano (ORE-03) and garlic (AIL-03) EOs against two strains of *L. monocytogenes* and *L. grayi*. **Fig. 3.9 & 3.10** shows the results of the disk diffusion tests after 24 h and 48 h of incubation. The values for the control group in these graphs correspond to the diameter of the disk. The diameter of the inhibition zone for the *L. monocytogenes* 523 strain was significantly larger than that for *L. grayi* in the tests with carvacrol, CIN-03 and ORE-03 ($P \leq 0.05$). The same result was obtained with the *L. monocytogenes* 519 strain in the case of CIN-03; however, in the tests with carvacrol and ORE-03 the diameter of the inhibition zone was not significantly different from that for *L. grayi* ($P > 0.05$). The lower diameter of the inhibition zone for *L. grayi* indicates that *L. grayi* was more resistant than the two *L. monocytogenes* strains to carvacrol, CIN-03 and ORE-03 essential oils. In contrast, Delaquis *et al*. (2004) found that *L. grayi* was highly susceptible to the antilisterial activity of cilantro essential oil and its cell viability decreased fastest among strains of *L, monocytogenes, L. grayi, L. innocua* and *L. seeligeri*. Nevertheless, crude cilantro oil inhibited the growth of all the tested strains at a level of 0.01% (v/v), and no difference in MIC was found among the *Listeria* species.

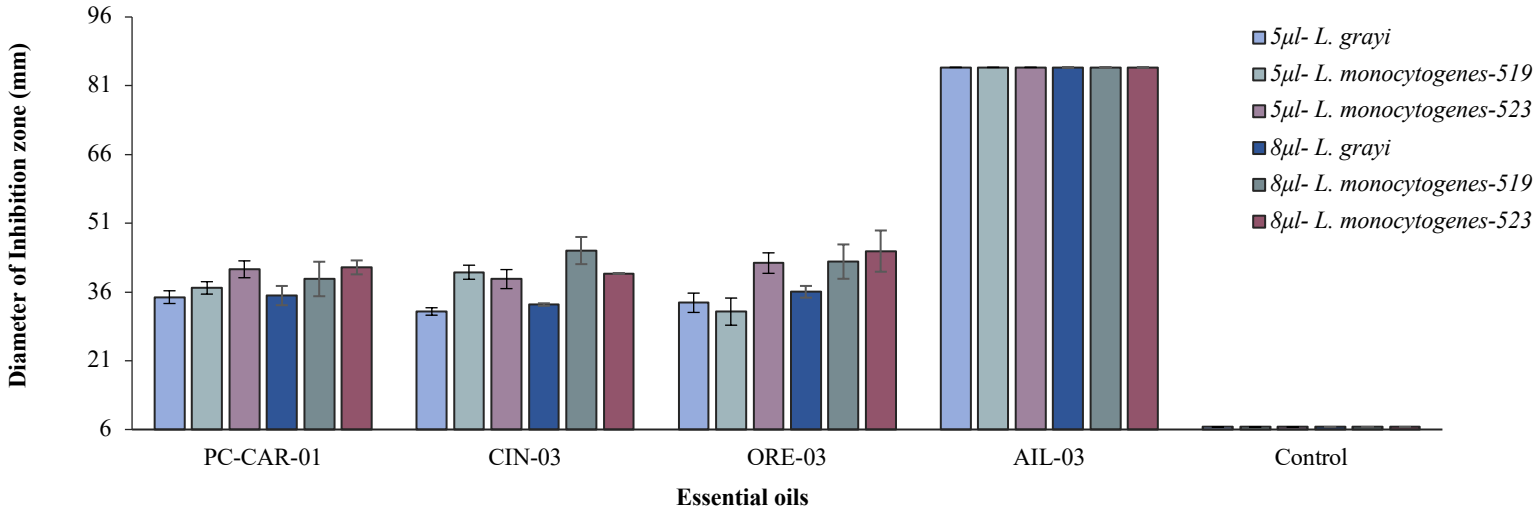


Figure 3.9 Diameter of inhibition zone in disk diffusion tests measuring inhibitory activities of four EOs in different concentrations (5 and 8 µL of EO per disk) against *L. grayi* and two strains of *L. monocytogenes* after 24 h of incubation.

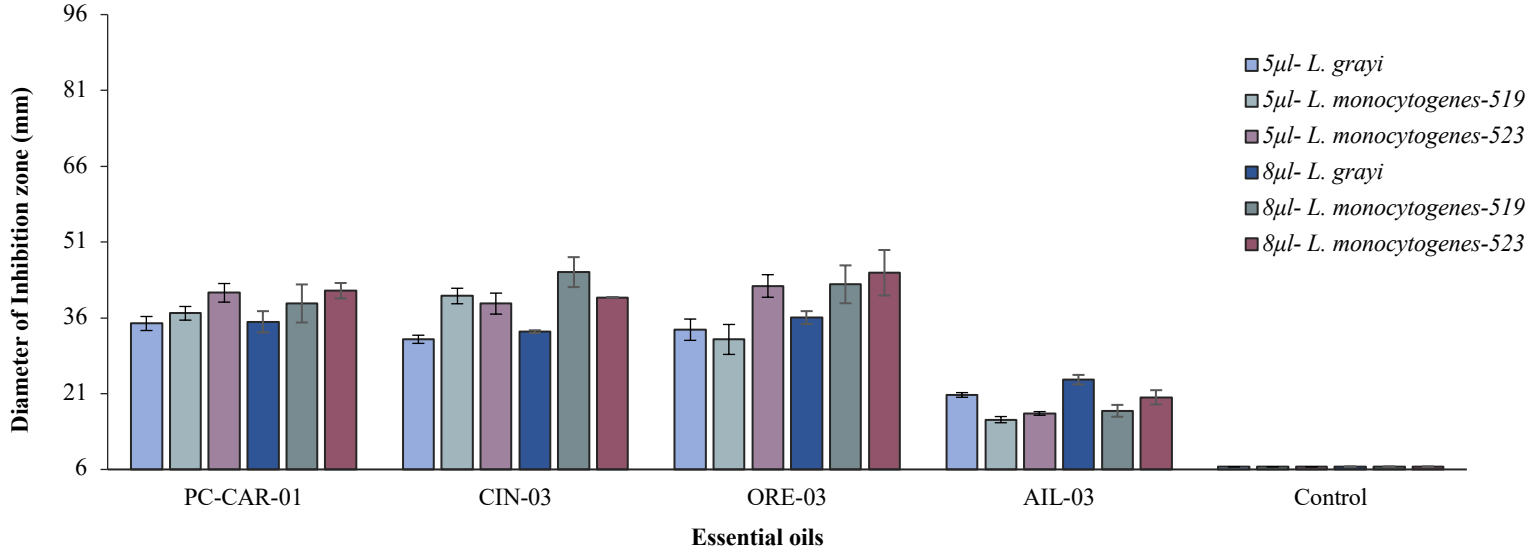


Figure 3.10 Diameter of inhibition zone in disk diffusion tests measuring inhibitory activities of four EOs in different concentrations (5 and 8 µL of EO per disk) against *L. grayi* and two strains of *L. monocytogenes* after 48 h of incubation.

Besides, **Fig. 3.9 & 3.10** show that the diameter of the inhibition zone was unchanged between day1 and day 2 in all cases except in the tests with AIL-03, which produced large inhibition zones after 24 h of incubation but much smaller zones of inhibition after 48 h of incubation. This result was also noted above in the previous tests with *L. grayi* and indicates that AIL-03 essential oil was not bactericidal and only showed bacteriostatic effects for 24 h. The mode of action of the active ingredient, allicin (diallyl thiosulfinate), in garlic essential oil can be attributed to the reversible inhibition of selected intracellular enzyme and transientuly inhibition of DNA repliation and protein synthesis in some bacteria leading to an extended lag phase for bacteria, resulting in a large inhibition zone on first day (Hyldgaar *et al.*, 2012).

In the research of Troxler *et al*. (2000), the susceptibilities of six *Listeria* species, including *L. monocytogenes* and *L. grayi*, to each of 71 antimicrobial drugs were examined. While the antimicrobial effects of many drugs were the same across all species, many interspecies differences in susceptibilities to some classes of drugs were documented. For instance, the natural resistance of *L. grayi* to all antifolates was unique, and it was less susceptible to the antibiotic rifampicin than all the other *Listeria* species but more susceptible to the quinolone class of antibiotics than the other species (Troxler *et al*., 2000). Similarly, it is likely that the antimicrobial activities of different essential oils against different *Listeria* species may not follow any particular trend, and this could account for the discrepancy noted above between the results reported by Delaquis *et al*. (2004) and the findings of the present study.

3.3.1.7.1.2 Results of vapor diffusion test

The results of vapor diffusion tests with different concentrations of EOs after incubation for 24 h and 48 h were shown in **Fig. 3.11** and **Fig. 3.12**, respectively. In **Fig. 3.11**, the diameter of the inhibition zone obtained with CIN-03 at all concentrations was significantly lower for *L. grayi* as compared to both strains of *L. monocytogenes*, and the same phenomenon was also observed with CAR at doses of 2 and 5 μL ($P \leq 0.05$). These differences indicate that *L. grayi* was more resistant than the two *L. monocytogenes* strains to carvacrol and CIN-03 essential oil in the vapor phase for first day. However, in the case of ORE-03 and AIL-03 essential oils there was no significant difference in the diameter of the inhibition zone among *Listeria* species ($P > 0.05$), and all the tested strains were highly susceptible to the vapor of AIL-03 initially.

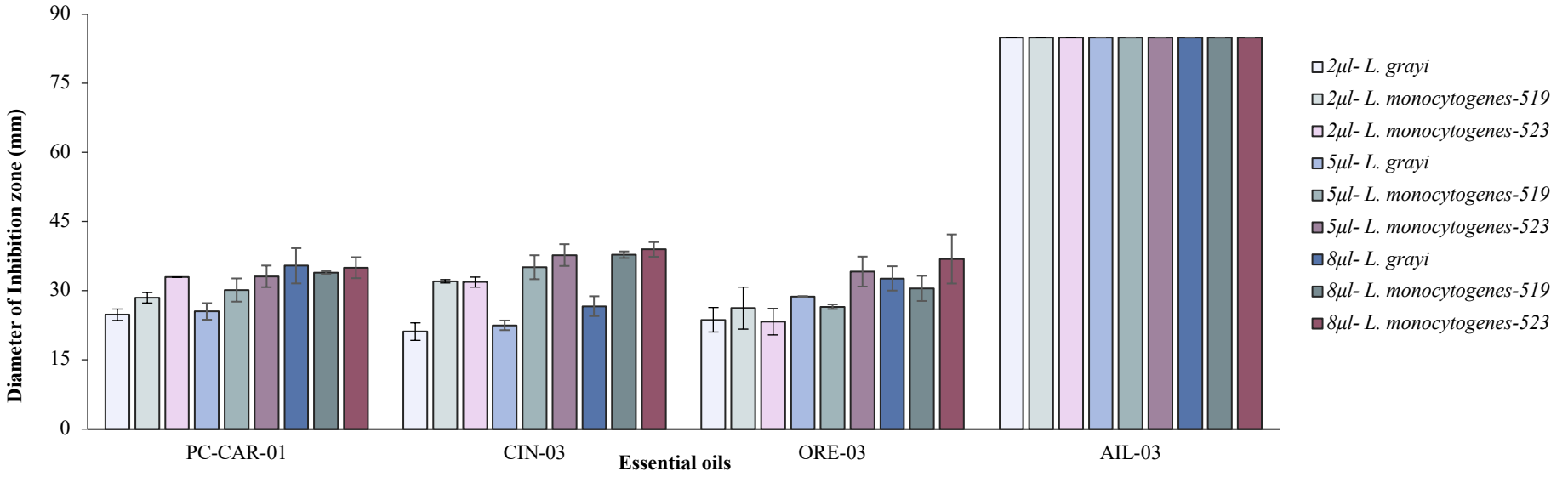


Figure 3.11 Diameter of inhibition zone in vapor diffusion tests measuring inhibitory activities of four EOs in different concentrations (2, 5 and 8 µL of EO per disk) against *L. grayi* and two strains of *L. monocytogenes* after 24 h of incubation.

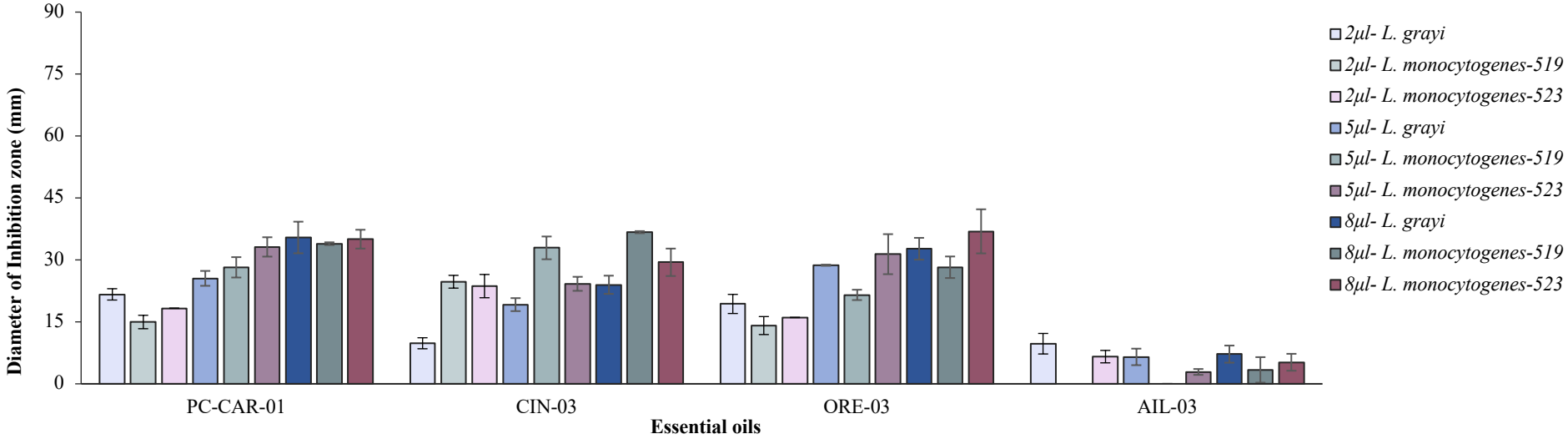


Figure 3.12 Diameter of inhibition zone in vapor diffusion tests measuring inhibitory activities of four EOs in different concentrations (2, 5 and 8 µL of EOs per disk) against *L. grayi* and two strains of *L. monocytogenes* after 48 h of incubation.

In all cases, the diameter of the inhibition zone was smaller after 48 h of incubation (**Fig. 3.12)**, indicating some loss of EO vapor from the sealed Petri plates. In particular, the inhibition zones produced by AIL-03 EO vapor were much smaller than after 24 h of incubation, and it was the least effective EO in inhibiting the growth of all the *Listeria* strains during 48 h of incubation. In the case of CAR, CIN-03, and ORE-03, the changes in the size of the inhibition zone for the two *L. monocytogenes* strains were larger than for *L. grayi.* The inhibition zones produced by CIN-03 were still significantly smaller for *L. grayi* compared to both strains of *L. monocytogenes* ($P \leq 0.05$) in all but one case (*L. monocytogenes* strain 519 at an EO dose of 5 μL), indicating that *L. grayi* was more resistant to CIN-03 EO vapor than the *L. monocytogenes* strains tested in these experiments. In the case of ORE-03, the measurements of the diameter of the inhibition zone after 48 h of incubation indicated that *L. monocytogenes* 519 was slightly more resistant than the other strains. Thus, the antimicrobial abilities of each essential oil were various depend on the species of the *Listeria* strains and the performance of *L. grayi* might slightly lower susceptible to carvacrol and CIN-03 EO than the other two *L. monocytogenes* strains.

3.3.1.7.2 *Escherichia coli* strains

3.3.1.7.2.1 Results of disk diffusion test

Escherichia coli is a Gram-negative bacterium, which is motile, nonsporulating, rod-shaped, and a facultative anaerobe. Most E. coli strains are non-pathogenic, including the *E. coli* K12 strain employed in the work described above. Foodborne pathogenic *E. coli* strains can be categorized into different pathotypes according to their abilities to produce toxins, adhere to epithelial cells, and invade epithelial cells, including adherent invasive *E. coli* (AIEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), and enteropathogenic *E. coli* (EPEC), etc. Shiga toxin- producing *E. coli* (STEC) is defined by the presence of Shiga toxin genes (stx_1 or stx_2), acquired phage-mediated horizontal gene transfer. STEC infections can result in bloody diarrhea and life-threatening haemolytic uremic syndrome (HUS), and lesions on the microvilli are produced by strains that contain the *locus of enterocyte effacement* (LEE) genes. *E. coli* O157:H7 is the most common serotype of STEC while O26:H11 has emerged as the most common non-O157 serotype associated with human illness (Croxen *et al.*, 2013).

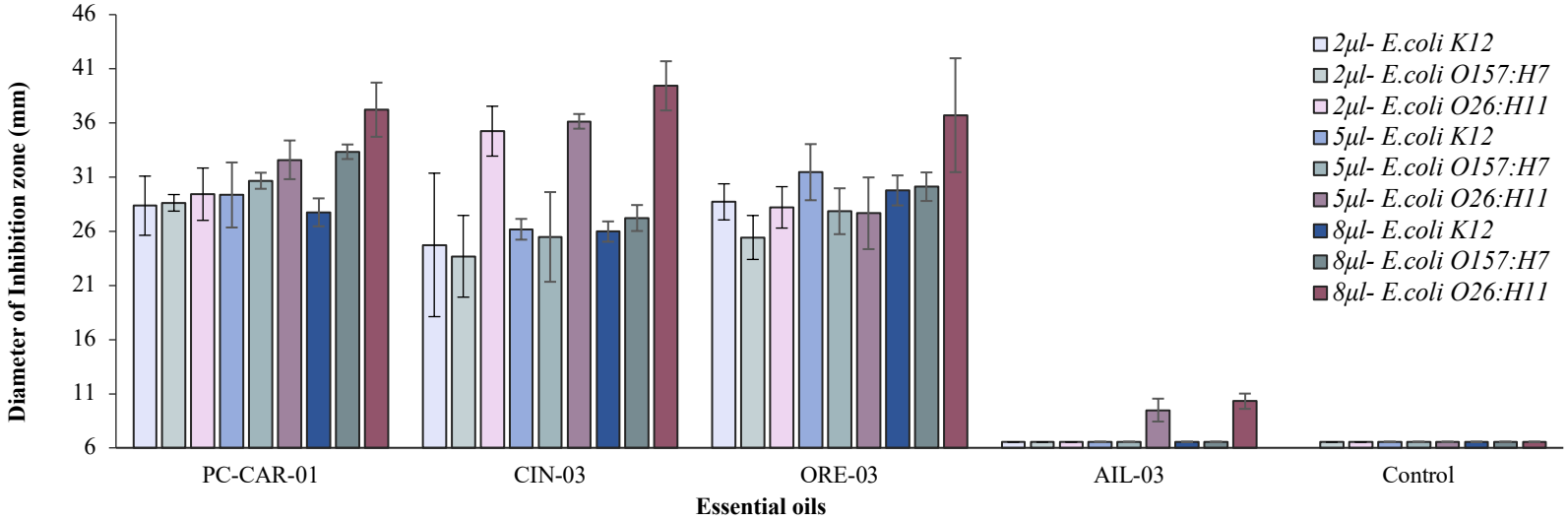


Figure 3.13 Diameter of inhibition zone in disk diffusion tests measuring inhibitory activities of four EOs in different concentrations (2, 5 and 8 µL of EOs per disk) against *Escherichia coli* strains after 24 h.

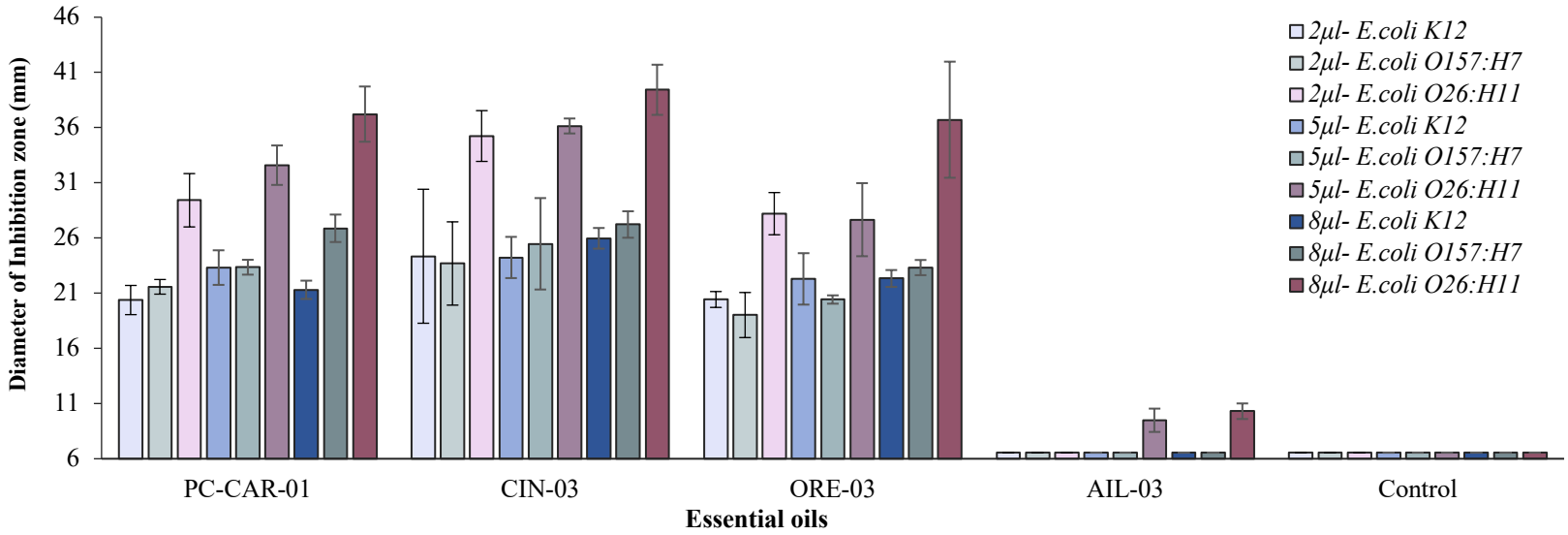


Figure 3.14 Diameter of inhibition zone in disk diffusion tests measuring inhibitory activities of four EOs in different concentrations (2, 5 and 8 µL of EOs per disk) against *Escherichia coli* strains after 48 h.

The results of disk diffusion tests performed with *E. coli* K12, *E. coli* O157:H7 and *E. coli* O26:H11 strains and different doses of carvacrol and CIN-03, ORE-03, and AIL-03 EOs after incubation for 24 h and 48 h were shown in **Fig. 3.13** and **Fig. 3.14**, respectively. For all three strains, the diameter of the inhibition zone after 24 h of incubation (**Fig. 3.13)**, increased with increasing dose of all test substances. There was no significant difference between the results of *E. coli* K12 and *E. coli* O157:H7 except in the tests with carvacrol at a dose of 8 μL ($P > 0.05$). AIL-03 EO had no antimicrobial activities against any of the tested bacteria at the lowest dose and just exhibited slight activity against *E. coli* O26:H11 when the dose was increased. The inhibition zone obtained with CIN-03 EO and *E. coli* O26:H11 was significantly larger at every dose than that for the other two tested *E. coli* strains ($P \leq 0.05$), indicating that *E. coli* O26:H11 was more susceptible than the other target strains to CIN-03 EO.

As shown in **Fig. 3.14**, in all cases the inhibition zone was smaller after 48 h of incubation, but the density of bacterial colonies near the inhibition zone was less than on the rest of the agar plate, which means that only a small amount of bacteria were able to grow under the EO diffusion environment. The differences in the size of the inhibition zone between *E. coli* O26:H11 and the two other strains were more obvious after 48 h than after 24 h and the diameter of the inhibition zone for *E. coli* O26:H11 was significantly larger than that for the other tested *E. coli* strains in all cases ($P \leq 0.05$). No significant difference between the results for *E. coli* K12 and *E. coli* O157:H7 were found ($P > 0.05$).

In the study of Khalifa *et al*. (2016), the extracts of olive leaf and olive exhibited higher activity against *E. coli* O26:H11 than against *E. coli* O157:H7, which is consistent with our results. However, Duoss-Jennings *et al*. (2013) investigated the antimicrobial effects of various concentrations of citrus byproducts (CBPs) containing essential oil against the STEC serotypes O157:H7, O145:H28, O111:H8, O103:H8, and O26:H11 *in vitro*. They found no changes in the STEC population in media supplemented with powdered or pelleted CBP except in the case of O103:H8 and suggested that variations exist in the effect of CBP on different STEC serogroups. In research comparing the effects of various spices on E. coli O157 and non-pathogenic *E. coli* strains, Takikawa *et al*. (2002) found that 0.1% concentration of extracts from cinnamon, clove, thyme and star anise reduced the population of all tested *E. coli* strains and there was no difference between the O157 and non-pathogenic strain in –their tolerance to spices other than nutmeg which was found to have strong antibacterial activity against *E. coli* O157. Based on detailed analyses of the genome of *E.*

coli O157:H7 in comparison with that of the non-pathogenic *E. coli* K12 (Perna *et al*., 2001; Hayashi *et al*., 2001), it was suggested that the structure of plasma membranes in O157 cells may be different from that in cells of *E. coli* K12), resulting in different susceptibilities to antimicrobial activity of essential oils (Takikawa *et al*., 2002). In addition, Bouhdid *et al*. (2008) have studied the antimicrobial activity of *Origanum compactum* essential oil against *E. coli* K12 and *E. coli* O157:H7. From the GC-MS analysis, carvacrol (30.53%), thymol (27.50%) and their precursor γ-terpinene (18.20%) were the main components of this essential oil. The inhibition zone for these *E. coli* strains was the same, which is consistent with the results of the present study. However, the MIC and MBC of *E. coli* O157:H7 were higher than those of *E. coli* K12, indicating the slightly lower susceptibility of *E. coli* O157:H7 to *Origanum compactum* essential oil.

3.3.1.7.2.2 Results of vapor diffusion test

The disk diffusion tests simulate real-life situations in which essential oils are going to be added to the food per se rather than the packaging (Lopez *et al*., 2005). Though essential oils in some kinds of packaging may exert their effect via direct contact, such as wrapping of meat, the releasing volatiles in essential oils from packaging material are still occurred in real cases, which can effectively extend the shelf life of food products. In addition, the lipophilic molecules in the vapor phase were reported to be freely attached to the organism, whereas in an aqueous phase micelle formation suppresses the attachment between EO and organism (Laird & Phillips, 2012).

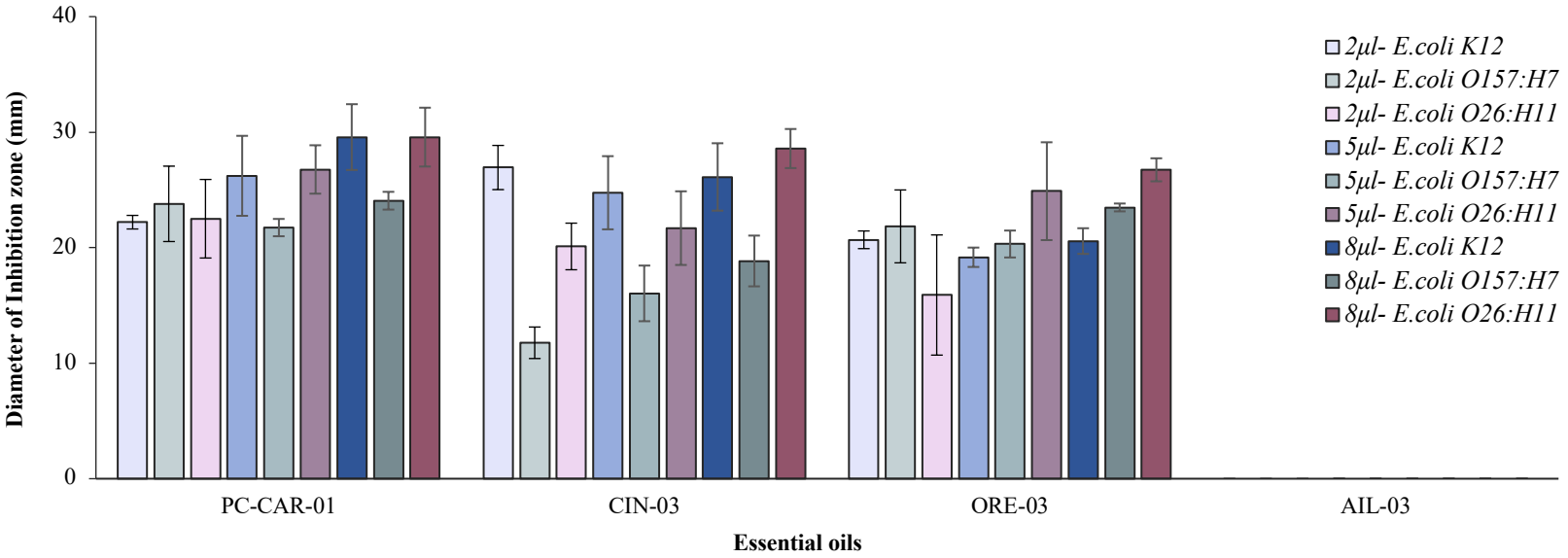


Figure 3.15 Diameter of inhibition zone in vapor diffusion tests measuring inhibitory activities of four EOs in different concentrations (2, 5 and 8 µL of EOs per disk) against *Escherichia coli* strains after 24 h.

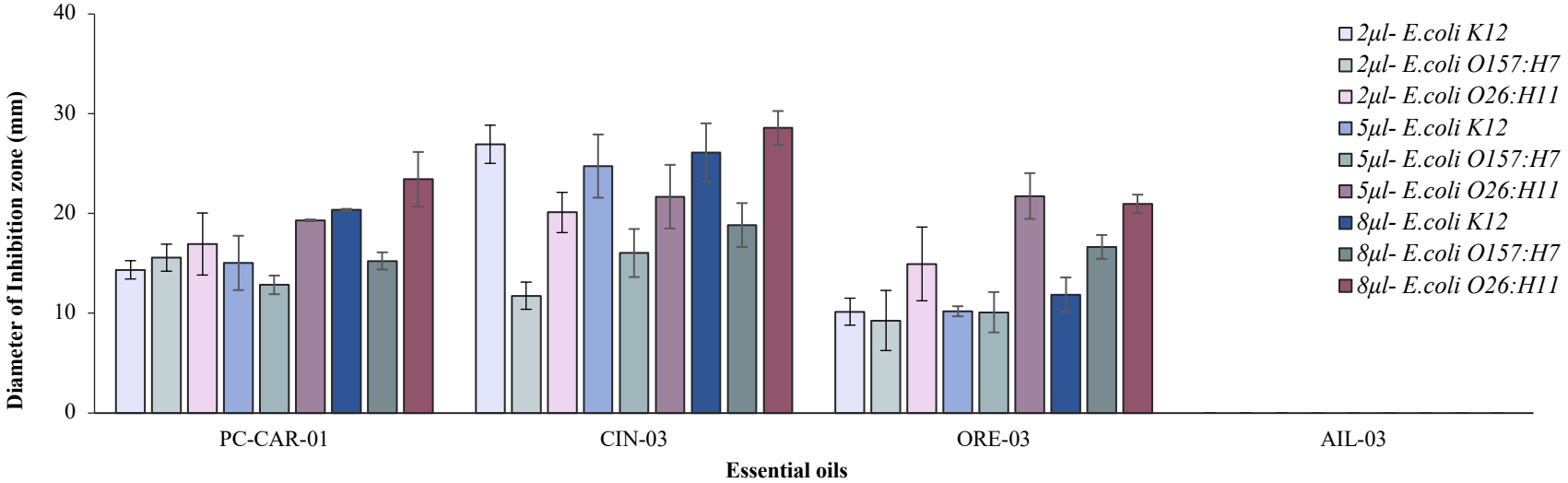


Figure 3.16 Diameter of inhibition zone in vapor diffusion tests measuring inhibitory activities of four EOs in different concentrations (2, 5 and 8 µL of EOs per disk) against *Escherichia coli* strains after 48 h.

The results of vapor diffusion tests in which the antimicrobial activities of different concentrations of EOs in the vapor phase against the three *E. coli* strains after incubation for 24 h and 48 h were shown in **Fig. 3.15** and **Fig. 3.16**, respectively. The results of the vapor diffusion tests were somewhat unpredictable relative to disk diffusion tests. In contrast to the similar inhibition of *E. coli* K12 and O157:H7 by EOs via direct contact, the inhibition zone for *E. coli* O157:H7 was smaller than that for the other strains in the vapor diffusion tests with CIN-03 and with the higher concentration of carvacrol during the incubation. After 24 h incubation (**Fig. 3.15)**, the inhibition zone for *E. coli* O157:H7 was significantly smaller than that of the other strains in the tests with CIN-03 and CAR at a dose of 8 μL ($P < 0.05$), while there were no significant differences among the strains in the vapor diffusion tests with ORE-03 and lower concentrations of CAR ($P > 0.05$). These results indicated that *E. coli* O157:H7 exhibited high resistance to CIN-03 EO vapor by comparison with the other tested strains; however, all *E. coli* strains showed the same susceptibility to ORE-03 and its active compound carvacrol in the vapor phase. After the incubation time was increased to 48 h, the diameter of the inhibition zone of CIN-03 for all strains stayed the same compared to those after 24 h (**Fig. 3.16)**. However, in the case of ORE-03 and carvacrol, the diameter of the inhibition zone decreased, as usually observed with an increase in the incubation time from 24 to 48 h, while the relative susceptibilities of the tested strains did not change. In addition, the inhibition zone of *E. coli* O26:H11 was significantly larger than that of *E. coli* K12 and O157:H7 in the tests with CAR and ORE-03 at doses of 5 μL and 8 μL ($P < 0.05$), corresponding to our previous results from the disk diffusion tests, which demonstrated the higher susceptibility of *E. coli* O26:H11 to carvacrol and oregano and cinnamon EOs. In the case of AIL-03, no antimicrobial activity against any of the strains was observed during the incubation period.

The antimicrobial activities of essential oils in the vapor phase against *E. coli* strains have been much less studied than their activities in direct contact with the microorganisms. In a study of the effectiveness of food packaging containing cinnamon or oregano EO in inhibiting the growth of *E. coli*, Becerril *et al*. (2007) found that the antimicrobial activities of oregano EO, cinnamon EO and cinnamaldehyde in the vapor phase were similar while tcarvacrol was more effective than the others after 20-h incubation. López *et al*. (2007) found that oregano essential oil was more effective than cinnamon essential oil against *E. coli*, for which the MIC values of oregano and cinnamon EOs in the vapor phase were 13.1 and 17.5 μL/L, respectively. According to our results, the antimicrobial activities of ORE-03 and CIN-

03 essential oils were not much different after 24 h. Such differences between different studies may be caused by different concentrations of the main active compounds in essential oils of any given type due to factors such as harvest season and geographic location, as well as differences in extraction methods (Jerković *et al*., 2001).

3.3.1.8 **Minimum inhibitory doses (MID) of EOs in a vapor-phase test**

To estimate minimum inhibitory doses (MID) of essential oils and pure carvacrol in the vapor phase, the different inhibitory concentration and their MID of some essential oils in a certain volume were conducted in petri dish, showing in **Fig. 3.17**. As the concentration of each essential oil in the vapor phase increased, the inhibition% of all EOs and carvacrol were increased. In particully, carvacrol and ORE-03 showed the most effective inhibition against the growth of *L. grayi*, exhibiting the 100% inhibition area, which was defined as MID, at the concentration of 241 and 362 µl/L, respectively. In following, ORE-02, CIN-01, CIN-02, CIN-03 also showed higher inhibition% area as concentration increasing and MID values of all essentail oils and carvacrol in this figure were summarized in **Table 3.2**.

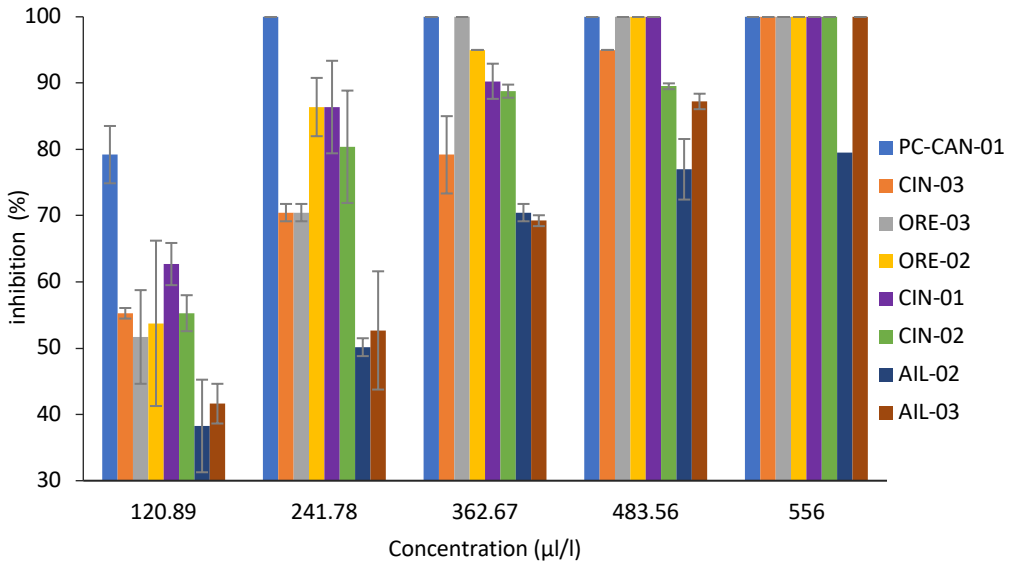


Figure 3.17 Vapor-phase activity of various EOs and pure carvacrol against *L. grayi*. % Inhibition = T/C × 100, where T is the diameter of the inhibition zone and C is the diameter of the petri dish (90 mm).

Table 3.2 MID values of various EOs and pure carvacrol based on vapor-phase activity against *L. grayi.*

Test substance	Lowest volume producing complete inhibition (µl)[a]	MID (µl·L^{-1} of headspace)
Carvacrol	10	241.78
ORE-03	15	362.67
CIN-01	20	483.56
ORE-02	20	483.56
CIN-03	23	556
CIN-02	23	556
AIL-03	23	556
AIL-02	>23	>556

[a] Volume of test substance added to filter paper.

Similarity, carvacrol and ORE-3 EO were the most effective among all the tested substances followed by CIN-01 and ORE-02 EOs, which is consistent with the results of the vapor diffusion tests. Garlic essential oils (AIL-02 and AIL-03) could also completely inhibit the growth of *L. grayi* in which the concentration of the bacterial suspension was lower (10^4 CFU/ml) than that in vapor diffusion test above. As mentioned above, the garlic EOs inhibited the growth of *L. grayi* in the first 24 h of incubation but the bacteria grew in the next 24 h, so their antimicrobial activity was much lower than that of other EOs. However, according to the MIC measurements above, the efficiency of some EOs, including ORE-02, were gradually weakened over time, and their quite low antilisterial abilities made them not a good choice for further research due to their unstable active componets. To consider the lower usage approved by FMEA of the cinnamon oleoresin for practical food industry (Burdock, 2016), we would like to choose the CIN-03 instead of CIN-01 (oleoresin).

In the studies of Lopez *et al* (2005, 2007), the inhibitiory activities in the vapor phase of cinnamon and thyme EOs against *L. monocytogenes* in a similar assay were 18% and 33% at a concentration of 175 µl/l, while 75% inhibition was obtained for oregano EO at a concentration of 131 µl/l. In addition, Goni *et al*. (2009) reported a MIC for cinnamon EO of 54 mg/l, which is higher than the presented above. Carvacrol, cinnamon, and oregano have been considered as Generally Recognized As Safe (GRAS) by FDA and approved for using in food. Thus, carvacrol, ORE-03 and CIN-03 were chosen for further application in the vegetables packaging research.

3.3.2 **Application of essential oils on vegetables**

In the first set of tests of the antimicrobial effects of carvacrol on vegetables, fresh potatoes, broccoli and green peppers on which an inoculum of *L. grayi* had been spot-applied were pre-sprayed with a 1% solution of carvacrol (in 95% ethanol) and then stored for 2 weeks at room temperature. As shown in **Fig. 3.18**, there was a substantial difference in the appearance of the potatoes between the control and treatment groups after 14 days of storage. While the control potatoes were obviously spoiled, the surface of the treated potatoes was still dry and no mold or spoilage had occurred. In contrast, pre-spraying with the 1% carvacrol solution was not effective in retarding microbial growth on broccoli and green peppers. These results were attributed to the low concentration of carvacrol in the spraying solution and the high microbial population on the surface of the vegetables.

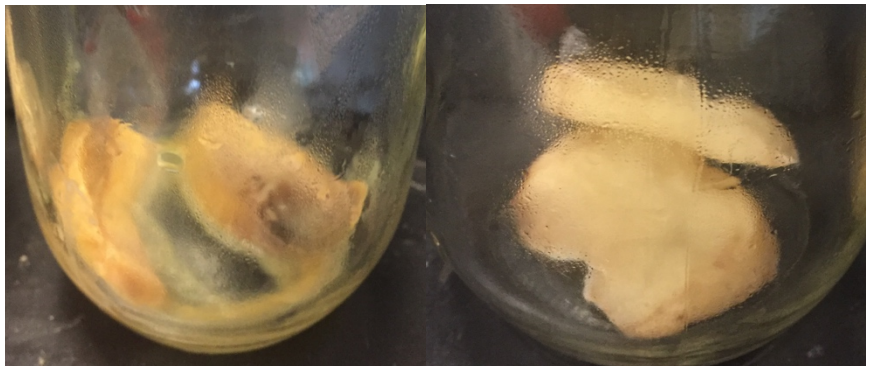


Figure 3.18 Effectiveness of pre-spraying with a 1% carvacrol solution (in 95% ethanol) in preventing spoilage of fresh potatoes during a storage period of two weeks at room temperature (left :control group; right: pre-sprayed group).

In the next set of experiments, small pieces of green peppers inoculated with *L. grayi* were stored in Petri dishes together with a filter paper disk impregnated with carvacrol, which was attached to the inside of the lid of the Petri dish without touching the green pepper pieces. In these experiments, the green pepper pieces were placed into several Petri dishes so that samples could be taken for microbial analysis during the storage period without loss of essential oil vapor. **Fig. 3.19** shows photos of the green pepper pieces stored in the presence of carvacrol at room temperature. From day 0 to day 5, samples treated with carvacrol did not change in appearance except for a slight change in colour due to enzymatic browning, which was prevented in subsequent experiments by blanching the green peppers before treatment with the EO. In the case of the control samples, some small mold colonies were observed at the beginning of day 3 and became larger as time went on. After 5 days, all the control peppers had changed in color and became spoiled.

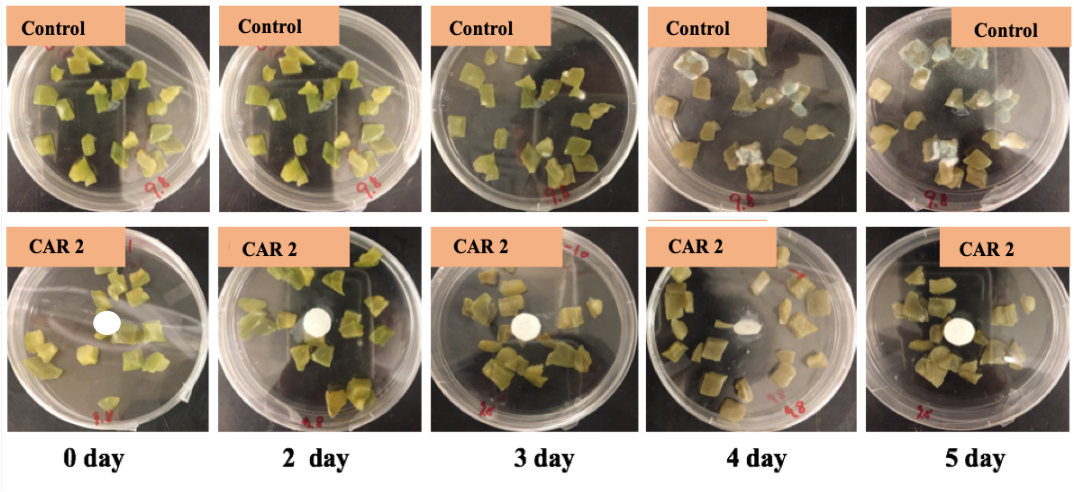


Figure 3.19 Storage of green pepper pieces in the presence of carvacrol at room temperature for 5 days in comparasion with control.

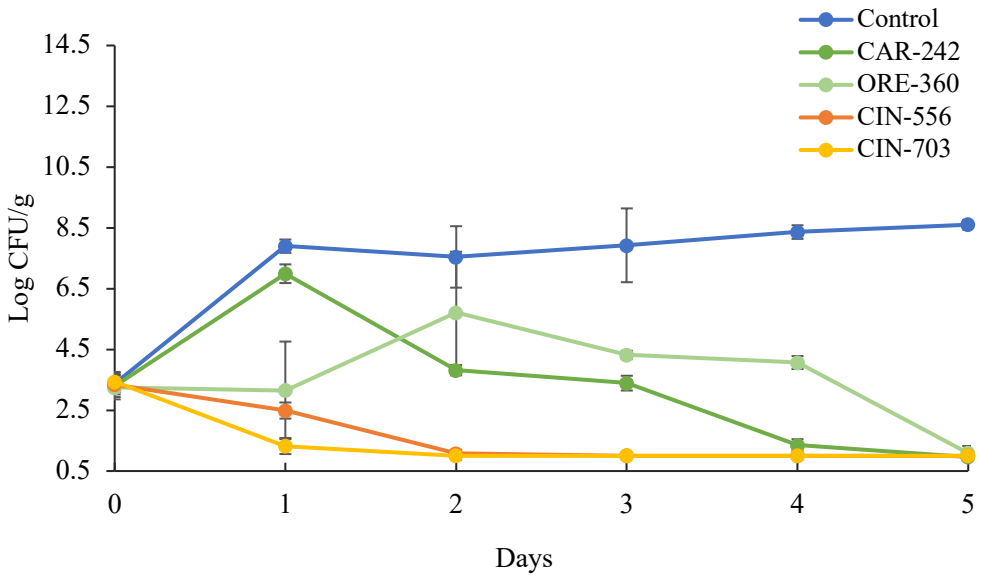


Figure 3.20 Growth of *L. grayi* on green peppers at room temperature in the presence of filter paper impregnated with 17 µL of carvacrol, 26 µL of oregano, and 40 or 50 µL of cinnamon essential oils corresponding to 242, 360, 556 or 703 µl per liter of headspace in the Petri dish.

The effectiveness of carvacrol, oregano and cinnamon EOs against *L. grayi* in the sealed system is shown in **Fig. 3.20**. As the results of carvacrol shown in it, we found that the total count of *Listeria* increased rapidly in the control group from 3.73 to 8.60 log CFU/g during 5 days of storage and that the bacteria started to be in stationary phase after 1 day. For samples treated with carvacrol at levels of 242 µl per liter of headspace, the colony count of *Listeria* reached 1.36 log CFU/g after 4 days of storage, demonstrating that carvacrol vapor can effectively inhibit the growth of *Listeria*.

Similar experiments were subsequently conducted with oregano and cinnamon essential oils. From the results of the vapor diffusion tests, the minimum inhibitory concentration of oregano and cinnamon essential oils corresponded to the amount of oregano and cinnamon essential oils released into the vapor phase from filter paper impregnated with 360 and 556 µl of the essential oil per liter of headspace, respectively. Accordingly, these were the lowest concentrations chosen for the experiments with the green peppers. The results of oregano show that the population of *Listeria* on treated vegetables slightly increased to 4.07 log CFU/g after storage of 4 days and decreased to around 1 log CFU/g at 5th day, indicating the inhibition effect of oregano EO against the growth of *Listeria* on vegetables at level of 360 µl/L headspace. The results of cinnmaon EO in **Fig. 3.20** show that the colony count of *Listeria* on the treated samples decreased rapidly in 1 day from 3.44 to 2.49 or 1.32 log CFU/g at the level of 556 or 703 µL of cinnamon EOs, respectively, at room temperature. After 2 days, the colony count was at the limit of detection (10 CFU/g) whereas the colony count for the controls had risen to 7.54 log CFU/g. To establish whether the cinnamon essential oil vapor had bactericidal effects, after 8 days of storage the samples were transferred into tryptic soy broth (TSB) and incubated for 24 h at 37 ^{o}C, and then a 100-µl inoculum was tested on listeria-selective agar (LSA). No bacterial growth was observed on the LSA, demonstrating that exposure to cinnamon essential oil vapor had killed the *Listeria* present on the green peppers at the beginning of the storage period. Therefore, cinnamon essential oil placed inside packaging at a level of 556 µL per liter of headspace can inhibit the growth and kill *Listeria* during storage. It may be noted that in ready-to-eat vegetables for which the refrigerated shelf life is less than 5 days, the Canadian Food Inspection Agency requires no greater than 100 CFU of *Listeria* per gram. In the case of frozen vegetable products without cooking instructions, the product must be rejected if any of five 10-g samples exceed 100 CFU of *Listeria* per gram, with the standard deviation of the five analyses being no greater than 0.25 log CFU/g (CFIA, 2016). The results presented in **Fig. 3.20** indicate that the cinnamon essential oil at the levels tested would keep the number of *Listeria* below 100 CFU/g, in compliance with these CFIA regulations.

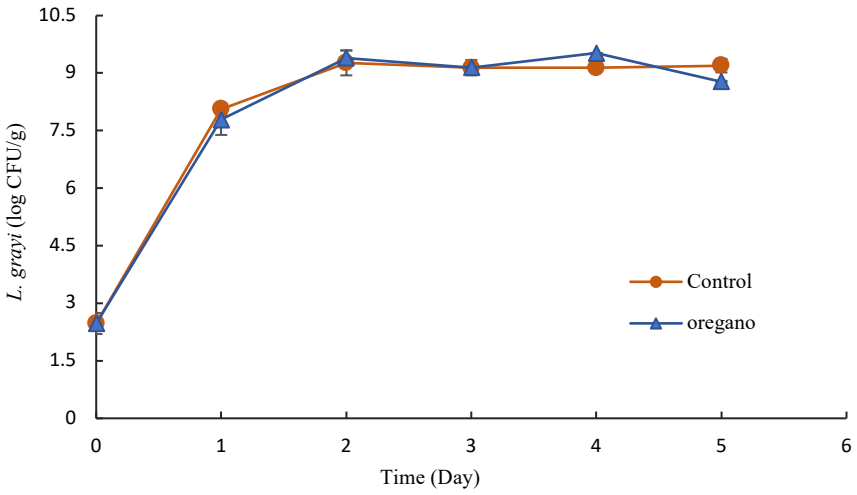


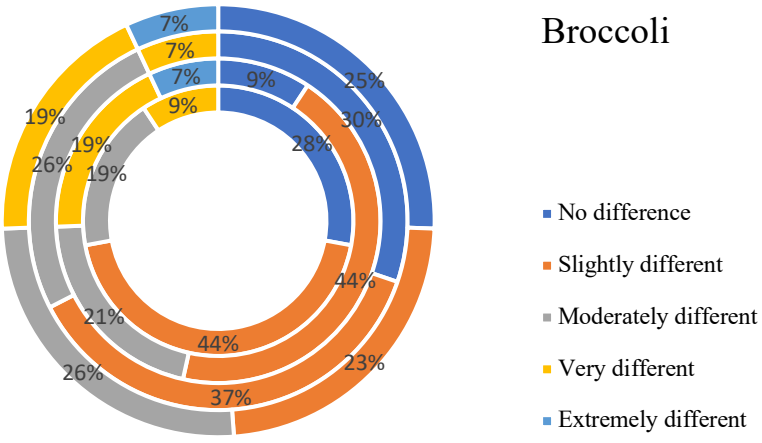
Figure 3.21 Growth of *L. grayi* on broccoli at room temperature in the presence of filter paper impregnated with 15 µL of oregano essential oil, corresponding to 360 µl per liter of headspace in the glass jar.

As the above approach was very effective in inhibiting the growth of *L. grayi* on green peppers, it was subsequently tested with broccoli florets because of their much larger surface. **Fig. 3.21** shows that similar *Listeria* growth curves were obtained for broccoli florets stored at room temperature in glass jars in the presence or absence of a sticker impregnated with oregano essential oil. Accordingly, the treatment of broccoli florets with the vapor of oregano essential oil under these conditions had no antilisterial effect. Although the filter paper was impregnated with the volume of oregano essential oil established to yield the MIC in the vapor phase, it appears likely that the amount of essential oil vapor distributed inside the jar was insufficient to cover the large surface area of the floret. In most studies reported in the literature, antimcirobial agents have been applied directly on the surface of broccoli florets via coating or spraying, or non-thermal treatments such as γ-irradiation have been employed to ensure the safety of broccoli florets (Ben-Fadhel *et al.,* 2017; Takala *et al.,* 2011). In research conducted by the research group of Dr. Hosahalli Ramaswamy at McGill, a pulsed-light non-thermal treatment did not effectively decrease the total population of *Escherichia coli* O157:H7 on the surface of broccoli florets compared with other food, such as cherry, french fries, red pepper and mango.

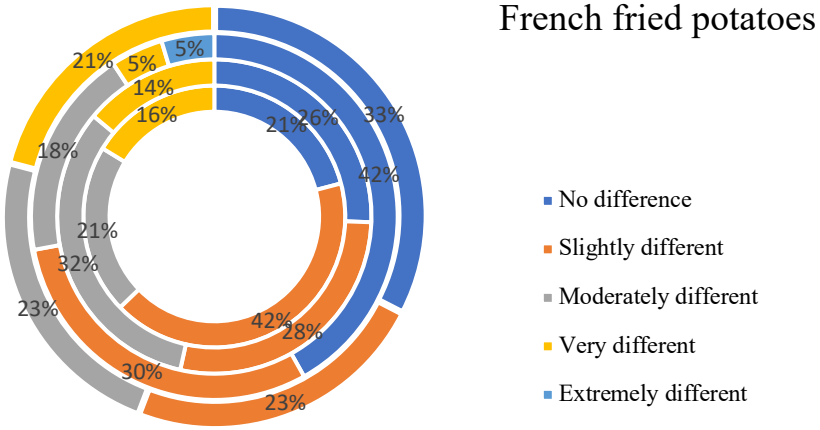
3.3.3 **Sensory evaluation**

3.3.3.1 **Difference test**

The vegetables used for sensory evaluation were prepared by exposing the frozen broccoli and french fried potato to oregano and cinnamon EO vapor (delivered from a filter paper impregnated with the EO and placed inside the package without making contact with the contents) during storage at 4ºC for 24 h (results were shown in **Fig.3.22**). The broccoli and french fired were served as a sample of food to be exposed under EOs for sensory evuluation as the taste of frozen pepper is not suitable in raw or after steaming for panelists. The broccoli samples were removed from the package and steamed for 10 min. The french fried potatoes were warmed in the oven. **Fig.3.22**(A) shows that only 9% of the panelists described 'no difference' between broccoli samples that had been exposed to oregano EO vapor at the higher treatment level (180 µl/L of headspace) and control samples, which was less than the others. In addition, 7% of panelists described these samples as "extremely different" , and this was also the case for as well as broccoli samples that had been exposed to cinnamon EO vapor at the higher treatment level (556 µl/L). However, more panelists described the 275 µl/L cinnamon samples (shown in **Fig 3.22**(B)) had no difference with control, which meant the concentration of the essential oil did not affect the taste of french fried potatoes compared with broccoli samples.



(A)

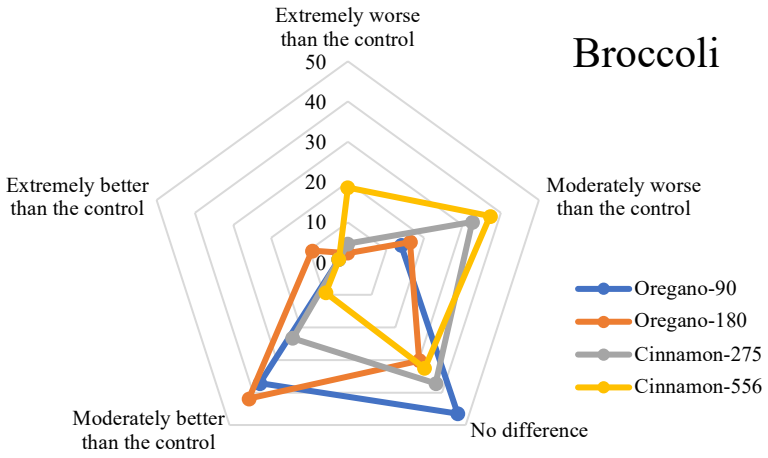


(B)

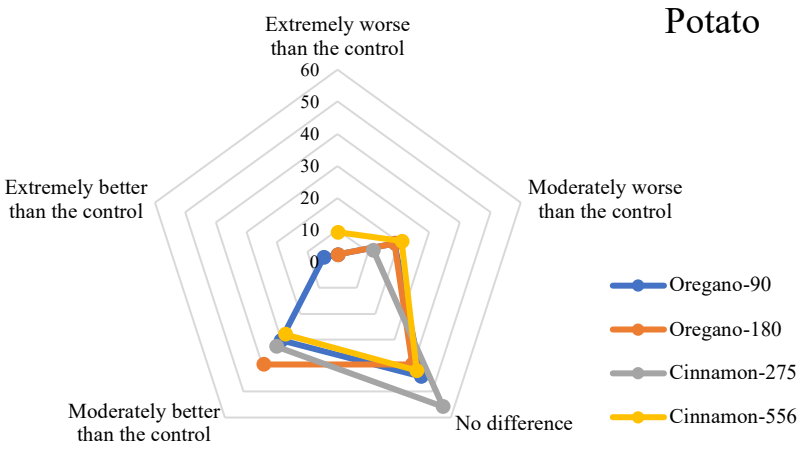
Figure 3.22 Plots showing the results of the difference test for the sensory evaluation of frozen broccoli(A) and french fried potatoes (B) exposed to oregano and cinnamon vapor. (The circles from the inner to the outer represent the products that had been packaged together with a filter paper impregnated with 90 µl/L oregano, 180 µl/L oregano, 275 µl/L cinnamon, and 556 µl/L cinnamon essential oil).

3.3.3.2 **Liking test**

The results of the liking test in the sensory evaluation of broccoli and french fried potatoes treated with vapor of oregano and cinnamon EO in packaging were shown in **Fig.3.23**. The broccoli groups exposed to cinnamon essential oil vapor were tasted moderately worse than the control compared to that to vapor of oregano essential oil. For the french fried potatoes, no significant differences were observed between samples exposed to oregano or cinnamon essential oil vapor in **Fig. 3.23** (B). Overall, panelists preferred the oregano groups.



(A)



(B)

Figure 3.23 Liking test of the sensory evaluation of broccoli (A) and french fried potatoes (B) with oregano and cinnamon essential oils in packaging.

Table 3.3 Mean score of difference and liking tests for broccoli and french fried potatoes exposed to oregano and cinnamon essential oil In the headspace of packaging.

Vegetable	Essential oil	Concentration (µl/L)	Difference test	Liking test
Broccoli	Oregano-03	90	2.09	3.28^{b}
		180	2.70	3.40^{b}
	Cinnamon-03	275	2.09	2.86^{ab}
		556	2.58	2.40^{a}
French fried potatoes	Oregano-03	90	2.33	3.16^{b}
		180	2.35	3.16^{b}
	Cinnamon-03	275	2.00	3.21^{b}
		556	2.33	2.88^{ab}

Descriptor of **difference test**: 1- No difference; 2- Slightly different; 3- Moderately different; 4- Very different; 5- Extremely different.
Descriptor of **liking test**:1- Extremely worse than the control; 2- Moderately worse than the control; 3- No difference; 4- Moderately better than the control; 5- Extremely better than the control.
Different letters represent significant differences, $P \leq 0.05$.

The results of the difference and liking tests are summarized in in **Table 3.3**. Statistical analysis was carried out using SPSS software to do multiple comparisons by Tukey' HSD at 5% significance level. The results of sensory tests showed slight differences between the controls and the samples stored in packaging containing filter paper impregnated with EOs, while there were no significant differences among the different treatment groups ($P > 0.05$). On average, panelists described 'no difference' between samples treated with oregano EO and the controls in the liking test. However, the samples stored with cinnamon EO tasted moderately worse than the controls, except for french fried potatoes stored with cinnamon

EO at the lower concentration. Meanwhile, the score for broccoli treated with cinnamon EO at the higher level (556 μL/L of headspace) was significantly lower than that for all the oregano EO groups. Thus, cinnamon EO at the higher concentration was less acceptable to panelists than oregano EO on both types of samples. Accordingly, oregano EO vapor in low concentation might be a better choice for use in vegetable products as antimicrobial agent. Besieds, carvacrol, cinnamon, and oregano have been considered as Generally Recognized As Safe (GRAS) by FDA and approved for usie in food. From the Flavor and Extract Manufacturer's Association (FEMA), the dialy intake of carvacrol is 0.3μg/kg in USA. Maximum use level of flavoring ingredients, such as carvacrol, cinnamon, oregano, in some food under the food additive amendment are collected in GRAS substancere, in which maximum level of carvacrol in ice cream and baked goods are 34 and 120 ppm; the maximum use level of oregano in baked goods and meat are 400 and 540 ppm; and the maximum use level of cinnamon in ice cream, baked goods and meat are 53, 1900 and 880 ppm. In addition, the toxicity of essential oil swallowed were all tested on animals, and the ingestion of a large quantity of pure EO can cause burning sensation in the mouth and throat and sometimes causes tsymptoms of vomiting, nausea, and diarrhea. The LD_{50} (animal) and lethal dose for a 70-kg adult of carvacrol, oregano (*Origanum majorana*) essential oil, and cinnamon (bark) essential oil are 0.81 g/kg and 63 ml; 2.24 g/kg and 174 ml; and 3.4 g/kg and 164 ml (Price & Price, 2011), which are far higher than the quantity in food packaging and also higher than our MID.

Our results were also similar to the study of De Azeredo *et al*.(2011), their sensory score of the most evaluated attributes fell in the range between "slightly like" and "neither like nor dislike" on the hedonic scale for lecture, beet and rocket treated with oregano and rosemary EOs alone and their mixtures, but their applied volume of EO on vegetables products (1.25–5 and 20–40 μL/mL for oregano and rosemary EO, respectively) were far higher than what we used in our study. However, a negative effect of EOs directly applied on vegetables was reported by Scollard *et al*. (2013). They found thyme, oregano and rosemary EOs (0.5 g/25 g) sprayed on lecttuce and cabbage led to unacceptable damage to appearance during storage, and similar results were reported by Uyttendaele *et al*.(2004), who found that thyme EO caused softening of bell peppers tissue and moisture loss. These effects were probably due to the phytotoxic or cytotoxic effect of the thyme or oregano EOs. Thus, the concentration of EOs or their vapor in packaging has to be taken into consideration to eliminate the changes in appearance and taste of vegetable products by EOs in food packaging.

3.3.4 **Antimicrobial effect of aerosolized essential oils**

3.3.4.1 ***In vitro* assessment of antimicrobial activity of aerosolized essential oils against *L. grayi* and *E. coli* K12**

As the delivery of EO vapor from stickers impregnated with oregano-03 EO proved ineffective in reduction of *Listeria* counts on broccoli florets in accelerated tests, a method based on aerosolization of EOs was developed as a means of treating vegetables that have a very large surface, such as broccoli florets, with EO prior to packaging and freezing. This aerosolization method is a modification of the method described by de Oliveira *et al*. (2018) and employs ultrasound (delivered by a commercially available ultrasonic aroma diffuser, producing an oscillation frequency of 2.4 million oscillations/s) to aerosolize essential oil, producing billions of particles as fine as fog. Furthermore, this micro-pulverization is done without heat, thereby not changing the components of the essential oil. By placing unpackaged vegetables in a container connected to the ultrasonic aroma diffuser, the essential oil can be delivered as an aerosol and deposit on the surface of the vegetables prior to packaging and freezing. This may be regarded as a form of active packaging incorporating essential oil to inhibit bacterial growth during thawing of frozen vegetable products.

To investigate the potential effectiveness of this method in retarding bacterial growth, agar plates inoculated with suspensions of *L. grayi* and *E. coli* K12 diluted to 10^2 CFU/ml and 10^3 CFU/ml were treated with aerosolized carvacrol in a sealed container connected to the ultrasonic aroma diffuser mentioned above. **Fig. 3.24** shows the viability of *L. grayi* and *E. coli* K12 after continuous treatment with aerosolized carvacrol in the container for different amounts of time. The results show that bacterial viability decreased with increasing time, with the exception of the first time point (10 min) in the case of *E. coli* K12 (inoculum size of 10^2 CFU/ml). After 1 hour of treatment, there was almost no survival of *L. grayi* and *E. coli* K12 on the agar, indicating that aerosolized carvacrol could effectively inhibit the growth of *L. grayi* and *E. coli* K12 *in vitro*.

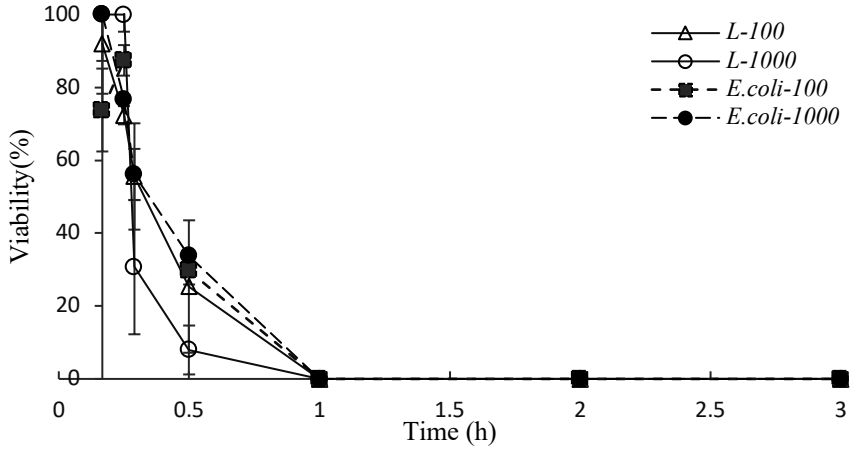


Figure 3.24 Viability of *L. grayi* and *E. coli* K12 during treatment with aerosolized carvacrol.

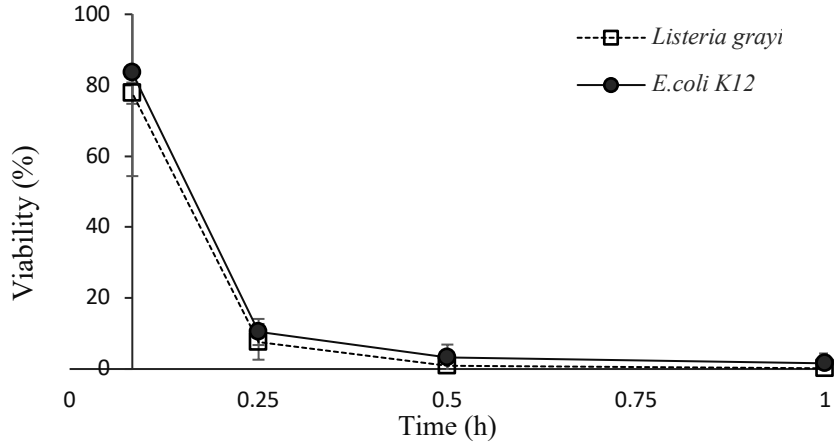


Figure 3.25 Viability of *L. grayi* and *E. coli* K12 during treatment with aerosolized oregano-03.

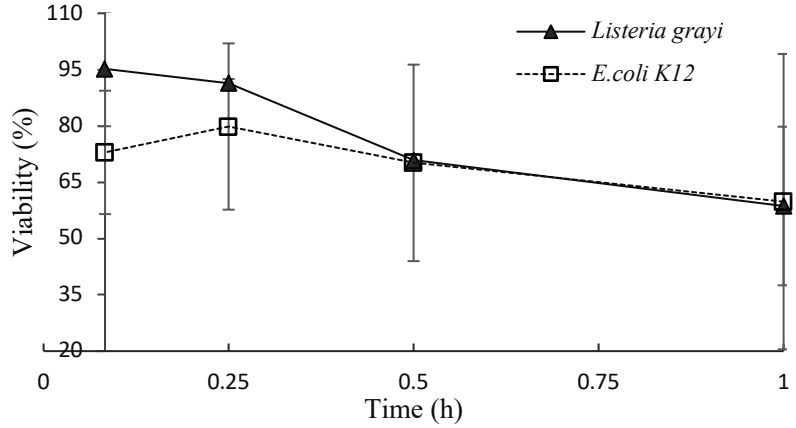


Figure 3.26 Viability of *L. grayi* and *E. coli* K12 during treatment with aerosolized cinnamon-03 EO.

The results of viability of *L. grayi* and *E. coli* K12 after treatment with aerosolized oregano-03 and cinnamon-03 essential oils for different amounts of time are shown in **Fig. 3.25 & 3.26**. In these experiments, plates inoculated with suspensions of *L. grayi* or *E. coli* K12

diluted to 10^2 CFU/ml were treated with aerosolized EO for 5, 15, 30 and 60 minutes. The results for oregano-03 EO show that bacterial viability decreased with increasing treatment time. When treated with aerosolized oregano-03 EO vapor for around 30 minutes, there was almost no survival of *Listeria* and *E. coli* K12 on plates. However, 58% of bacteria were still alive on plates after treatment with aerosolized cinnamon essential oil for 1 hour. This result indicates that the effectiveness of the aerosolization method in inhibiting bacterial growth was much lower with cinnamon EO than with oregano EO and carvacrol.

The results achieved by our aerosolization method compare favorably with those reported by Gaunt *et al*. (2005), who investigated the antibacterial activities of candles containing oregano EO and β-pinene against *E. coli*. A 20% decrease in viability of *E. coli* was observed after 5 h due to the volatilized components in the candle flame as well as ionization products. In addition, the increasing aerial concentration of volatiles in the chamber also decreased the viability of *E. coli*. However, the slow rate of evaporation of EOs and ion production from the flame made this approach less effective than our aerosolization method, with which there were almost no survival of *E. coli* after 1 h. A diffuser and sealed chamber were also used for dispersing EO vapor in the study of Fisher *et al*. (2009), who found significant reduction of vancomycin-susceptible and resistant enterococci (*E. faecalis* and *E. faecium*) in number after exposure to citrus vapor for 15 min. However, the citrus vapor was produced by a heating element in the diffuser instead of vibration, which could alter the distribution of active compounds relative to the liquid essential oil.

3.3.4.2 *In vitro* assessment of aerosolized essential oils against *L. grayi* and *E. coli* K12 after 1-hour accumulation

In the next set of experiments, the agar plates inoculated with *L. grayi* or *E. coli* K12 were placed into the container after aerosolized carvacrol or EO had accumulated inside the container for a defined amount of time. Based on the results presented above, a 1-hour accumulation time was used. **Fig. 3.27** shows the viability of *L. grayi* and *E. coli* K12 as a function of time in the presence of aerosolized carvacrol. The results show that bacterial viability decreased with increasing exposure time. When the agar plates were exposed to aerosolized carvacrol for around 15 minutes, there were only 0.204±0.35% (10^2 CFU/ml) and 0.179±0.24% (10^3 CFU/ml) of *L. grayi* alive, but there were still 7.61±3.5% and 1.5±0% of *E. coli* K12 surviving on the agar. The higher killing rate of the *Listeria* strain is consistent with the higher susceptibility of *L. grayi* to carvacrol and EOs by comparison with *E. coli* K12 in the disk diffusion and vapor diffusion tests conducted in this study.

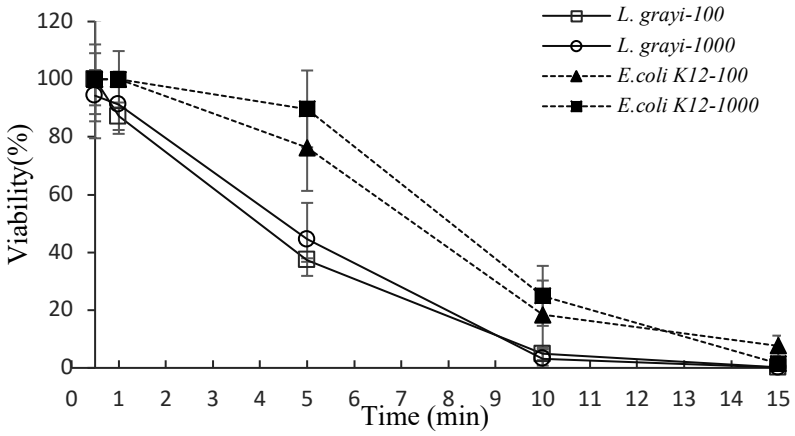
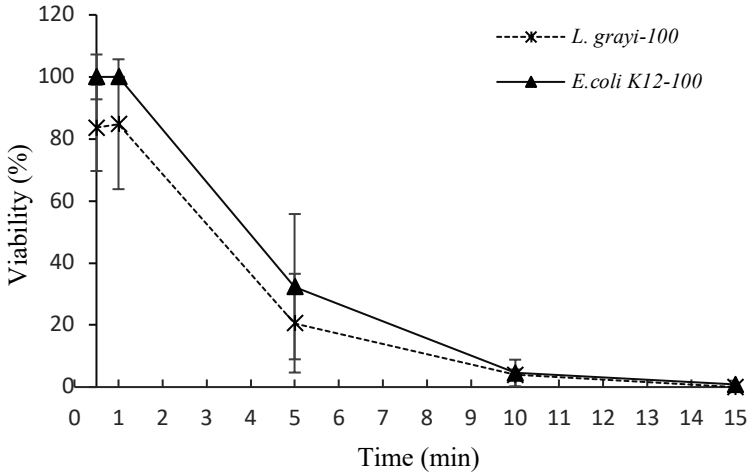
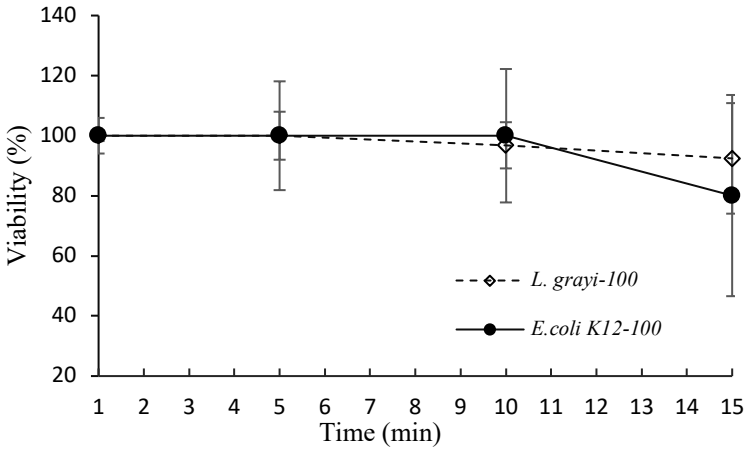


Figure 3.27 The viability of *L. grayi* and *E. coli* K12 in the presence of aerosolized carvacrol in a sealed container. Aerosolized carvacrol was delivered continuously into the container by an ultrasonic aroma diffuser for 1 h prior to introduction of plates inoculated with the bacteria.



(A)



(B)

Figure 3.28 The viability of *L. grayi* and *E. coli* K12 as a function of exposure to aerosolized oregano-03 EO (A) and cinnamon-03 EO (B) in a sealed container. Aerosolized EO was delivered continuously into the container by an ultrasonic aroma diffuser for 1 h prior to introduction of plates inoculated with the bacteria.

Fig. 3.28 shows the viability of *L. grayi* and *E. coli* K12 on agar plates exposed to aerosolized oregano-03 EO and cinnamon-03 EO for different amounts of time after 1-hour accumulation of the aerosolized EO in the sealed container. The experiments with aerosolized oregano EO gave similar results to those obtained with aerosolized carvacrol. Bacterial viability decreased with increasing exposure time, and there was almost no survival of *L. grayi* (0%) and *E. coli* K12 (0.85±1.2%) on the plates after 15-min exposure. However, in the case of aerosolized cinnamon EO, 80-90% of bacteria were still alive on the plates under the same conditions. Accordingly, even though cinnamon-03 EO was found to have high antimicrobial activity in the disk diffusion and vapor diffusion tests conducted in the present study, it was not very effective in these tests of the aerosolization method, Furthermore, its lack of effectiveness by comparison with oregano-03 EO is not consistent with the findings of Sato *et al*. (2006), who studied the antimicrobial effects of cinnamaldehyde and carvacrol on airborne microorganisms using an airwasher to vaporize the pure compounds diluted in water to 5.0 mg/m^3. The results showed the reduction of the microbial count by 45% after 1-h accumulation of cinnamaldehyde vapor and by 34% after 1-h accumulation of carvacrol vapor. The dilution of the antimicrobial agents in water may have contributed to the lower effect of carvacrol owing to the low solubility of this compound in water and reduced volatility due to solvation of its hydroxyl group in the aqueous phase, resulting in decreased antimicrobial activity in the vapor phase. However, the high antimicrobial activity of vaporized cinnamaldehyde in the study of Sato *et al*. (2006) is in contrast to our finding that aerosolized oregano EO and carvacrol were effective against *L. grayi* and *E. coli K12* while aerosolized cinnamon EO was not.

A different type of aroma diffuser from the one used in the present work was employed to generate EO vapor in the study of Doran *et al*. (2009), who investigated the effects of EO dispersed by an ST Pro^{TM} machine against antibiotic-sensitive and resistant bacteria. According to the authors, this machine has been used in UK hospitals as a fragrance generator, and release of vapor is by means of negative and Venturi airflow, without vibration and heat. The dispersing of an EO blend containing lemongrass and geranium EOs via the ST Pro^{TM} machine reduced 89% of airborne microbes in 15 h while natural evaporation of the blended EOs effected a 38% reduction of bacteria (suspension of 200 CFU) after 20 h of exposure. These results revealed that the EO vapor dispersed by the ST Pro^{TM} machine differed from the vapor produced by natural evaporation of the same EO blend in the extent to which it retarded growth of airborne microbes. Based on the results of TD-GC-

MS analysis, the authors suggested that this difference resulted from differences in the rates at which different components of the EO vapor were dispelled by the ST Pro^{TM} machine, leading to differences in relative proportions of limonene, α-pinene, β-myrcene, camphene, and 2-carene by comparison with the EO vapor produced by natural evaporation. Analogously, the results obtained by our aerosolization method may be affected not only by differences in the antimicrobial activities of different EOs but also by differences in their characteristics when aerosolized. Such differences, which might account for the low effectiveness of aerosolized cinnamon EO in the present study, would be highly dependent on the nature and conditions of the aerosolization process.

3.3.5 **Preliminary tests of aerosolization treatments with vegetables inoculated with *L. grayi***

The reduction in the total amount of residual *Listeria* on broccoli treated with aerosolized oregano-03 essential oil in a container connected to the ultrasonic aroma diffuser for different amounts of time (after 1 h accumulation of the aerosolized EO inside the container) after storage for 0 or 8 days were shown in **Fig. 3.29**. When broccoli samples were tested immediately after treatment (after storage of 0 day in **Fig. 3.29**), the total count of *Listeria* showed no significant differences between the treated samples and the controls ($P > 0.05$), indicating that the aerosolized oregano essential oil had no immediate bactericidal effect on *L. grayi*. Based on this result, additional samples were treated with aerosolized oregano-03 essential oil for different amounts of time and then stored at 4 ^{o}C for 8 days before determination of their total population of *Listeria*. Results showed that the reduction in the total count of *Listeria* after 8 days of storage increased with increasing treatment time except for 60 min, which indicated the effectiveness of aerosolized oregano essential oil in inhibiting the growth of *L. grayi* during storage at 4 ^{o}C; especially a large reduction was found in samples exposed to aerosolized oregano EO in 45 min. These results demonstrated the potential utility of aerosolization of oregano essential oil as a method of ensuring contact between the active compounds present in the essential oil and the broccoli floret surfaces.

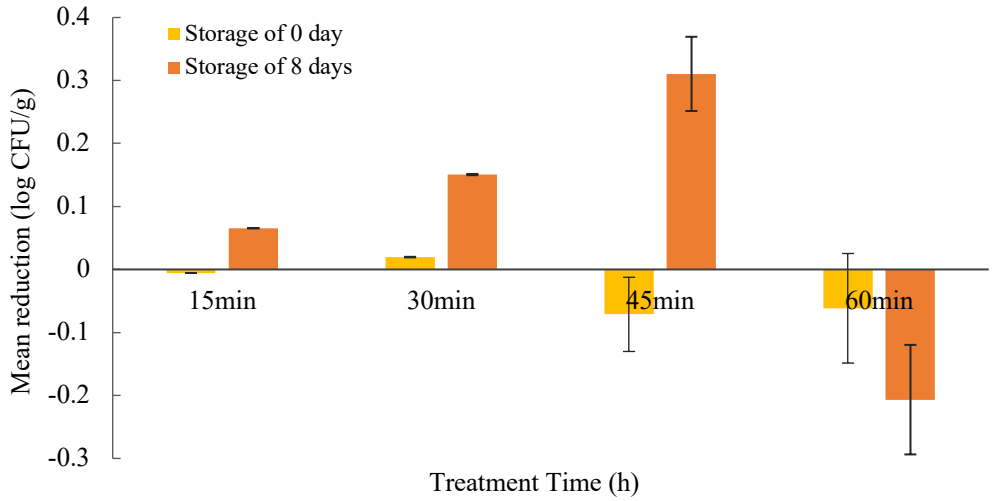


Figure 3.29 Reduction in total count of *Listeria* on broccoli exposed to aerosolized oregano EO for different amounts of time after different storage periods at 4 ºC. Reduction values were calculated by subtraction from the total count of *Listeria* on the controls.

3.3.6 **Preliminary storage tests of vegetables inoculated with *L. grayi* and treated with aerosolized oregano essential oil**

In the next set of experiments, broccoli samples were again treated with aerosolized oregano essential in the same manner as previously. Based on the previous results shown in Fig. 3.30, the treatment time employed was 45 min. Following the treatment with aerosolized oregano essential oil, samples were randomly separated into different sterilized bags and stored at 4 ºC. Over the course of 3 weeks, randomly selected bags were periodically removed to determine the total population of *Listeria*. The results obtained are compared with those for the matched controls (i.e., inoculated broccoli samples stored under identical conditions for the same amounts of time) in **Fig. 3.30.** In the first 15 days of the storage at 4 ºC, the *Listeria* population grew rapidly in the control group, increasing from 2.9 to 8.05 log CFU/g during 20 days of storage; however, the bacteria were not in stationary phase until 15 days. Over the same period, the population of *Listeria* in samples that had been treated with aerosolized oregano essential oil increased from 2.9 to 6.15 log CFU/g, corresponding to a reduction of the *Listeria* count by almost 2 log CFU/g in comparison with controls after 12 days of storage ($P<0.05$) and a reduction of *L. grayi* by 1.9 log CFU/g compared with control after 20 days of storage. Moreover, the growth rate of *L. grayi* was really low, which was in a 99% reduction of *L. grayi* load during the first 12 days of storage, demonstrating the aerosolized oregano EO was highly effective in inhibiting the growth of *L. grayi* at 4 ºC.

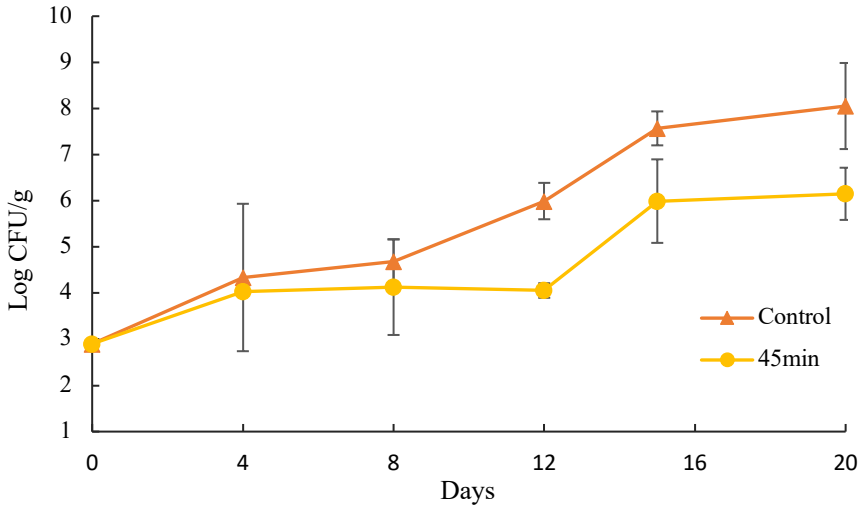


Figure 3.30 Long-term effectiveness of treatment with aerosolized oregano essential oil for 45 min immediately prior to packaging: tests with broccoli inoculated with *Listeria grayi* and stored at 4 °C for 20 days.

The results of this study indicated that treatment with aerosolized oregano-03 EO for 45 min can effectively inhibit the growth of *Listeria grayi* on packaged broccoli for 3 weeks at 4 °C. Similar investigations of the duration of antimicrobial effects of essential oils beyond an initial treatment period were performed by Fisher *et al*. (2009). In their study, samples of cucumber skin and lettuce leaf that had been inoculated with *Enterococcus* sp. were treated in a 600-L vapor chamber saturated with citrus vapor produced by heating a blend of citrus essential oils inside the chamber. Although these authors reported a 99% reduction of microbial load after 45-s exposure to the citrus vapor, testing of samples at various times following treatment showed continuous growth of *Enterococcus* sp., and between 6 and 8 h post-treatment, the growth rate was approximately the same as for the control samples. This study revealed the time limitation on the effects of treatment with citrus vapor on *Enterococcus* sp.in salad products, which would make it necessary to apply this treatment very soon before the product is consumed. In contrast, under the conditions employed in our study, treatment of broccoli florets with aerosolized oregano essential oil had inhibitory effects against the targeted bacteria (*Listeria*) for up to 3 weeks of storage at 4 °C.

3.4 **Conclusion**

Based on the results of *in vitro* tests, cinnamon (CIN-01 and CIN-03), thyme (THY-02), and oregano (ORE-02 and ORE-03) essential oils and carvacrol were very effective in inhibiting the growth of *L. grayi* and *E.coli* K12 through direct contact and in the vapor phase. Cinnamon-03 and oregano-03 essential oils and carvacrol proved to be effective in the vapor phase in inhibiting the growth of *L. monocytogenes* strains; the slightly lower susceptibility of *L. grayi* makes it a suitable non-pathogenic surrogate in this study. Cellulose stickers impregnated with these EOs were found to be effective in inhibiting the growth of bacteria *in vitro* and cinnamon essential oil at a concentration of 556 µl per liter of headspace completely inhibited the growth of *L. grayi* and was shown to have bactericidal activity. In a sensory evaluation of frozen vegetable products that had been stored at 4 ^{o}C in active packaging incorporating oregano or cinnamon EO, products had been exposed to cinnamon EO vapor were generally less acceptable to panelists than those exposed to oregano EO vapor. In addition, delivery of aerosolized oregano EO onto broccoli in a sealed container was found to be effective in inhibiting the growth of *L. grayi* in a reduction of 2 log CFU/g in comparison with control after storage at 4 ^{o}C after 12 days.

CONNECTING STATEMENT 2

Chapter III completed the study of the effectiveness of essential oils in inhibiting the growth of *L. grayi in vitro* and in storage tests with vegetables. Cinnamon and oregano essential oils and carvacrol were found to be effective in the vapor phase in inhibiting the growth of *Listeria* and *E. coli* strains. Cellulose stickers impregnated with these essential oils and placed inside packaging were found to be effective in reducing the *Listeria* count on vegetables during storage. In the research presented in Chapter IV, conditions for the preparation of soy protein films as carriers for these essential oils in active-packaging applications were investigated. The effects of incorporating these essential oils in soy protein films on the film characteristics, including mechanical, moisture barrier and optical properties, were explored, and antimicrobial activities of the films were evaluated. Furthermore, Fourier transform infrared (FTIR) spectroscopy was used to study changes in protein structure in films incorporating carvacrol and oregano and cinnamon essential oils.

This work will be first presented as an oral presentation at the 2020 AOCS Annual Meeting & Food-American Oil Chemists' Society. This chapter will subsequently be submitted as a manuscript for publication in the journal *Food Control*.

Tao, R., Sedman, J., & Ismail, A. (2020) Characterization of Biopolymer Films Incorporating Essential Oils Exhibiting Antilisterial Activity as Active-Packaging for Ready-to-Eat Foods. 2020 AOCS Annual Meeting & Food Expo, Montreal, Canada, April 26- 29.

Tao, R., Sedman, J., & Ismail, A. (2020) Preparation and Characterization of Biopolymer Films Incorporating Essential Oils Exhibiting Antilisterial Activity as Active Packaging for Ready-to-Eat Foods. *To be submitted*.

CHAPTER 4 PREPARATION, CHARACTERIZATION AND *IN VITRO* ANTIMICROBIAL STUDY OF BIOPOLYMER FILMS INCORPORATING ESSENTIAL OILS

Abstract

Soy protein, which is a by-product of soybean oil extraction, has been used in several studies as the carrier of essential oils in active packaging, such as edible films and coatings. In the present study, conditions for the preparation of soy protein films as carriers for essential oils in active-packaging applications were investigated. The mechanical properties of films cast from solutions of soy protein isolate (SPI; 5% w/v) depended on the concentration of glycerol added to the solution as a plasticizer, the pH of the solution, and the heating temperature/time applied. These experiments resulted in the selection of the following conditions for film preparation: 2% glycerol, pH 10 for the film-forming solution and heating of this solution at 85 °C for 10 minutes. These conditions were used for the preparation of soy protein films incorporating carvacrol, oregano essential oil, and cinnamon essential oil. The mechanical properties, water vapor permeability (WVP), and opacity of these SPI films were influenced by the type of essential oil, the concentrations of glycerol and essential oil, and the emulsification treatment applied. The emulsified carvacrol and oregano essential oil acted as plasticizers like glycerol to reduce resistance and rigidity of SPI films and increase their extensibility, resulting in increases in elongation at break (EAB) coupled with decreases in tensile strength (TS) and Young's modulus of SPI films. In contrast, incorporation of cinnamon essential oil in SPI films had the opposite effect on the mechanical properties of the films. The addition of glycerol and essential oils was found to increase the WVP of SPI films, whereas the effect of the essential oils on the rate of water vapor transmission through the films depended on the type and concentration of essential oil. High concentrations of glycerol and essential oils improved the transparency of SPI films, which was also affected by the emulsification treatment employed. Fourier transform infrared (FTIR) spectroscopic analysis of protein secondary structure in the films revealed the transformation of β-sheet to α-helical structure with addition of carvacrol and oregano essential oil, which may have been a contributing factor to the increased flexibility of the films with higher content of carvacrol and oregano essential oil. In addition, the vapor-phase release of essential oil active compounds from the films was evaluated by determining the effectiveness of the films in inhibiting microbial growth *in vitro*. Films prepared with ≥1.5% oregano EO or ≥2% carvacrol in the film-forming solution exhibited high antimicrobial activities against *L. grayi* in the vapor phase and could be employed in active packaging to improve the safety of vegetable products.

Keywords：Soy protein film, essential oils, glycerol, mechanical properties, water vapor permeability, opacity, FTIR spectroscopy.

4.1 **Introduction**

Plastic, the most common packaging material, is increasingly produced; however, only 5% of plastic packaging is recycled, causing an enormous accumulation of plastic in the environment. Environmental pollution caused by synthetic packaging materials has triggered the development of novel eco-friendly materials. Biopolymer films and coatings are thereby increasingly studied because of their biodegradability (Espitia *et al*, 2014). Unlike coatings, films are self-supporting and are generally located on the food surface or between different food components and are used to wrap food products (Guilbert *et al*., 2005; Salgado *et al*., 2015). Edible films are traditionally used to improve food appearance and prolong shelf life. Edible films as food packaging materials can protect foods from physical, chemical and biological deterioration in order to enhance food quality and prolong shelf life. For this purpose, they should have good barrier properties against gas, moisture, oils and solutes. Furthermore, their mechanical properties can provide protection against mechanical damage during transportation. Like all edible materials, edible films should be safe in accordance with their intended use and processed under good manufacturing practices. Currently, edible films or coatings are mainly produced from edible biopolymers and food-grade additives. Many materials can be used to manufacture edible films, including polysaccharides, lipids, and proteins as well as mixtures of two or more of these components (Khwaldia *et al*., 2004).

Soybean protein is one of the most widely available plant proteins and has been used in food products for a long time, especially in Asian countries. It has been cultivated in China for more than 3000 years and can be utilized in many forms such as tofu, natto, and miso (Nishinari, 2014). Soybeans contain approximately 20% oil and 40% protein on a dry matter basis. Soy protein isolate (SPI) is obtained from soybeans by removing the oil at low temperature. SPI is a mixture of proteins, which can be classified into four types according to their sedimentation coefficients 2S, 7S, 11S and 15S (Nishinari, 2014). Studies have shown that soy proteins have excellent film-forming ability and that their films have good oxygen barrier properties (Miller and Krochta. 1997; Cho *et al*., 2007) and are low cost and biodegradable. In the case of edible films made of the subunits of SPI, 11S-based films had greater tensile strength than 7S-based films due to the high concentration of cysteine groups in glycinin (Kunte *et al*., 1997). However, edible films made only of soy protein are brittle and exhibit poor moisture resistance. Incorporation of other materials, such as polysaccharides, lipids or plant extracts, can improve the mechanical or physical properties of soy protein-based films. Sivarooban *et al*. (2008) reported that the incorporation of grapeseed

extract (GSE), nisin and EDTA significantly increased the thickness and puncture and tensile strengths of soy protein films. Friesen *et al*. (2015) investigated the mechanical, optical and water vapor barrier properties of SPI-based edible films with addition of rutin and epicatechin as cross-linking agents and he found that rutin can be used to improve specific properties of SPI films. Special components, such as antimicrobials, nutraceuticals, and flavors in the films, can prevent food from oxidation, inhibit microbial growth and bring some additional functional properties to films (Han and Scanlon, 2014).

Food degradation is mainly caused by spoilage microorganisms and growth of foodborne pathogens. Spoilage microorganisms in food can produce toxins, accelerate oxidation processes, decompose nutrients, and produce changes in the structure and sensory properties of food products (Saggiorato *et al*., 2012), whereas foodborne pathogens can result in illnesses in humans. To solve this problem, antimicrobial packaging, which could extend shelf life and ensure the quality of food products, has been proposed. Essential oils (EOs) are volatile and aromatic oily liquids extracted from some herbs and spices, such as flowers, leaves, seeds, roots and fruits, and have been recognized as antimicrobial agents (Burt, 2004). The antimicrobial mechanisms of EOs involve various effects on microbial cells, including disrupting enzyme systems, attacking the phospholipid bilayer of the cell membrane, and compromising the genetic material of bacteria (Burt *et al*., 2007). Several studies reported antibacterial or antifungal activity of EOs incorporated in edible films. Seydim *et al*. (2006) found that the whey protein films incorporated with oregano EO were the most effective against growth of *Staphylococcus aureus, Escherichia coli* O157:H7*, Listeria monocytogenes, Salmonella enteritidis* and *Lactobacillus plantarum*. Arancibia *et al*. (2014) developed soy protein, lignin and formaldehyde films with citronella EO. With 3% citronella EO in the film-forming solutions, the films showed good antifungal activity against *Fusarium oxysporum* in bananas. In addition, there was a large reduction in total molds, yeast and aerobic mesophiles on bananas covered by these films during storage. Emiroglu *et al*. (2010) reported that soy edible films with oregano EO, thyme EO or a mixture of both had higher inhibitory effects against *E. coli, E. coli* O157:H7 and *S. aureus*. However, when these antimicrobial films were applied on ground beef patties, there was no significant effect on total viable counts. While the effects of EOs incorporated in soy protein films on various bacterial and fungal species have been reported, there is little information about their efficacy in inhibiting the growth of *Listeria* spp. *in vitro* or on food.

Likewise, incorporation of EOs in protein films also has various impacts on the physical properties of the films, including their mechanical properties, water vapor permeability (WVP) and transparency. Pires *et al*. (2011) found that the effects of incorporation of EOs in films on tensile properties were quite complex and could be influenced by the characteristics of the EO and its interaction with the protein matrix. Both positive and negative effects of EOs were reported on TS and elongation of protein films. Tongnuanchan *et al*. (2012) found that addition of citrus oil caused a decrease in TS but an increase in extensibility of fish skin gelatin films (Limpisophon, 2010), however, increases in TS were reported by Atarés *et al*. (2010a) for soy protein isolate films and by Ojagh *et al*. (2010) for chitosan films with cinnamon EO. Similarly, owing to the complex composition of EOs, various effects on WVP have been reported for different EOs and polymer matrices. Thus, how the EO could have such complex effect on structure of protein need to be studied well. Fourier-transform infrared – attenuated total reflection spectroscopy (FTIR-ATR) were usually used to collect the FTIR spectrum for protein films, however, most of the film spectrum were only analyzed and discussed by their original spectrum without any further analyzed processes, especially for their secondary structure in amide bands. Some changes of secondary structure in original spectrum were not shown clearly and even hidden under the overlapped peaks. Furthermore, the interference effect of some EOs on analyzing the secondary structure of protein films were attributed to the appearance of some high intensity peaks in the 1700-1600 cm^{-1} region for EO. Therefore, the FTIR spectrum of protein films have to been analyzed well after removing these interfere information.

To date, such high concentration of carvacrol, oregano and cinnamon essential oil incorporated into soy protein films and their efficiency of retarding growth of *L. grayi in vitro* were rarely reported in many studies of biopolymers packaging material, meanwhile, the different concentration of glycerol have not been studied well within the soy protein film incorporated with different types of essential oils. Furthermore, the secondary structure of soy film incorporated with EO were rarely studied and further analyzed by FTIR-ATR. Therefore, this chapter was aimed to optimize the parameters of soy protein film preparation and investigate the effects of different concentrations of glycerol and essential oils on the film characteristics, and comparison of efficiency of different essential oils and emulsified treatments on film properties. Lastly, the effectiveness of vapor-phase release of essential oil active compounds from the films in inhibiting the growth of *L. grayi* was determined *in vitro*.

4.2 Materials and methods

4.2.1 Microorganisms and chemicals

Microorganisms: The Gram-positive bacterium *Listeria grayi* (ATCC 25401) and Gram-negative bacterium *Escherichia coli* K12 were obtained from the Laboratoire de santé publique du Québec (LSPQ) and maintained on 5% sheep's blood agar at 4 °C. All microorganisms and their inoculum were stored and prepared as previous described in **3.2.1.1**.

Chemicals: The cinnamon and oregano essential oils employed in this chapter were supplied by BSA Frutarom (Saint-Leonard, QC, Canada) and Novo-taste Corporation Inc. (Dollard-des-Ormeaux, QC, Canada) and correspond to those designated as CIN-03 and ORE-03 in Chapter III. Carvacrol (98% purity) was obtained from Sigma Aldrich (St. Louis, MO, USA). Soy protein isolate (SPI) (85%) was purchased from Cargill Corporation. (Minneapolis, MN, USA). Hydrochloric acid, sodium hydroxide, glycerol, sodium chloride, peptone water, trypsin soya broth (TSB), brain heart infusion agar (BHIA), Mueller Hinton agar (MHA) and cheesecloth (grade 40) were purchased from Fisher Scientific.

4.2.2 Film Preparation

4.2.2.1 Soy protein-based films

Film-forming solutions were prepared by stirring soy protein isolate (SPI) (5%, w/v) and different concentrations of glycerol (1%, 1.5%, 2%, and 2.5%, w/v) in distilled water for 15 min. The pH values of the solutions were adjusted to 4.5, 7, 8.5 and 10 by addition of 2M sodium hydroxide. Subsequently, the solutions were placed in a water bath (HB-250, IKA Works Inc. Wilmington, NC, USA) set at 75 °C or 85 °C. After reaching the temperature of the water bath, solutions were kept in the water bath for 0, 2, 5, 10, 20, or 30 min. Solutions were then filtered through cheesecloth (grade 40) to remove foam and undissolved impurities. The filtered solutions were poured into Petri plates (100 mm × 15 mm) and left to dry at room temperature for about 48 h. Cast films were then peeled off the plates and stored in a desiccator at 25 °C and 52% RH.

4.2.2.2 Incorporation of carvacrol into soy protein films

A solution of SPI (5%, w/v) and glycerol (0%, 1%, 1.5%, 2%, 2.5%, or 3% w/v) in distilled water was stirred for 15 min. After adjusting the pH to 10, the solution was heated in a water bath at 85 °C for 10 min and then cooled down and filtered through cheesecloth (grade 40). Carvacrol was added to the filtered solution at a concentration of 1%, 2%, or 3% (w/v) and

mixed in by magnetic stirring for 10 min. Then different emulsification methods were applied, including homogenization with a high-shear Polytron homogenizer (Westbury, NY, USA) for 1.5 min, addition of Tween 80 and stirring for 10 min, or the combination of these treatments; control samples received no emulsification treatment The emulsion was left to degas for 1 h and then were poured into Petri plates and left to dry at room temperature for 48 h. Cast films were then peeled off the plates and stored in a desiccator at 25 °C and 52% RH.

4.2.2.3 **Incorporation of oregano and cinnamon essential oils into soy protein films**

Based on the results from the previous experiment, a glycerol concentration of 1.5% was chosen for the preparation of films incorporating essential oils. A solution of SPI (5%, w/v) and glycerol (1.5% w/v) in distilled water was stirred for 15 min. After adjusting the pH to 10, the solution was heated in a water bath at 85 °C for 10 min and then cooled down and filtered through grade 40 cheesecloth. Then oregano essential oil (0.5%, 1%, 1.5%, or 2% w/v) or cinnamon essential oil (0.5%, 0.75%, 1%, or 1.25% w/v) was added and mixed into the solution for 10 min followed by homogenization with a high-shear mixer for 1.5 min. The emulsion was left to degas for 1 h and then poured into Petri plates and left to dry at room temperature for 48 h. Cast films were then peeled off the plates and stored in a desiccator at 25 °C and 52% RH.

4.2.3 **Characterization of films**

4.2.3.1 **Light transmission and transparency**

Light transmission through the films was quantified at selected wavelengths in the range between 325 and 700 nm by a spectrophotometer (Thermo Scientific, USA). Films were cut to a size of 0.8 cm x 3 cm and placed in cuvettes; an empty cuvette was used as blank. Opacity of films as a function of wavelength was determined by the following equation (1):

$$\text{Opacity } (\lambda) = A_{\lambda} / x \qquad (1)$$

where A_{λ} is the absorbance at wavelength λ, and x is the film thickness (μm). Transparency of films is commonly evaluated on the basis of light transmission at 600 nm (Han and Floros, 1997), where a lower opacity value corresponds to greater transparency.

4.2.3.2 **Tensile testing**

All films were conditioned for 48 h in an environmental chamber at 25 °C and 52% RH before testing. Tensile testing was performed with an Instron Universal Testing Instrument (model 4500, Instron Corporation, Canton, MA, USA) on film strips (6.4 × 0.96 cm). Initial distance of separation was set at 30 mm, while crosshead speed was set at 5 mm/min. Tensile strength (TS) (MPa) was calculated by dividing maximum (peak) load developed during the test by initial cross-sectional area of the film specimen. Percentage elongation at break (EAB%) was calculated by dividing film extension at the moment of rupture by 30 mm (initial gauge length) and multiplying by 100. Young's modulus was calculated by the Young's modulus equation: E = tensile stress/tensile strain = (FL) / (A * change in L), where F is the applied force, L is the initial length, A is the square area, and E is Young's modulus in units of pascal.

4.2.3.3 **Water vapor permeability**

Water vapor permeability (WVP) was determined by the ASTM method E 96-95 (ASTM, 1995) with slightly modification (Wu *et al*., 2013 & 2017). Glass cups were filled with 12 ml of distilled water to a height of 20 mm from the top edge and sealed by film samples onto the circular mouth of the cups (2.713 x 10^{-4} mm^2 of film area) with silica gel. The sealed cups were weighed and then placed in a desiccator at 24 °C and 30% RH with anhydrous calcium chloride. The water transported through the film and adsorbed by the desiccant was determined from the weight loss of the glass permeation cell (every 1 h over a period of 24 h). Weight loss over time was plotted to obtain the slope ($r^2 \geq 0.99$):

$$WVP\ (g\ mm/m^2\ h\ kPa) = WVTR(L/\Delta P) = \Delta m \cdot L\ /(A \cdot t \cdot \Delta P) = \Delta m \cdot L\ /[A \cdot t \cdot S \cdot (R_1 - R_2)] \qquad (2)$$

where WVTR is the water vapor transmission rate ($g/h \cdot m^2$) through a film, L is film thickness (mm), $\Delta m/t$ is the slope of the plot of weight (0.0001 g) loss of the cup vs. time (g/h), A is the area of the exposed film on the cup (m^2), and ΔP is the partial water vapor pressure difference between the two sides of the film (kPa), S is the saturation vapor pressure at the test temperature ([mm Hg (1 mm Hg = 1.333 x 10^2 Pa)], R_1 is relative humidity at the source expressed as a fraction, and R_2 is relative humidity at the vapor sink expressed as a fraction.

4.2.3.4 **Water solubility**

To determine the water solubility of films (Kavoosi *et al.,* 2013), 20 mm x 20 mm portions of films were dried in an oven at 104 °C for 24 h and their initial dried weight (W_i) measured. Then, the dried films were separately immersed in 25 ml of distilled water and gently shaken at room temperature for 24 h. Thereafter, the insolubilized film portions were taken out and dried in the oven again for 24 h. The weights of the final films were measured as W_f. All films were tested in triplicate. The solubility percentage (S%) or the weight loss percentage was calculated from the following equation (3):

$$S\ (\%) = [(W_i - W_f) / W_i] \times 100 \quad (3)$$

4.2.3.5 **Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy**

Infrared spectra were recorded in the range between 4000 and 400 cm^{-1} with an Excalibur FTIR spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an attenuated total reflectance (ATR) accessory fitted with a 2-mm-diameter diamond ATR crystal (Specac, Orpington, UK). Films were placed on the surface of the diamond ATR crystal and scanned in triplicate at a resolution of 4 cm^{-1} with co-addition of 64 scans. Spectra were baseline-corrected using OMNIC 7.0 software (Thermo Electron Corp.) prior to further analysis.

4.2.3.6 **The antimicrobial activities of soy protein films incorporating carvacrol, oregano essential oil and cinnamon essential oil**

Films prepared in previous section (**4.2.2.2** and **4.2.2.3**) with different concentration of carvacrol, oregano and cinnamon essential oils were cut into a disk shape, and both sides of the film disks were sterilized under ultraviolet light for 15 min prior to use. All the plates had been smeared with 100 µl of a standardized inoculum containing 10^6 CFU/ml of *L. grayi* or *E. coli* K12, respectively. For disk diffusion tests, the film disks were then placed on BHIA or MHA plates, while, for vapor diffusion test, each film disk was laid on the inside surface of the upper lid of the Petri dish, with no direct contact between the disk and the plated bacteria. Plates were incubated at 37°C for 48 h. At the end of that period, the diameter of inhibition zone was measured with a caliper (mm).

4.2.4 **Statistical analysis**

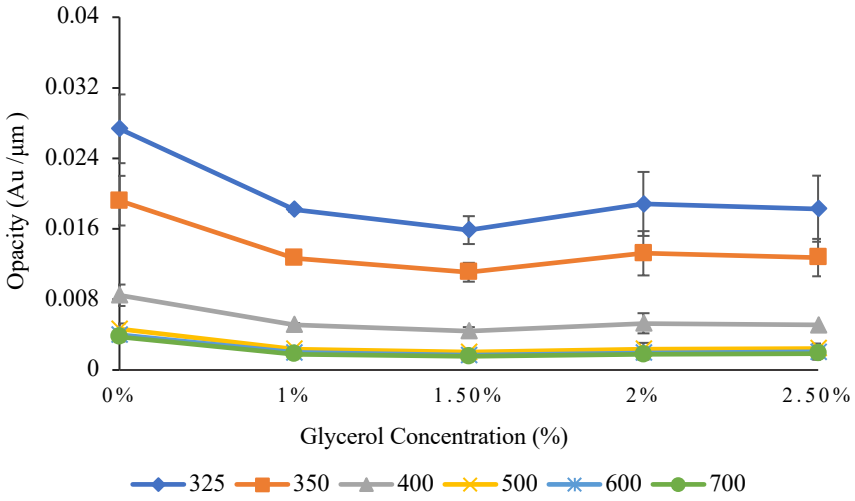
All experiments were performed in triplicate. The data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine statistical significance of differences ($p < 0.05$) between means. SPSS statistics program was applied for the statistical analysis.

4.3 **Results and discussion**

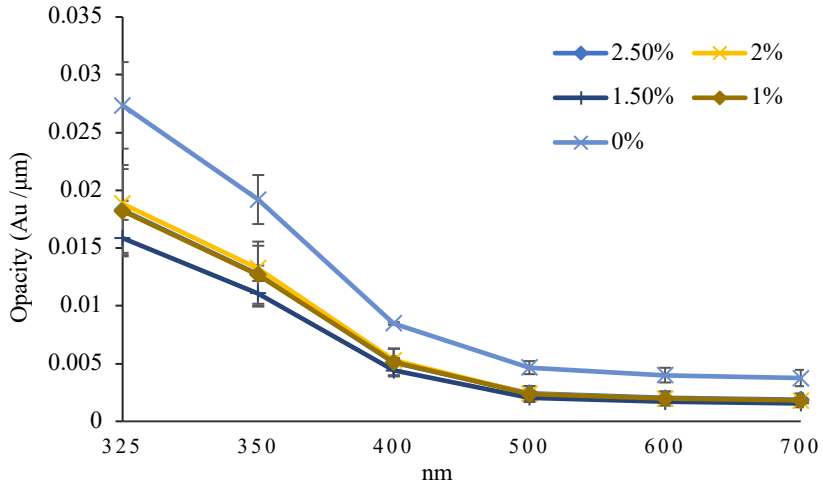
4.3.1 **Optimization of conditions for preparation of soy protein films**

4.3.1.1 **Evaluation of opacity of SPI films**

The opacity or transparency of the films is one of the important properties of packaging material, as they enhance product appearance and provide protection against light during storage (Lee *et al.*, 2015). Opacity of films prepared from solutions of soy protein isolate (SPI) with different glycerol concentrations as well as without addition of glycerol was measured spectrophotometrically at 325, 350, 400, 500, 600 and 700 nm. All the film-forming solutions were prepared at pH 10 and heated for 10 min at 85 °C.



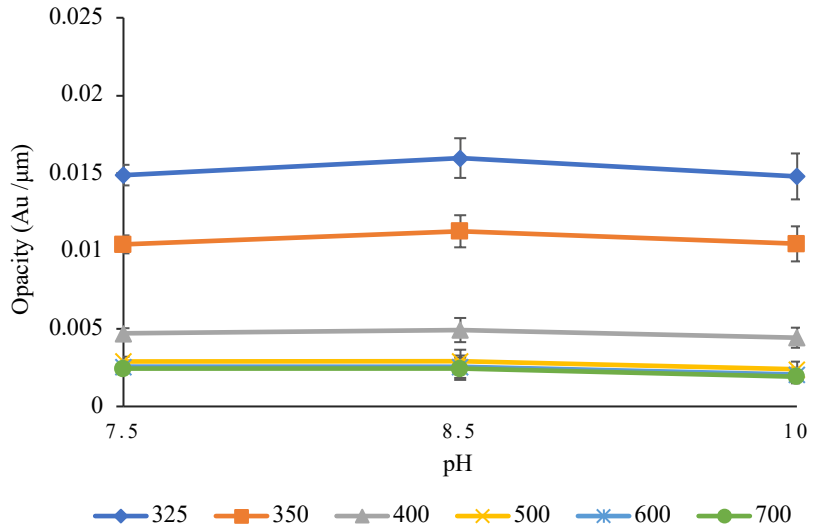
(A)



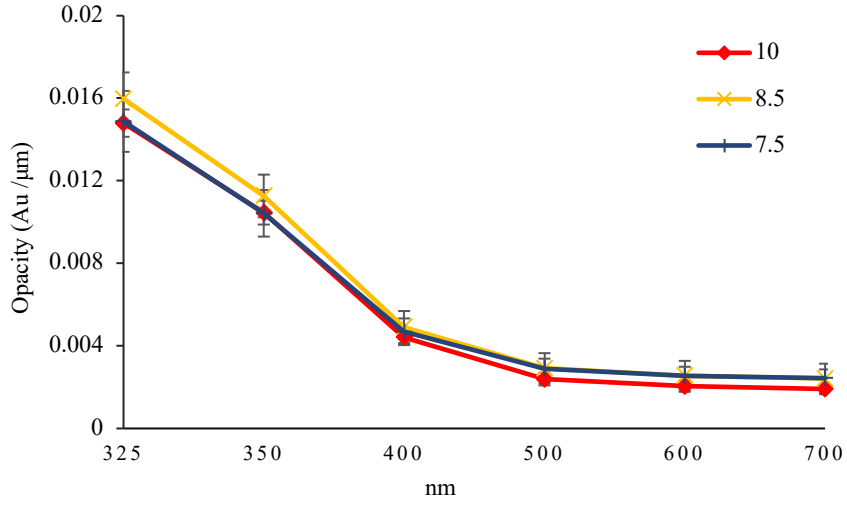
(B)

Figure 4.1 Effect of concentration of glycerol in the film-forming solution on opacity of soy protein-based films.

When the glycerol concentration increased from 0 to 1%, the opacity of the films significantly decreased (P≤0.05) at all wavelengths (in **Fig. 4.1**). However, there was no significant decrease with further increase in the glycerol concentration. The opacity at short wavelengths, especailly in the ultraviolet range, was higher than in the visible range for all the films. Li *et al.* (2008) found that the amount of aromatic amino acid groups in SPI peptides resulted in UV light-barrier properties of SPI films. The opacity of the films was fairly constant between 500 and 700 nm. Transparency of films is commonly evaluated on the basis of light transmission at 600 nm. The results presented here, showing that the presence of glycerol in the SPI films led to a reduction in opacity values at all wavelengths, indicate that glycerol can improve the transparency of SPI films.



(A)

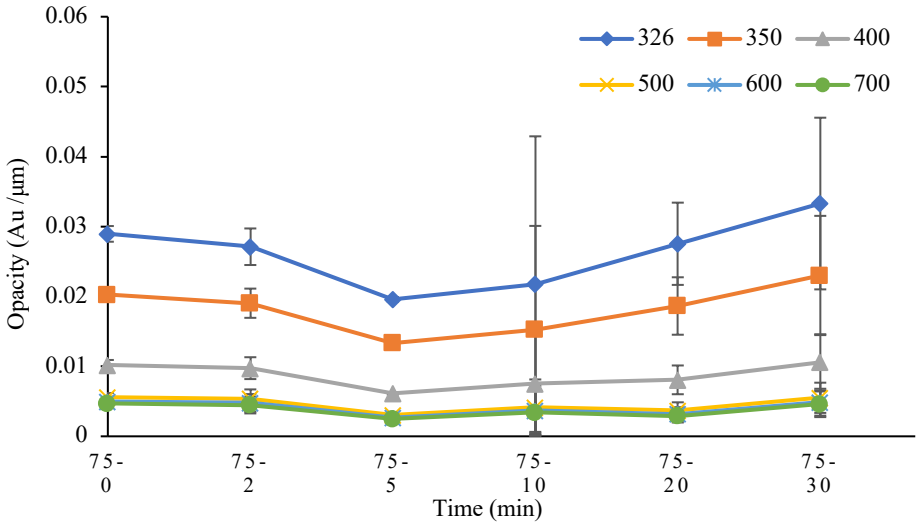


(B)

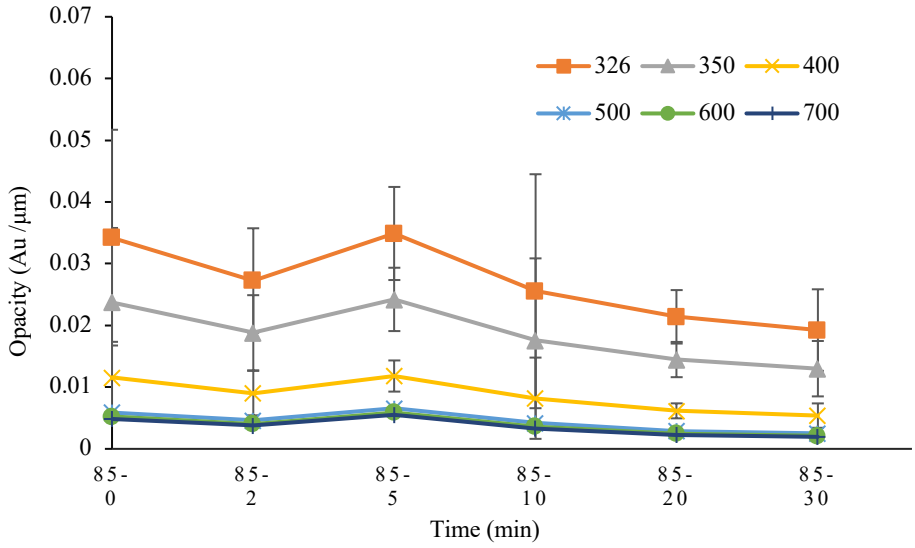
Figure 4.2 Effect of pH of film-forming solution on opacity of SPI films.

The opacity of SPI films can be affected by the pH of the film-forming solution. For example, Brandenbury *et al*. (1993) found SPI films formed at pH 6 were opaque with many insoluble particles in them, owing to the limited solubility of soy protein at that pH. Similarly, Cho *et al*. (2006) demonstrated that the surface of SPI films prepared at neutral pH was uneven and contained unsolubilized particles, while the solubility of the protein was increased when the pH was made alkaline. In the present study, there were no significant differences ($P>0.05$) in opacity among the films obtained when the pH of the SPI film-forming solution was 7.5, 8.5 and 10 (**Fig. 4.2**). When the pH of the film-forming solution was reduced to 4.6, the proten precipitated during heating of the film-forming solution because this pH corresponds to the isoelectric point of soy protein. Gennadios *et al*. (1993) reported that formation of soy protein films was hindered by poor protein dispersion in the film-forming solution when the solution pH was near the isoelectric point of soy protein. Generally, pH is a very important paremeter with respect to dispersion and solubilization of proteins, and higher pH makes the protein disperse more easily in solution. A high content of ionized polar amino acids contributes to a protein's sensitivity to pH (Swain *et al*., 2004). Treatment of soy protein at high pH denatured the protein and changed its structure from globular to extended chain, and thus more protein-protein interactions would occur (Brandenburg *et al*., 1993; Cao *et al*., 2007). Based on the analysis above, higher pH could be chosen for the preparation of soy protein edible films.

Heating temperatures of 75 ^{o}C and 85 ^{o}C are commonly used in making SPI films. **Fig. 4.3** shows the opacity of films cast from alkaline (pH 10) film-forming solutions containing 2% glycerol as a function of heating time at these temperatures. When the solutions were heated at 75 ^{o}C, there was no trend with heating time. However, when the heating temperature was increased to 85 ^{o}C, the opacity of the films decreased with increasing heating time. Furthermore, the films were more homogeneous and transparent to the naked eye when the film-forming solution had been heated for a longer period of time. The longer heating time favoured the dispersion of soy protein in the solution and increased the solubility of SPI (Cao *et al*., 2007). Similarly, Stuchell and Krochta (1994) found that heat treatment produced soy protein films that were more transparent and smoother than those obtained from film-casting solutions that had not received any heat treatment.



(A)



(B)

Figure 4.3 Effect of heating time of film-forming solution at 75 ºC (A) and 85 ºC (B) on opacity of SPI films.

4.3.1.2 **Mechanical properties of SPI films**

The mechanical properties of edible films are mainly determined by tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus, which are linked to intermolecular and intramolecular polymer interactions. **Figs. 4.4** and **4.5** show the tensile strength and percentage elongation at break of the soy protein-based films cast from film-forming solutions with various glycerol concentrations and pH values. From **Fig. 4.4**, it can be seen that there was no signifcant difference in TS with change in the pH of the film-forming solution, whereas the percentage elongation at break increased when the pH was adjusted from neutral to alkaline. The films cast from pH 8.5 and pH 10 solutions had significantly larger values of elongation at break than those cast from pH 7.5 solutions. This finding is consistent with the results obtained by Brandenburg *et al*. (1993) in comparing the mechanical properties of SPI films prepared at pH values in the range of 6-12. The films prepared at pH 6 had the lowest tensile strength and elongation at break, while slightly higher values were obtained for films prepared at pH 8 and pH 10.

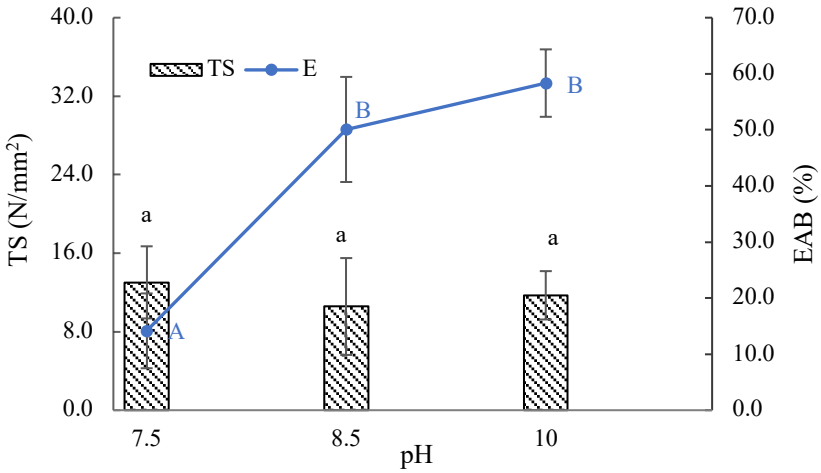


Figure 4.4 Effect of pH of the film-forming solution on tensile strength (TS) and elongation at break (EAB) of SPI films. (Different letters represent significant differences, $P \leq 0.05$).

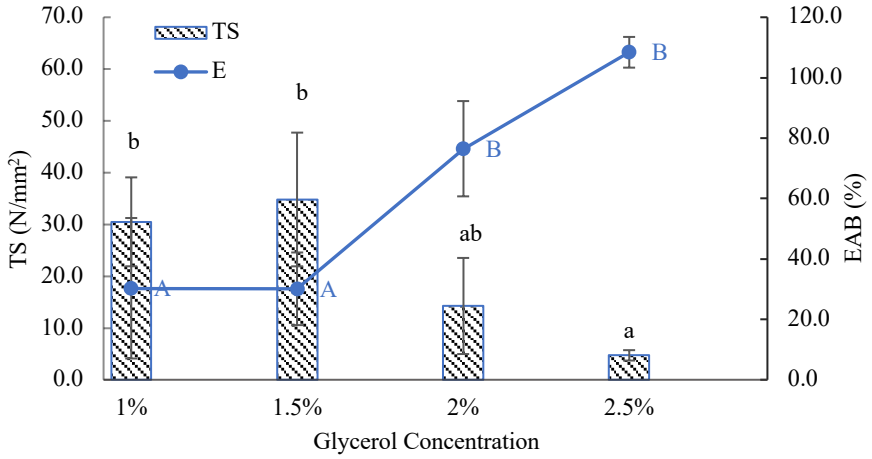


Figure 4.5 Tensile strength (TS) and percentage elongation at break (EAB) of SPI films prepared with various concentrations of glycerol in the film-forming solution. (Different letters represent significant differences, $P \leq 0.05$).

Fig. 4.5 shows that the TS of the films decreased while the EAB value increased with addition of glycerol to the film-forming solution. The TS of films cast from 2.5% glycerol solutions was significantly lower than that of films cast from 1% and 1.5% glycerol solutions. Meanwhlie, films cast from solutions with glycerol concentrations of 2-2.5% had significantly higher EAB values than the other films. Therefore, adding glycerol into the film-forming solution decreased tensile strength and increased extensibility of SPI films. Similarly, Cho and Rhee (2002) found that increasing glycerol content decreased TS of soy protein-based films from around 20 MPa to 5 MPa, while the elongation at break increased at 50% relative humidity. Kim *et al*. (2003) also reported that the TS of soy protein films decreased with increasing glycerol content while EAB% showed the opposite trend. The soy protein-based films with low glycerol content were very brittle because SPI was in a glassy state (Lim *et al*., 1999). The addition of glycerol as a plasticizer can reduce hydrgen bonding between protein chains and also increase the spacing of protein molecules (Lieberman *et al*., 1973). Meanwhile, the presence of a hydrophilic plasticizer such as glycerol leads to large increases in the amount of water absorbed by soy protein films, resulting in large decreases in TS (Song *et al*., 2011). Therefore, considering the flexibility of films, a glycerol concentration of 1.5%-2.5% in the film-forming solution would be optimal.

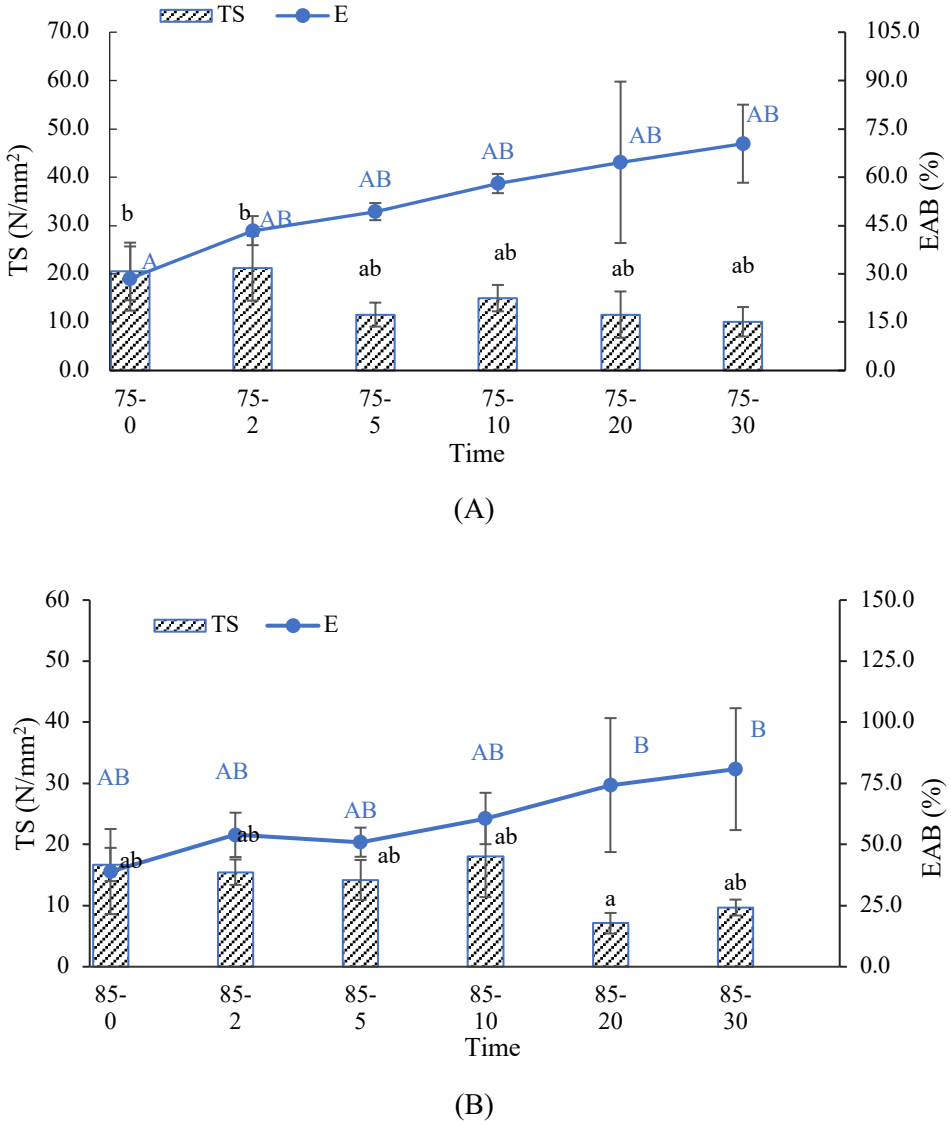


Figure 4.6 Tensile strength (TS) and elongation at break (EAB%) of SPI films cast from solutions that had been heated at 75 ° C (A) and 85 °C (B) for different amounts of time. (Different letters represent significant differences, $P \leq 0.05$).

Fig. 4.6 shows the effects of different thermal treatments of the film-forming solution on the tensile strength and elongation at break of the soy protein-based edible films. The heating time in **Fig. 4.6** refers to the amount of time the film-forming solution was heated after reaching the specified temperature. Heating the film-forming solution at 75 °C for ≥5 min resulted in a decrease in the TS of the films. In contrast to TS, the percentage elongation of films at break showed an upward trend when the heating time of the film-forming solution was longer, but no statistically significant differeces were detected among the EAB values of the films. The same trends were observed for films cast from solutions heated at 85 °C for different amounts of time. The TS of films cast from solutions heated at 85 °C for 20 and 30 min was lower than that of films cast from solutions heated for ≤10 min. Moreover, the

percentage elongation at break was higher when the film-forming solution had been heated for 20-30 min. Pérez-Gago and Krochta (2001b) and Pérez-Gago *et al*. (1999) studied the effect of heating time and temperature on mechanical properties of whey protein isolate (WPI) films. The results showed that heating of the film-forming solution at 70-100 ºC for 5-20 min resulted in stronger and more extensible WPI films, although the differences in the TS and EAB values of films cast from solutions heated at 80 and 90ºC were not statistically significant. In the case of soy protein, several groups have reported on the effects of heat treatment of the film-forming solutions in relation to the mechanical properties of films (Cao & Chang, 2001; Cao *et al*., 2007; Sabato *et al*., 2001; Stuchell & Krochta, 1994). Heat treatment of SPI disrupted the quatemary structures of the 7S and 11S protein fractions. Moreover, due to unfolding of the three–dimensional structure of 11S, some hydrophobic groups buried inside the protein, including sulfhydryl groups, disulfide bonds and hydrophbic residues, were exposed to water. Under the action of heat, intermolecular polymeriztion occurred via formation of S-S bonds and hydrophobic interactions, and thus the mechanical properties of films were improved. Kunte *et al*. (1997) reported that the TS of soy protein films cast from solutions of commercial and lab-prepared SPI were 8.53 ± 0.48 MPa and 10.68 ± 0.95 MPa, respectively. In our experiments, the TS of films cast from solutions that had been heated for 20-30 min was similar to the values reported by Kunte *et al*. (1997), but higher values were obtained for films cast from solutions that had been heated for ≤10 min. Accordingly the conditions we selected for treatment of the film-forming solution were heating at 85 ºC for 10 min.

4.3.1.3 **Water vapor permeability of SPI films**

Protein films generally display poor resistance to water permeation because of their hydrophilic character (Krochta *et al*., 1997). In the case of soy protein films, the large number of polar amino acids in soy protein makes the films highly sensitive to water vapor (Rhim & Lee, 2004). As a result, the WVP values for soy protein-based films are high in comparison with the values reported for some whey protein-based films, (Ferreira *et al*., 2009).

As shown in **Table 4.1**, no significant differences were detected among the WVP values of soy protein films cast from solutions heated at 75 ºC and 85 ºC for different amounts of time. The WVP values are similar to those reported for soy protein films by Kunte *et al*. (1997) and Otoni *et al*. (2016) and lower than the WVP values of soy protein films studied by Jiang *et al*. (2012). In the latter study, the WVP of films slightly decreased as the heating temperature of

the film-forming solution increased, and this reduction was attributed to the exposure of hydrophobic groups or the aggregation of the hydrophobic protein (Jiang *et al*., 2010). This trend is also observed in **Table 4.1** except in the case of films cast from solutions cooled immediately upon reaching the targeted heating temperature (i.e., heating time = 0).

Table 4.1 Water vapor permeability (WVP) of soy protein-based edible films cast from solutions subjected to different heat treatments.

Heat treatment (°C-min)	Thickness (mm)	WVP ($mm \cdot g/m^2 \cdot h \cdot kPa$)
75-0	0.0456±0.0020 ^{bc}	1.2066±0.0459 ^{a}
75-2	0.0422±0.0039 ^{abc}	1.1505±0.1513 ^{a}
75-5	0.0456±0.0051 ^{bc}	1.2368±0.1665 ^{a}
85-0	0.0545±0.0039 ^{c}	1.4084±0.0036 ^{a}
85-2	0.0378±0.0069 ^{ab}	1.0335±0.1620 ^{a}
85-5	0.0300±0.0067 ^{a}	0.8135±0.1655 ^{a}
85-10	0.0344±0.0059 ^{ab}	0.8816±0.2816 ^{a}
85-20	0.0342±0.0012 ^{ab}	0.9391±0.0254 ^{a}
85-30	0.0417±0.0001 ^{abc}	1.0138±0.0378 ^{a}

Different letters represent significant differences, $P \leq 0.05$

4.3.1.4 **FTIR spectroscopic study of SPI films**

The FTIR spectra of pure glycerol, soy protein isolate powder, and an SPI film are presented in **Fig. 4.7**. The spectrum of glycerol exhibits the typical bands of alcohols, including the O-H stretching absorption band in the 3600-3000 cm^{-1} region and the absorption bands at 1029 and 1108 cm^{-1} associated with the carbon-oxygen (C-O) bonds of primary and secondary alcohols, respectively. In addition, bands in the 1100-1000 cm^{-1} range are assigned to the H_2C-OH bending vibration while bands in the 1150-1100 cm^{-1} range are assigned to the HC-OH bending vibration. In-plane bending vibrations of hydroxyl groups are observed in the 1400-1150 cm^{-1} range. Lastly, peaks at 2878 and 2931 cm^{-1} can be assigned to symmetric and asymmetric stretching vibrations of C-H bonds (Pretsch *et al*., 2000; Arrieta *et al*., 2013).

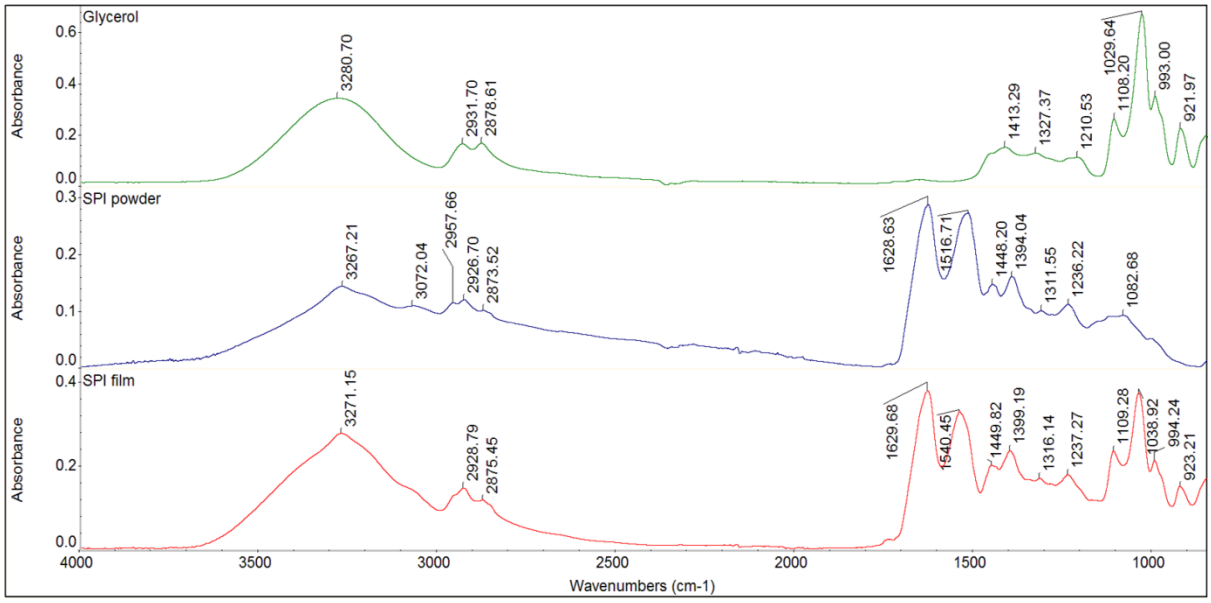


Figure 4.7 FTIR spectra of pure glycerol, soy protein isolate (SPI) powder and an SPI film in the range of 4000-400 cm^{-1}.

The FTIR spectrum of pure SPI powder in the middle panel of **Fig. 4.7** exhibits a broad band in the 3500-3000 cm^{-1} range, which is indicative of protein-bound water. The peak at 3267 cm^{-1} superimposed on this broad band and the band at 3072 cm^{-1} are, respectively, the amide A and amide B bands characteristic of proteins, which are assigned to stretching of the N-H bonds in the peptide linkages (Karnnet *et al*., 2005). Other characteristic infrared absorption bands of proteins include the amide I (1700-1600 cm^{-1}), amide II (1600-1500 cm^{-1}) and amide III (1330-1220 cm^{-1}) bands, which have their peak maximum at 1628, 1516 and 1236 cm^{-1}, respectively, in the spectrum of the SPI powder. These three bands are assigned to mixed vibrational modes of the peptide linkage, with the predominant contributions being the C=O stretching (amide I band), N-H bending (amide II band) and C-N stretching (amide III band) vibrations (Sui & Zhang *et al*., 2016; Guerrero *et al*., 2010; Denavi *et al*., 2009). Lastly, the absorption bands at 2957 and 2926 cm^{-1} correspond to the C-H stretching vibration of CH_3 and CH_2 groups of amino acid side chains.

The spectrum of the SPI film in the bottom panel of **Fig. 4.7** is similar to the SPI powder spectrum except for some differences due to the spectral contributions of the glycerol added to the film as a plasticizer. The intensity of the peak at 3271 cm^{-1} in the spectrum of the SPI film appears higher than in the spectrum of SPI powder because it is superimposed on the broad absorption band observed in the 3600-3000 cm^{-1} region in the spectrum of glycerol. Similarly, the higher intensities of the peaks at 1109 and 1039 cm^{-1} in the spectrum of the SPI film compared with the SPI powder spectrum may be attributed to overlap with the

absorption bands of glycerol observed in these ranges. There are, however, no readily discernible spectral changes attributable to effects of glycerol on the protein structure.

4.3.2 **Incorporation of carvacrol into SPI-based edible films**

4.3.2.1 **Preparation of SPI films incorporating carvacrol**

Based on the investigations of the effects of film preparation conditions on various properties of SPI films described above, all the film-forming solutions employed for the casting of SPI films incorporating carvacrol were prepared at pH 10 and heated for 10 min at 85 °C. **Table 4.2** lists the concentrations of carvacrol and glycerol in the 5% (w/v) SPI film-forming solutions that were employed in the initial set of experiments. It also shows the ease of peeling off the cast films from the Petri plates in these experiments. With increasing content of glycerol in the film, it was more and more difficult to peel off intact films from the plastic Petri plate. The same trend was observed with addition of increasing amounts of carvacrol into the film-forming solution. Thus, the higher concentrations of both carvacrol and glycerol were found to reduce the rigidity of the films and caused them to be easily fractured**.** Thus, for the subsequent experiments, an upper limit of 3% was set for the concentration of glycerol and the concentration of carvacrol in the film-forming solution. In addition, the volume of film-forming solution added to each Petri dish was increased so as to increase film thickness, which made it easier to peel off intact films. However, the film prepared with 1% carvacrol in the absence of glycerol was still too brittle to permit additional study.

Fig. 4.8 shows the FTIR spectra of the soy protein-based films cast from 5% SPI solutions containing 2% glycerol and up to 3% carvacrol. All the spectra except that of the film prepared without carvacrol exhibit a peak at 810 cm^{-1}, characteristic of carvacrol, and there was a large increase in the intensity of this peak with the increase in the concentration of carvacrol in the film-forming solution from 1% to 2%. However, a commensurate increase in peak intensity was not observed with a further increase of the carvacrol concentration to 3%, This phenomenon could be the result of inhomogeneous distribution of the film components, or it could indicate that the carvacrol added to the film-forming solution at the 3% level was not completely incorporated in the cast film. The latter possibility is further addressed in the next section, in which the optical properties of the SPI films prepared in these experiments are reported.

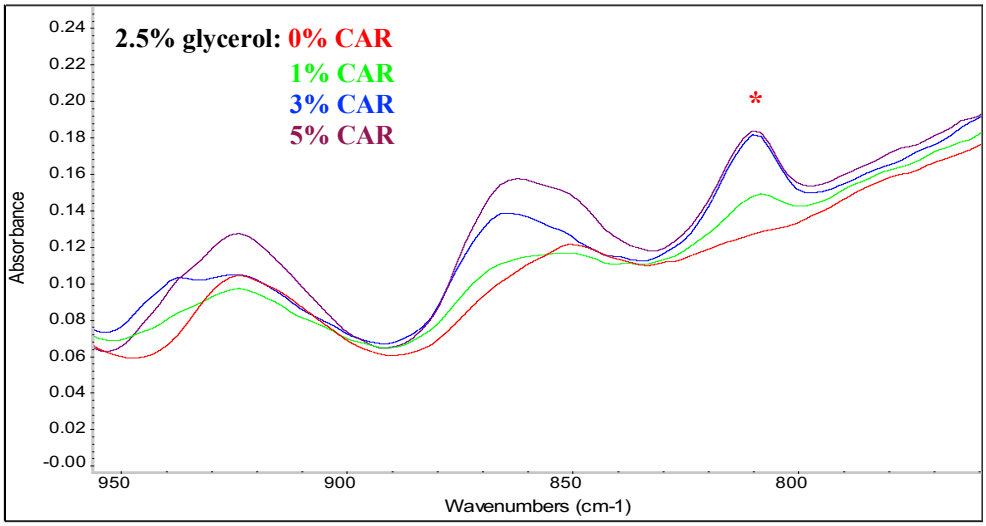


Figure 4.8 FTIR spectra of soy protein-based edible films incorporating different amounts of carvacrol. The asterisk marks the absorption band of carvacrol at 810 cm^{-1}.

Table 4.2 Peeling characteristics of soy protein films cast from 5% (w/v) SPI solutions containing different concentrations of glycerol and carvacrol.

Glycerol concentration (w/v)	Carvacrol concentration (w/v)	Ability to peel off film		
		Easy	Hard	Impossible
2%	0%	√		
	1%	√		
	3%			√
	5%			√
2.5%	0%	√		
	1%		√	
	3%			√
	5%			√
3%	0%	√		
	1%			√
	3%			√
	5%			√
3.5%	0%		√	
	1%			√
	3%			√
	5%			√

4.3.2.2 **Opacity of SPI films incorporating carvacrol**

Based on the results in **Table 4.2**, lower concentrations of carvacrol (up to 3%) and glycerol (up to 3%) were chosen to incorporate into the film-forming solution, and different emulsification treatments, including homogenization, addition of Tween 80 and the combination of these, were applied in these tests. The opacity of SPI films prepared by incorporation of 1% carvacrol and different concentrations of glycerol in the film-forming solution is shown as a function of wavelength in **Fig. 4.9**, In all cases, an emulsification treatment consisting of homogenization of the film-forming solution with a Polytron homogenizer for 1.5 min had been applied. A decrease in opacity of films with increasing concentration of glycerol is observed in **Fig.4.9**, indicating that glycerol could affect the transparency of films with incorporated carvacrol. Sothornvit & Krochta (2001) reported that plasticizers can enhance transparency of films in the visible region by interfering with protein-protein interactions. Furthermore, the opacity of each film decreased with increasing wavelength, in accordance with our previous results. The high opacity values at 325 nm revealed good UV barrier properties of the films, which might be due to the absorption of UV radiation by carvacrol as well as by aromatic amino acids in the protein (Limpan *et al*., 2010).

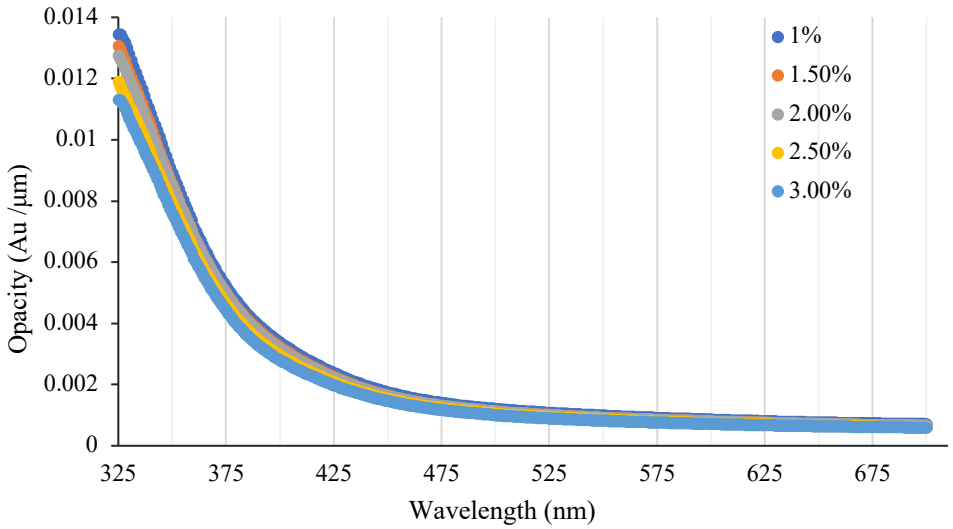


Figure 4.9 Effect of concentration of glycerol in film-forming solutions containing 1% carvacrol on opacity of SPI films.

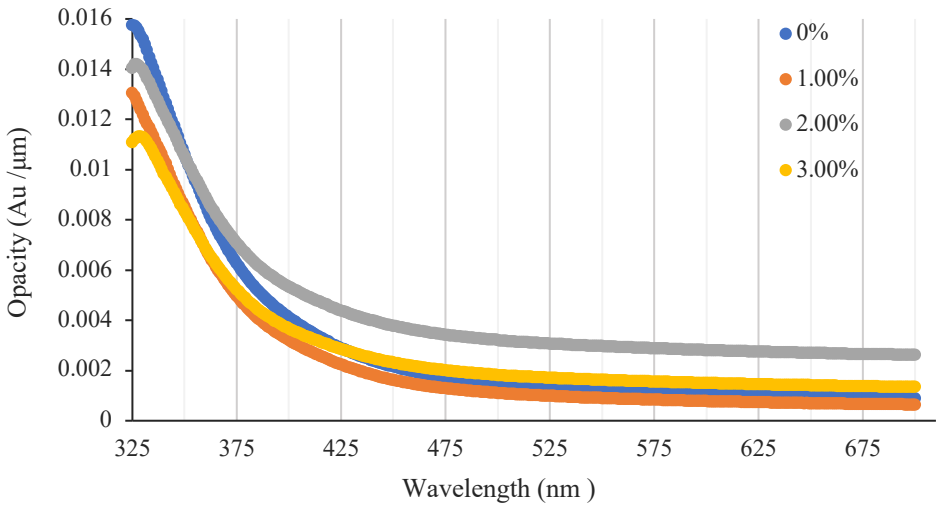


Figure 4.10 Effect of concentration of carvacrol in film-forming solutions containing 1.5% glycerol on opacity of SPI films.

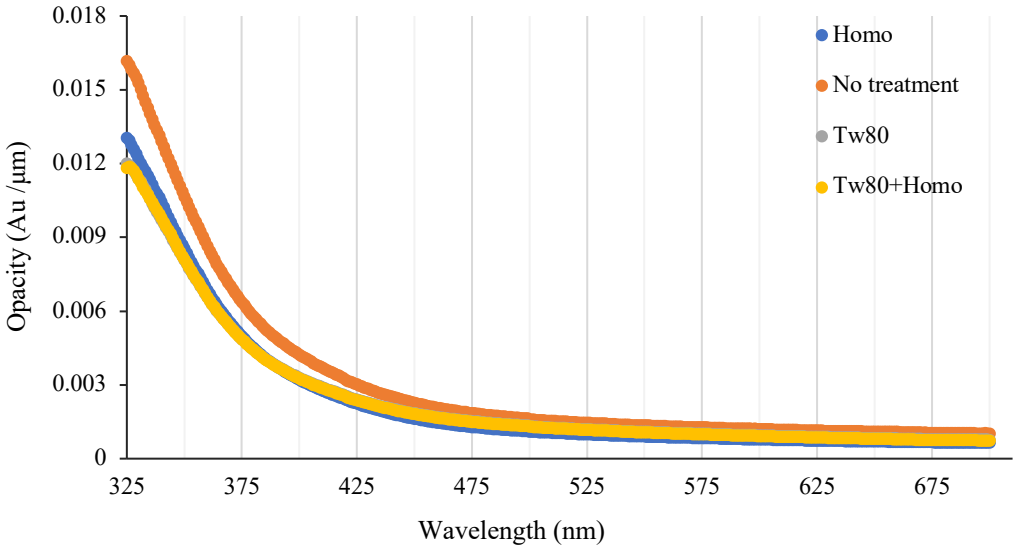


Figure 4.11 Effect of different emulsification treatments (following addition of 1% carvacrol and 1.5% glycerol to 5% SPI solution) on opacity of SPI films.

Fig.4.10 shows the effects of different concentrations of carvacrol on opacity of SPI films prepared with 1.5% glycerol and homogenization for 1.5 min. The opacity values of the films in the UV region dereased with addition of carvacrol. Similarly, Neira *et al*. (2019) observed a reduction in the opacity of fish gelatin films with addition of carvacrol, and Tunç & Duman (2011) also reported that the opacity of methylcellulose/montmorillonite nanocomposite films decreased with carvacrol addition. With regard to our results for the SPI films prepared with 2% and 3% carvacrol (w/v) in the film-forming solution, these concentrations are very high

in relation to the additive concentrations commonly employed in preparing soy protein films, and these films were hard to peel off, with some carvacrol not emulsified completely in the film still remaining on the surface of the Petri plate. These films were cloudy in appearance and had high opacity values in the visible range, and similar behavior was observed when films were prepared with high concentrations of oregano and cinnamon essential oils. Oregano essential oil was reported to increase the transparency of fish gelatin-chitosan films at 600 nm (Hosseini *et al*., 2015). However, Tongnuanchan *et al*. (2012) found that the oil droplets inserted in the polymer matrix can cause light scattering at the interface, resulting in the reduction of light transmission through the films when measured spectrophotometrically. The extent of dispersion and the concentration of essential oil droplets in the matrix are the main factors affecting the intensity of light scattering (Monedero *et al*., 2009; Hosseini *et al*., 2015), presumably explaining the high opacity values of the films prepared with 2% and 3% concentrations of carvacrol. However, in the UV range the opacity values of these films, like those of the other films prepared with carvacrol, were lower than that of the control film (0% carvacrol), indicative of the predominance of UV absorption over light scattering effects in this wavelength range.

Fig. 4.11 shows the effect of different emulsification treatments on opacity of SPI films prepared with 1% carvacrol and 1.5% glycerol. The control group (no emulsification treatment) showed higher opacity values than the other films across the wavelength range of 325 to 700 nm, Pérez-Gago & Krochta (2001a) found that the size of lipid droplets affected the lipid distribution in the film matrix and that films became opaque as the size of lipid droplets within the matrix became larger, producing a lipid-enriched layer when the film dried.Similarly, the low transparency of the control group (**Fig. 4.11**) can be attributed to the large non-emulsified droplets of carvacrol in the film-forming solution, as the incorporation of these large droplets in the film would cause light scattering. The opacity values of the homogenized group were slightly higher than those of the Tween 80 and comibined-treatment groups in the UV range, while the opposite trend was observed in the visible range, Overall, there was not much difference among the three treatment groups by comparison with the control group. These results demonstrated that homogenization and addition of Tween 80 were effective in reducing the size of carvacrol droplets, resulting in more translucent films.

4.3.2.3 **Mechanical properties of SPI films incorporating carvacrol**

The tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus of SPI films prepared with 1% carvacrol and various concentrations of glycerol in the film-forming solution were shown in **Fig. 4.12**. An SPI film with 0% glycerol was also made in this test but is not included in **Fig. 4.12** because the brittleness of this control film made it break into small pieces during drying at room temperature. **Fig. 4.12** shows the decrease of TS and Young's modulus and increase of the EAB with increasing concentration of glycerol. The TS of the SPI-carvacrol films decreased to 1.98 N/mm^2 as the concentration of glycerol increased, and the TS of films prepared with with 1% -1.5% glycerol were significantly higher than those of the other groups ($P \leq 0.05$). Similarly, the Young's modulus of these films was also significantly higher ($P \leq 0.05$). The elongation at break (EAB%) of the films increased with the addition of glycerol, but there was no further significant increase of EAB% when films were prepared with higher concentrations of glycerol ($P > 0.05$). Thus, for all the mechanical properties, there were no significant differences among the groups with 2%-3% glycerol ($P > 0.05$). Some studies found that increasing the glycerol concentration tended to decrease the TS and elongation at break of agar films and starch-chitosan films (Arham *et al*., 2016; Liu *et al*., 2013). The Young's modulus of amylose-enrichment cassava starch films was also reduced with addition of glycerol (Alves *et al*., 2007). Similarly, Maizura *et al*. (2007) reported the same phenomenon for sago starch-alginate films incorporating different concentration of lemongrass oil. Addition of glycerol into films can reduce the intermolecular forces and internal hydrogen bonding between polymer chains and increase molecular volume and mobility of the polymeric chains, resulting in more flexible films (Du *et al*., 2011). In the present study, the effects of glycerol on the mechanical properties of SPI films with incorporated carvacrol corresponded to those observed for SPI films without carvacrol, including reductions inTS and Young's modulus and increased elongation at break.

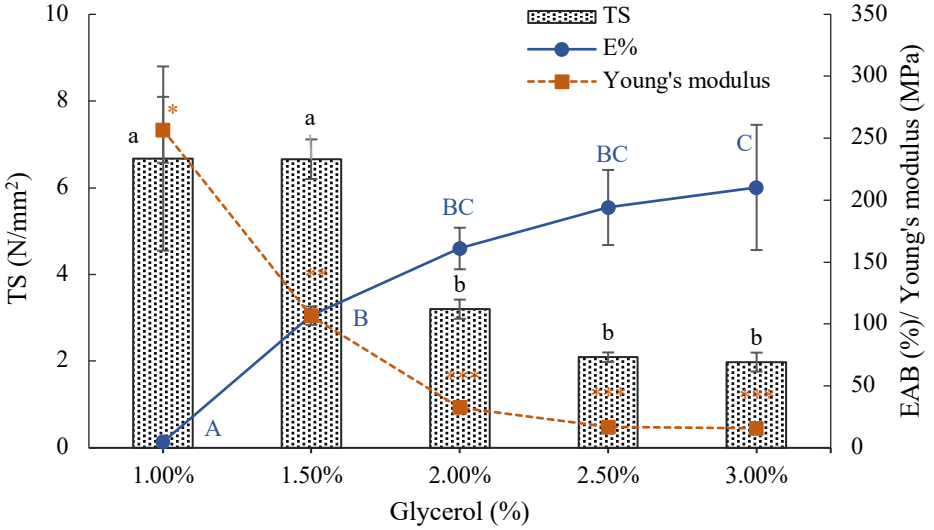


Figure 4.12 Tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus of SPI films prepared with 1% carvacrol and various concentrations of glycerol in the film-forming solution. (Different letters and symbols represent significant differences, $P \leq 0.05$).

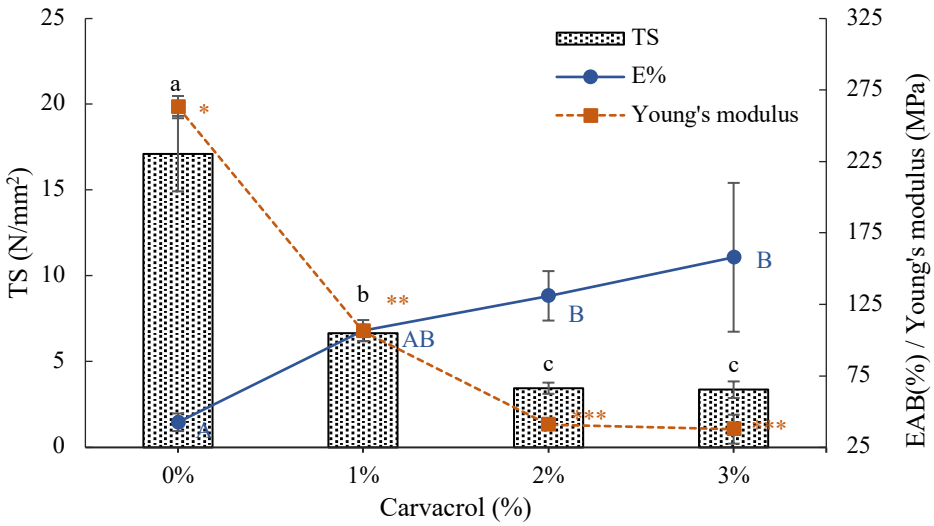


Figure 4.13 Tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus of SPI films prepared with various concentrations of carvacrol and 1.5% glycerol in the film-forming solution. (Different letters and symbols represent significant differences, $P \leq 0.05$).

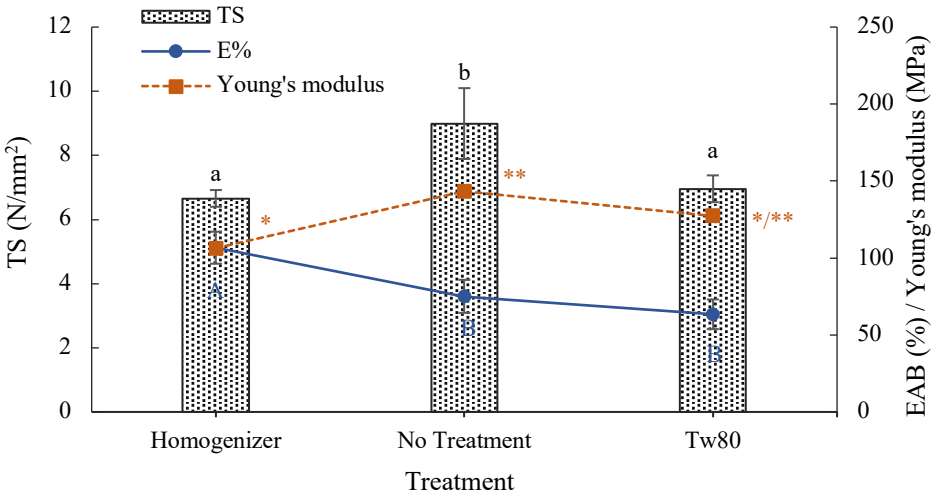


Figure 4.14 Tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus of SPI films prepared with 1% carvacrol and 1.5% glycerol in the film-forming solution and different emulsification treatments. (Different letters and symbols represent significant differences, $P \leq 0.05$).

The mechanical properties of SPI films prepared with different concentrations of carvacrol are shown in **Fig. 4.13**. The carvacrol added to the film-forming solution was emulsified with homogenization treatment, and a glycerol concentration of 1.5% (w/v) was employed. The effects of carvacrol addition on the mechanical properties of the SPI films followed the same trends as observed with glycerol addition. The TS and Young's modulus were decreased to 3.36 N/mm^2 and 37.75 MPa, respectively, with addition of 2%-3% carvacrol in the film-forming solution and were significantly lower than those of the other two groups in **Fig. 4.13** ($P \leq 0.05$). Elongation at break of the SPI films increased significantly with addition of carvacrol, but there was no significant difference in EAB% among films prepared with different concentrations of carvacrol ($P > 0.05$). These results indicated that carvacrol affects the mechanical properties and improves the flexibility of SPI films in the same manner as glycerol. Kavoosi *et al*. (2013) reported a significant decrease in TS with a simultaneous increase in EAB% of gelatin films upon incorporation of carvacrol into the films, and similar effects on TS and EAB% were obtained when citrus oil was incorporated in gelatin films (Tongnuanchan *et al*., 2012). The effect of carvacrol on mechanical properties of SPI films might be explained in terms of lowering the interaction between protein molecules and hindering the interaction between protein chains (Limpisophon *et al*., 2010). Thereby, emulsified carvacrol may act as a plasticizer like glycerol to reduce resistance and rigidity of SPI films and increase their extensibility (Otoni *et al*., 2016).

The effects of emulsification treatments on the mechanical properties of SPI films incorporating carvacrol are shown in **Fig. 4.14**. These films were prepared with 1% carvacrol and 1.5% glycerol in the film-forming solution. The carvacrol was emulsified by a homogenization treatment or by addition of Tween 80. The TS of the control group (films prepared without any emulsification treatment) was significantly higher than those of the other two groups, and the Young's modulus of the control group was significantly higher than that of the homogenization treatment group ($P \leq 0.05$) while the EAB% of the latter group was significantly higher than that of the other groups ($P \leq 0.05$). The reductions of TS and Young's modulus and increase of EAB% in the emulsification treatment groups indicated that increasing the surface areas of carvacrol droplets may lead to a more pronounced plasticizing effect (Otoni *et al*., 2016). Similar results were found in a study of soy protein films incorporating carvacrol or cinnamaldehyde. In the case of films with incorporated carvacrol, significant decreases of TS and Young's modulus were observed with decreasing droplet size, while EAB% significantly increased (Otoni *et al*., 2016), corresponding to our results. However, in the case of films with incorporated cinnamaldehyde, the opposite effects were observed with decreasing droplet size. Increased TS and EAB% of whey protein films at 60% concentration of beeswax were observed with decreasing particle size of the beeswax while particle size had little effect at lower concentrations of beeswax (Pérez-Gago & Krochta, 2001). These results demonstrate that the physical state, type and concentration of lipidcan influence the effect of decreasing particle size on mechanical properties of protein films.

4.3.2.4 **Water vapor permeability (WVP) of SPI films incorporating carvacrol**

Table 4.3 shows the water vapor permeability (WVP), thickness and rate of water vapor transmission (WVYR) of soy protein films prepared with different concentrations of glycerol and carvacrol and various emulsification treatments. The data shown in **Table 4.3** (top section) are for films prepared with 1% carvacrol and different concentrations of glycerol in the film-forming solution. As the amount of glycerol increased, film thickness increased. The WVTR significantly increased from 22.1 $g/m^2 \cdot h$ for films prepared without glycerol to 28.4 $g/m^2 \cdot h$ for films prepared with the highest concentration of glycerol ($P \leq 0.05$). Furthermore, the WVP values of films prepared with 1.5%-3% glycerol were significantly higher than those of films with a lower concentration of glycerol ($P \leq 0.05$) and were slightly lower than the WVP values of SPI films reported by Kim *et al*. (2003) and Kunte *et al*. (1997). Kokoszka *et al*. (2010) reported a similar increase of WVP for SPI films with increase of

glycerol content at RH 30-75%. According to Kim & Min (2012), the insertion of glycerol molecules between chains of polymers could lead to an increase of the interchain space, resulting in a higher rate of water vapor diffusion through the films and higher rates of water vapor transmission. It has also been suggested that clustering of glycerol molecules opens up the protein structure, thereby increasing the water vapor permeability of the films (Yang & Paulson, 2000). Additionally, the plasticizing effect and high hydrophilicity of glycerol can favor the absorption and adsorption of water molecules by the films, thereby substantially increasing the WVP value (Couplan*et al*., 2000). The absorption of more moisture in films with higher glycerol content could also lead to increased thickness due to swelling (Kokoszka *et al*., 2010). Thus, glycerol may affect the water vapor permeability of soy protein films in a number of ways.

Table 4.3 (middle section) also shows the moisture barrier properties and thickness of soy protein films prepared with 1.5% glycerol and different concentrations of carvacrol. Film thickness increased as the concentration of carvacrol increased to 3% ($P < 0.05$). Unlike the effect of glycerol on water vapor transmission rate (WVTR), the WVTR of SPI films significantly decreased to 27.9 $g/m^2 \cdot h$ with the addition of carvacrol ($P < 0.05$), while the WVP was non-significantly increased ($P > 0.05$). Kavoosi *et al*. (2013) reported that addition of carvacrol increased the WVP of gelatin films. However, in other studies, SPI films enriched with carvacrol were found to have slightly decreased permeance (WVTR/ΔP) and WVP (Otoni *et al*., 2016), and there was a significant reduction of WVP in hake protein films in which a high level of thyme essential oil had been incorporated ($P < 0.05$) (Pires *et al*., 2011). Various opinions about how essential oils affect water vapor permeability have been put forward in these and many other studies. One of the explanations was that the carvacrol can interact with the hydrophobic domains of proteins, leading to enhanced interfacial interaction of carvacrol and the film matrix. As a consequence, there would be less interaction between the protein and water, making it possible for water molecules to pass freely through the film, giving rise to the increased WVP (Zivanovic *et al*., 2005). According to another viewpoint, the plasticizing effect of essential oils on SPI films was attributed to the weakening of hydrogen bonding and disulfide bonds in these films, resulting in increased WVP (Otoni *et al*., 2016). On the other hand, since water vapor migrates through hydrophilic portions of a film, addition of increasing amounts of hydrophobic essential oils might decrease WVP of protein films (Pires *et al*., 2011), corresponding to the decreased WVTR of films in our study. However, other factors such as film thickness and relative humidity

conditions also influence WVP (McHugh *et al*., 1993). A positive slope relationship between WVP and film thickness was observed for hydrophilic films (Hagenmaier & Shaw, 1990). Thus, the plasticizing effect of carvacrol might be balanced by the increased content of this hydrophobic compound, leading to the decrease of water vapor transmission rate and slight increase of water vapor permeability.

Table 4.3 Water vapor permeability (WVP), thickness and rate of water vapor transmission (WVYR) of soy protein films cast from solutions containingdifferent concentrations of glycerol and carvacrol and subjected to different emulsification treatments.

Glycerol	Carvacrol	Emulsification treatment	Thickness (mm)	WVTR ($g/m^2 \cdot h$)	WVP ($mm \cdot g/m^2 \cdot h \cdot kPa$)
0%	1%	Homogenizer	0.0850±0.0071 [a]	22.1166±0.0000 [a]	1.3118±0.0000 [a]
1%			0.1075±0.0000 [ab]	24.3282±1.0426 [b]	1.8249±0.0782 [a]
1.5%			0.0988±0.0088 [a]	26.1713±0.5213 [bc]	1.8050±0.1973 [a]
2%			0.1313±0.0113 [bc]	27.0313±0.7673 [cd]	2.4883±0.3270 [b]
2.5%			0.1444±0.0101 [c]	27.5228±0.7673 [cd]	2.8637±0.0437 [b]
3%			0.1306±0.0075 [bc]	28.3829±0.5213 [d]	2.5881±0.1747 [b]
1.5%	0%	Homogenizer	0.0900±0.0321 [a]	35.8780±0.8513 [a]	2.1723±0.7521 [a]
	1%		0.0989±0.0084 [a]	34.1578±0.9276 [a]	2.2753±0.1697 [a]
	2%		0.1300±0.0088 [ab]	30.4717±1.1849 [b]	2.6748±0.2770 [a]
	3%		0.1425±0.0088 [b]	27.9221±0.3529 [c]	2.6809±0.1398 [a]
1.5%	1%	Homogenizer	0.1085±0.0114 [a]	31.5161±1.4276 [a]	2.1175±0.1761 [a]
		No treatment	0.1113±0.0025 [a]	31.5775±1.1261 [a]	2.1873±0.0862 [a]
		Tw80	0.1333±0.0014 [b]	31.3318±0.6384 [a]	2.5919±0.0711 [b]
		Tw80 and Homogenizer	0.1475±0.0001 [b]	29.6730±0.2606 [a]	2.7153±0.0239 [b]

Different letters represent significant differences, $P \leq 0.05$

As shown in **Table 4.3** (bottom section), none of the three emulsification treatments resulted in a significant decrease of the water vapor transmission rate of the SPI films in comparison with the no-treatment group ($P > 0.05$). Meanwhile, the thickness and WVP of films emulsified by homogenization treatment were not significantly different from those of the control group ($P > 0.05$), but they were pronouncedly lower than those for the Tween 80 treatment groups ($P<0.05$). These results indicated positive influence of film thickness on the WVP of SPI films, and the emulsification treatments or the particle size of lipid droplets might slightly influence ($P > 0.05$) the WVTR and WVP of films according to our data. The effects of droplet size on WVP were clearly evident in a study of emulsified SPI films prepared by addition of lipid (Acetem)/water micro- or nanoemulsions containing carvacrol or cinnamaldeyde to the film-forming solution (Otoni *et al*., 2016). As the droplet size reduced from micro to nano, the WVP of the cast films decreased dramatically ($P < 0.05$).

Thus, the reduction of WVP in edible films cannot simply be achieved by adding a hydrophobic component, since the amount added can impact the microstructure of the emulsified films, which is a decisive factor impacting WVP. In turn, the microstructure of films can also be affected by the distribution of the hydrophobic component in the matrix and by its physical state (Atarés and Chiralt, 2016). With regard to the effect of lipid distribution in the protein matrix, smaller oil droplets are more homogeneously distributed in the film matrix, resulting in lower WVP (Pérez-Gago and Krochta, 2001a). The type and concentration of lipid in the protein film also influences its effects on WVP. Thus, particle size had no effect ($P > 0.05$) on WVP of whey protein isolate-beeswax films when the beeswax content was low (20%), but at high beeswax content (60%) the WVP of the films decreased as particle size decreased (Pérez-Gago & Krochta, 2001). The hydrophilic protein may dominate the film matrix at low lipid concentration. Thereby, as the size of the lipid particles decreases, the immobilization of protein chains at the lipid interface could increase, resulting in a more tightly ordered cross-linked structure, which could pronouncedly influence the moisture barrier of films (Pérez-Gago & Krochta, 2001). In addition, the presence of hydrophilic groups and the low melting point of the lipid can lead to more moisture sorption and greater water migration (Gontard *et al*., 1994), which could explain the slight increase in the WVP of SPI films resulting from the addition of Acetem and Tween 60 (Otoni *et al*., 2016), corresponding to the increased WVP observed in our study with the addition of Tween 80. Therefore, to improve the WVP of SPI films, lower glycerol concentration and higher carvacrol content and emulsification of carvacrol in the film-

forming solution by homogenization could be chosen for further development of packaging films.

4.3.2.5 **FTIR spectroscopic investigation of SPI films incorporating carvacrol**

The FTIR spectra of soy protein films in which different concentrations of carvacrol were incorporated are shown together with that of pure SPI powder in **Fig. 4.15**. The spectra of all SPI films with incorporated carvacrol show similar characteristic peaks at 2958 and 2928cm^{-1} (C-H stretching vibrations) and 1535 cm^{-1} (protein amide II band). However, slight differences in the intensity and position of peaks were observed in the spectral range in which O-H and N-H stretching vibrations occur (3600-3000 cm^{-1}) and in the protein amide I (1700-1600 cm^{-1}) and amide III (1330-1220 cm^{-1}) regions. First, the maximum of the broad absorption band in the 3600-3000 cm^{-1} range slightly shifted to higher wavenumber (from 3272 to 3278 cm^{-1}), and the intensity at the peak maximum slightly decreased with addition of carvacrol. The gradual shift of this peak with increasing carvacrol content in the film may be attributed to overlap with the O-H stretching absorption observed in the spectrum of carvacrol at 3369 cm^{-1} while the decreased intensity of the peak may possibly be attributed to lower water content of the film (Fernandes Nassar *et al*., 2018), Such a reduction in water content would indicate a reduction in the hydrophilic character of the film resulting from incorporation of carvacrol and the formation of other bonds, likely via hydrophobic interaction, in SPI/carvacrol films, correlating to the results of SPI films enriched with chestnut bur extract (Wang *et al*., 2016) and sodium and calcium caseinate films with carvacrol (Arrieta *et al*., 2013). Second, the amide III band appeared to shift from 1239 cm^{-1} in the spectrum of the film prepared without addition of carvacrol to higher wavenumber with increasing content of carvacrol in the SPI film; at the highest carvacrol concentration, the peak maximum was at 1256 cm^{-1}. This apparent shift of the amide III band is likely due to overlap with the absorption band of carvacrol at 1250 cm^{-1} (in-plane O-H bending vibration of phenolic ring) (Arrieta *et al*., 2013).

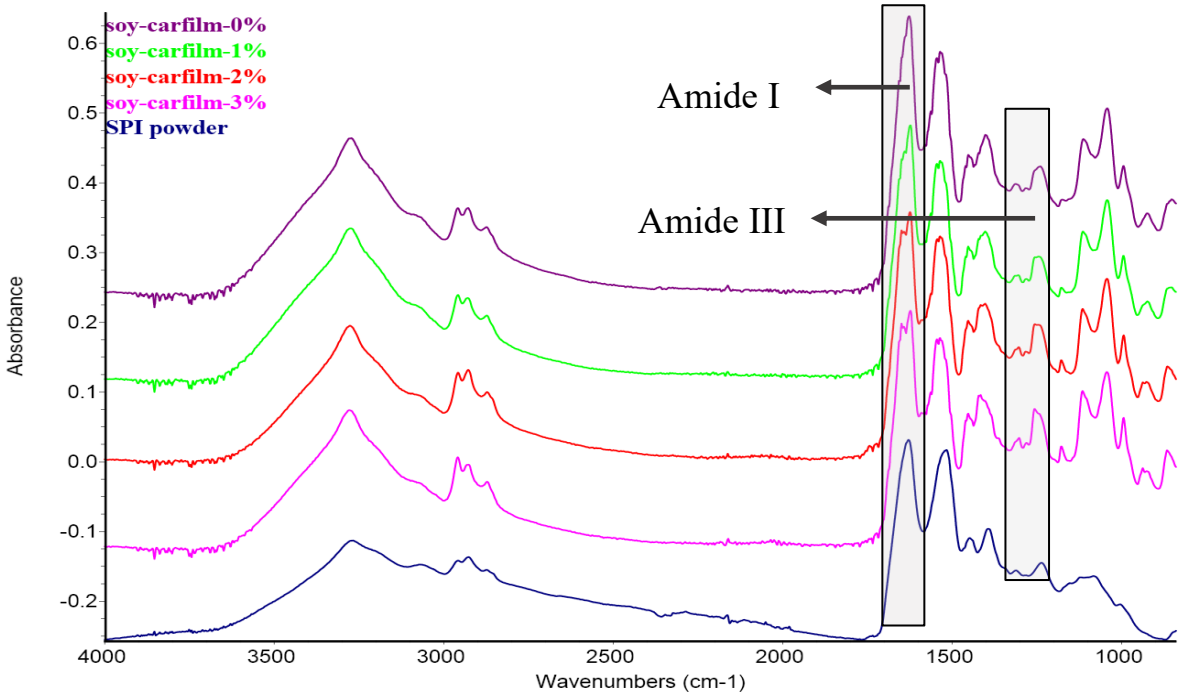


Figure 4.15 FTIR spectra of soy protein-based edible films incorporating different amounts of carvacrol [0-3% (w/v) in the film-forming solution] and pure SPI powder.

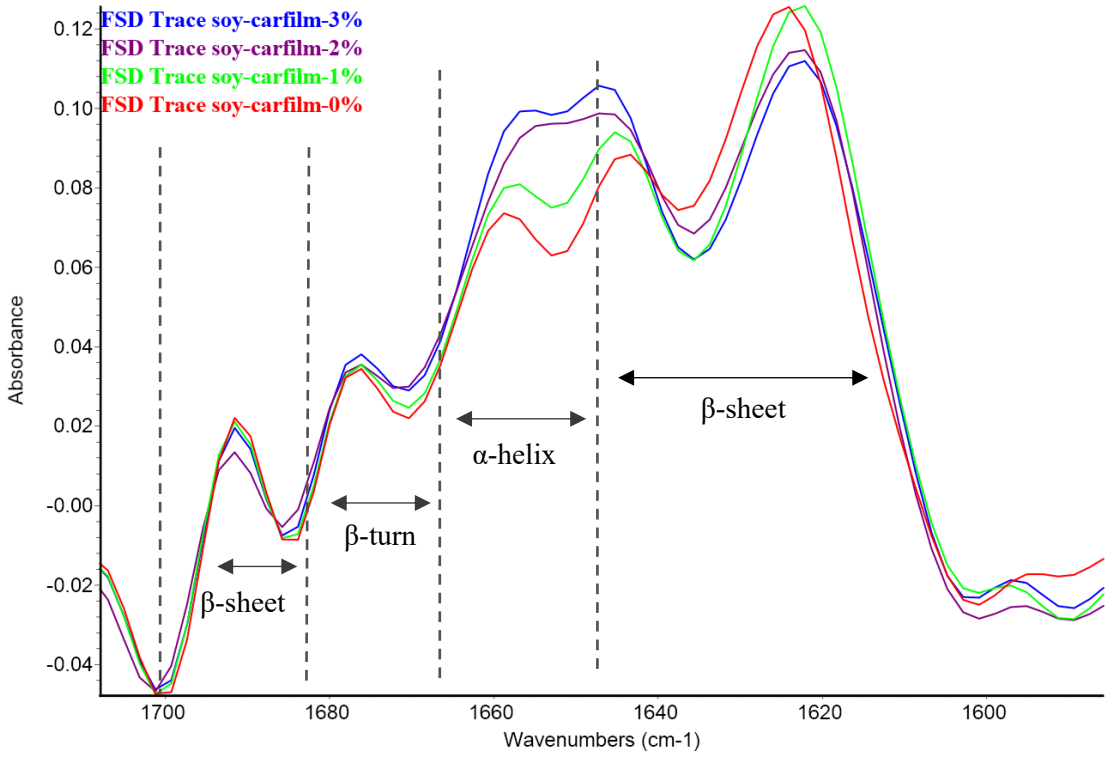


Figure 4.16 Fourier self-deconvoluted protein amide I region (1700-1600 cm^{-1}) in the FTIR spectra of soy protein films incorporating different amounts of carvacrol (0-3% in the film-forming solution). Prior to Fourier self-deconvolution, spectral contributions of carvacrol in this region were eliminated by scaled subtraction of the spectrum of pure carvacrol in the region of 850-750 cm^{-1}.

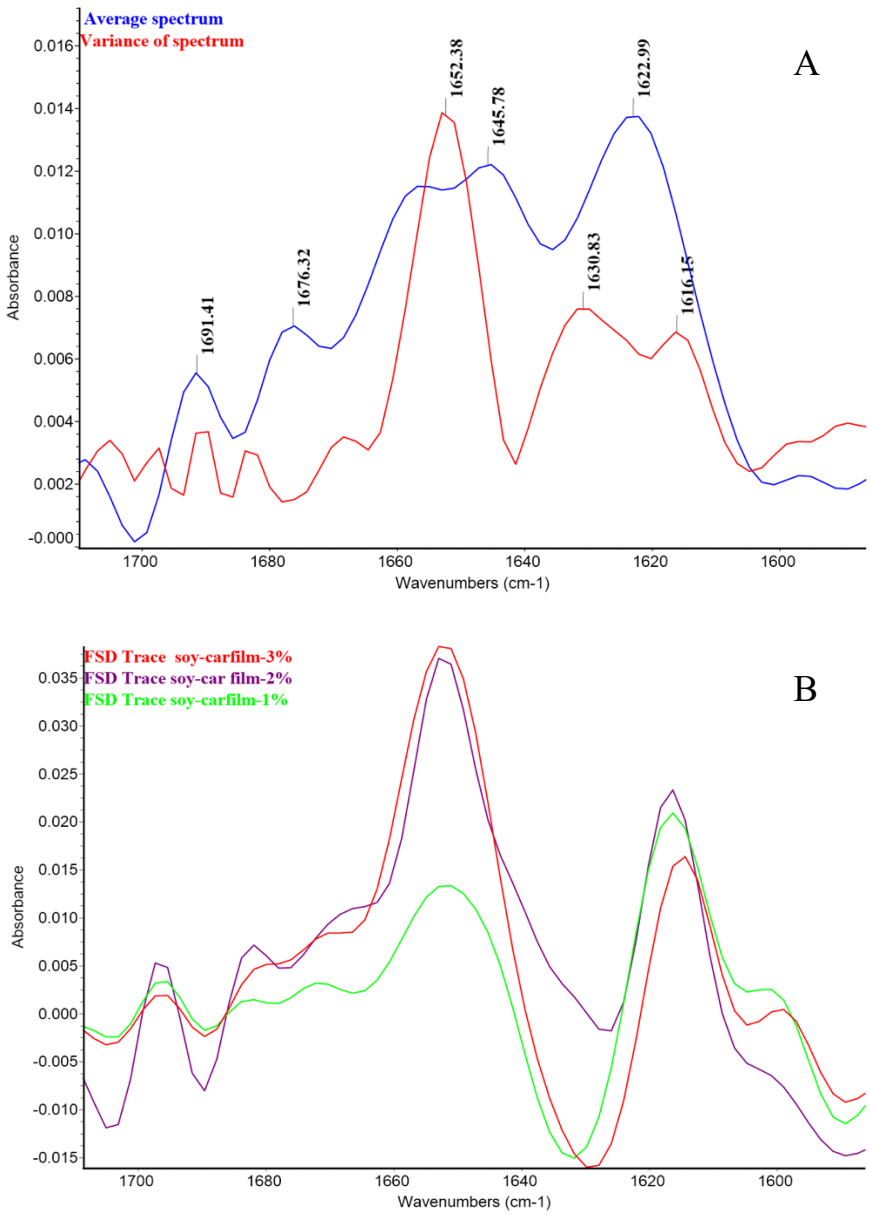


Figure 4.17 A: The average and the variance spectrum computed from the six FSD spectra shown in Figure 4.16; B: FSD protein amide I region (1700-1600 cm^{-1}) in the spectra of soy protein films prepared with different concentrations of carvacrol (1-3% in the film-forming solution) after subtraction of the spectrum of control SPI film.

Last but not least, **Fig. 4.15** shows that incorporation of carvacrol in the SPI films resulted in the appearance of a shoulder on the high-wavenumber side of the amide I band. As carvacrol does not have an absorption band in this region, this change in the secondary-structure-sensitive amide I band indicates that incorporation of carvacrol in the SPI films affected the secondary structure of the soy proteins. Thus, the spectra in **Fig. 4.15** were further analyzed by subtraction of the spectrum of pure carvacrol, scaled on the characteristic peak of carvacrol at 810 cm^{-1}, and vector normalization in the region of 1720-1490 cm^{-1} followed by mathematical enhancement of the spectral resolution in the protein amide I region (1700-

1600 cm^{-1}) by Fourier self-deconvolution (FSD) with a bandwidth of 18.1 and an enhancement factor of 1.8. The FSD spectra are shown in **Fig. 4.16.**

In resolution-enhanced spectra, the wavenumber ranges in which the amide I vibration occurs are characteristic of the different types of secondary structure and have been tabulated in the literature for proteins in aqueous (H_2O and D_2O) environments (Yang *et al*., 2015; Wang *et al*., 2016; Liu *et al*., 2011; Alvarez *et al*., 2008). In **Fig. 4.16**, the FSD spectrum of the control film (prepared without addition of carvacrol) exhibits five amide I band components within the 1700-1600 cm^{-1} region, with peak maxima at 1691, 1676, 1656, 1644 and 1622 cm^{-1}. For proteins in an H_2O environment, amide I band components in the ranges 1652-1602 cm^{-1} (peaks at 1622 and 1644 cm^{-1} in **Fig. 4.16**) and 1700-1685 cm^{-1} (peak at 1691 cm^{-1}) are normally identified as β-sheet bands, those in the range 1670-1652 cm^{-1} (peak at 1656 cm^{-1}) as α-helix bands, and those in the range 1685-1670 cm^{-1} (peak at 1676 cm^{-1}) as belonging to β-turns. With addition of carvacrol into the soy film matrix, the intensity of the peaks at 1691 and 1676 cm^{-1} associated with anti-parallel β-sheet structure and β-turns, respectively, gradually increased, indicating the increasing proportion of anti-parallel β-sheets and β-turns in the protein structure. In addition, the peak at 1622 cm^{-1} assigned to parallel β-sheet structure in the SPI films was slightly shifted to lower wavenumber, which might suggest more extensive hydrogen bonding in some β-sheets (Seo *et al*., 2017) or formation of intermolecular β-sheets due to the addition of carvacrol (Litvinov *et al*., 2012). Apparent shifts of the peaks at both 1656 and 1644 cm^{-1} toward 1650 cm^{-1} with increasing content of carvacrol are also observed but are due to the increasing intensity of a new peak between these two peaks, This new band is more clearly visualized in **Fig. 4.17**(A), which shows the mean and the variance of the six FSD spectra in **Fig. 4.16**, and may be identified by its position at 1652 cm^{-1} as a new α-helix band. The other intense peaks in the variance spectrum are observed at 1631 and 1616 cm^{-1}, and these peaks indicate changes in the β-sheet structure as the carvacrol content of the films increases.

To obtain a more detailed understanding of the changes in secondary structure of the soy proteins with addition of carvacrol in SPI films, the FSD spectrum of the control film was subtracted from each of the other spectra in **Fig. 4.16**, and the resulting difference spectra are shown in **Fig. 4.17** (B). These spectra show the appearance of the peak at 1652 cm^{-1} upon addition of carvacrol in the film as well as a large increase in the intensity of this peak on going from the spectrum of the films prepared with 1% carvacrol to those prepared with 3% carvacrol in the film-forming solution. The only bands with negative intensity in these

difference spectra are observed in the range between 1635 and 1625 cm^{-1}, corresponding to the peak observed at 1631 cm^{-1} in the variance spectrum, and are indicative of the loss of some β-structure. Taken together, these features in the difference spectra suggest that the interaction of carvacrol with soy proteins in the formation of these films results in transformation of some β-sheets to an α-helix (band at 1672 cm^{-1}) that differs from the α-helical structure associated with the band at 1656 cm^{-1} in the FSD spectra in **Fig. 4.16**. In addition, the peaks below 1620 cm^{-1} in the difference spectra and the variance spectrum indicate that incorporation of carvacrol in the SPI films resulted in rearrangement of some of the remaining β-sheet structure.

With regard to the transformation of some β-sheet structure in the SPI films to a newly formed α-helix with addition of carvacrol, several factors may be taken into consideration. First, it is likely that the high dipole moment (D =1.9475) of carvacrol (Andrade-Ochoa *et al*., 2015) could disrupt the intramolecular hydrogen bonds between amide C=O and N-H in β-sheets. A relatively low dielectric constant subsequently could possibly promote unfolding of these β-sheets and induce an α-helix arrangement, analogously to the effect of 2-chloroethanol (D = 1.90, ϵ_r = 25.8) solvent on the secondary structure of concanavalin A (Jackson & Mantsch, 1992). Second, hydrogen bonding between the hydroxyl group (-OH) of carvacrol and amide C=O or other interactions, such as hydrophobic interactions, π - π stacking, and van der Waals forces, may twist the β-sheet to a newly formed α-helix structure during the film-forming process. It was recently reported that amyloid-derived dipeptide assemblies were transformed from β-sheet to α-helix by electrostatic repulsion between the C-terminal charges (deprotonated COOH groups) of the dipeptides under alkaline conditions in combination with attractive forces, including hydrogen bonding, π - π stacking, and water bridge interactions, (Xing *et al*., 2018). Similarly, at the alkaline pH of the film-forming solution employed for preparation of the SPI films incorporating carvacrol, electrostatic repulsion between deprotonated side-chain COOH groups may have favored interactions between carvacrol and the protein that resulted in transformation of β-sheet to α-helical structure, Finally, it may be noted that Yang *et al*. (2015) found that the content of β-sheet in the blended films they studied could contribute to the strength of the films, while α-helix and β-turn portions contributed to the flexibility of the films. Correspondingly, the β-sheet → α-helix transformation indicated by the FTIR spectroscopic investigation described in this section (**4.3.2.3**) is consistent with the increase of EAB and decrease of TS of SPI films with addition of carvacrol.

4.3.3 **Soy protein films incorporating oregano or cinnamon essential oil**

4.3.3.1 **Opacity of soy protein films incorporating oregano or cinnamon EO**

The effects of different concentrations of oregano-03 and cinnamon-03 EOs on opacity of soy protein films are shown in **Fig. 4.18** and **Fig. 4.19,** respectively. Opacity of the films at all wavelengths in the range from 325 nm to 700 nm decreased with addition of oregano essential oil except for the highest concentration of oregano EO (2%). In the visible range, these decreases in opacity indicated that the films incorporating oregano-03 EO were more transparent, which is consistent with the results presented above for the films incorporating carvacrol. Lee *et al*. (2015) found that incorporation of essential oils, such as marjoram oil, coriander oil, and clove bud oil, increased the transparency of films made from chicken feet protein. Similar results were also reported by Sánchez-González *et al*. (2010), who explained their finding that incorporation of tea tree oil (TTO) increased the transparency of chitosan films in terms of the insertion of TTO droplets between chitosan chains and the interaction with water molecules. However, the films prepared with 2% were slightly opaque to the naked eye and their opacity values in the visible region increased with increasing wavelength. These increased opacity values may be attributed to the high concentration of oregano essential oil and inhomogeneous distribution of droplets in the film matrix (Pérez-Gago & Krochta, 2001). Ortiz *et al*. (2018) observed an increase in opacity value of SPI films when clove EO was incorporated, and similar results with oregano EO were reported for fish protein films (Teixeira *et al*., 2014). In addition to the effects of concentration and droplet size discussed previously, the type of essential oil, the film thickness, and the color of essential oils and biopolymers were also taken into consideration (Teixeira *et al*., 2014). Furthermore, unlike the hake fish and whey proteins and some polysaccharide polymers, soy protein has a yellowish color due to the presence of a small amount of isoflavone in soy protein.

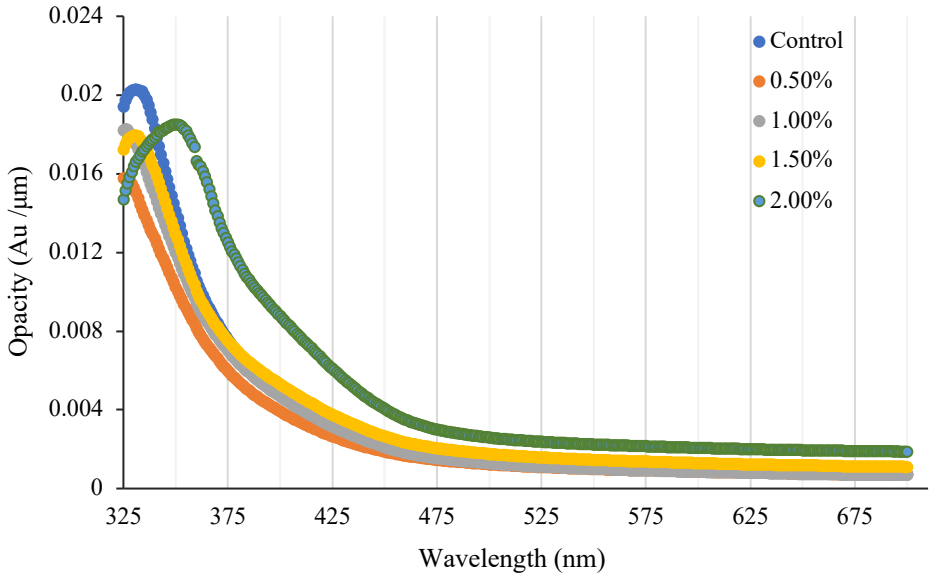


Figure 4.18 Effect of different concentrations of oregano-03 essential oil in the film-forming solution on opacity of SPI films prepared with 1.5% glycerol.

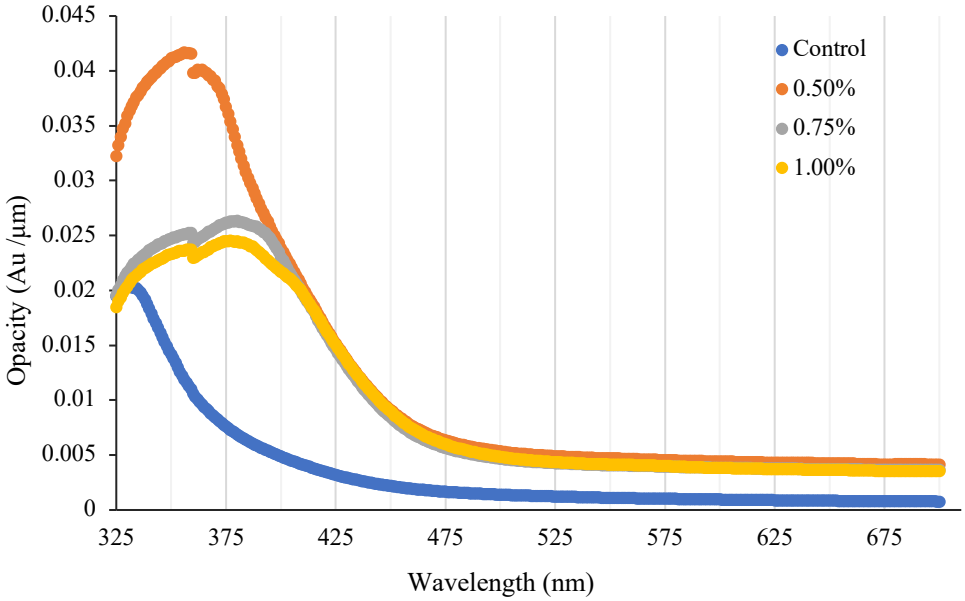


Figure 4.19 Effect of different concentrations of cinnamon-03 essential oil in the film-forming solution on opacity of SPI films prepared with 1.5% glycerol.

In this regard, the effects of the yellow color of cinnamon-03 EO were really obvious in our study. **Fig. 4.19** shows the effect of different concentrations of cinnamon-03 EO on opacity of SPI films prepared with 1.5% glycerol. The opacity values of films incorporating cinnamon-03 essential oil were larger than those of the control in all cases. Similar results were reported by Chen *et al*. (2016), who found lower transparency of chitosan films, measured at 600 nm, at higher concentration of cinnamaldehyde. Wu *et al*. (2017) also obeserved the reduction in light transmission of gelatin films at 600 nm with the addition of cinnamon EO, while the film's light transmission was slightly decreased between 400 and 600 nm with addition of cinnamon essential oil at a level of 0.5-2%, corresponding to our observation. Furthermore, with the addition of cinnamon EO, the opacity values of SPI films dramatically increased in the UV region in **Fig. 4.19**, indicating that cinnamon essential oil can effectively reduce UV light transmission in soy protein films, in concordance with the results reported by Wu *et al*. for gelatin films and SPI films incorporating cinnamon essential oil (Wu *et al*., 2017, 2019). Undoubtedly, food packaging materials that reduce exposure of the food to UV-visible light can effectively retard lipid oxidative deterioration and prevent nutrition losses to extend shelf life of food products (Liu *et al*., 2017). Such protective effects, which are generally not provided by plastic films, may be attained with incorporation of cinnamon EO in biopolymer films.

4.3.3.2 **Mechanical properties of soy protein films incorporating oregano or cinnamon essential oil**

Mechanical properties of soy protein films prepared with different concentrations of oregano and cinnamon essential are shown in **Fig. 4.20** and **Fig. 4.21,** respectively. All films were prepared with 1.5% glycerol, and the emulsification treatment applied was homogenization for 1.5 min. The significant decrease of tensile strength (TS) ($P \leq 0.05$) and Young's modulus ($P > 0.05$) and increase of elongation at break (EAB%) ($P > 0.05$) with increasing concentration of oregano essential oil observed in **Fig. 4.20** correspond to the results for SPI films incorporating carvacrol in Section 4.4.2.3. The decrease of Young's modulus with addition of oregano-03 essential oil is indicative of a plasticizing effect of oregano essential oil in SPI films. Similarly, the same trend of TS and EAB% in films with addition of oregano essential oil were observed in gelatin-chitosan films (Wu *et al*., 2014) and in red pepper seed meal protein films (Lee *et al*., 2016). A decrease of TS as essentail oil content increases has been reported in many diverse types of films, including partially hydrolyzed sago starch-alginate film enriched with lemongrass oil (Maizura *et al*., 2007) and hake protein films

incorporating thyme essential oil (Pires *et al*., 2011). These observations could be primarily explained in terms of the insertion of EO droplets between polymers in the film matrix (Atarés *et al*., 2010). The interactions between non-polar molecules and between non-polar and polar molecules were much weaker than those between polar molecules (Yang and Paulson, 2000). However, the characteristics of the lipid and its capacity to interact with the protein matrix also determined the effect of incorporated lipid on the mechanical properties of protein films (Gontard *et al*., 1994).

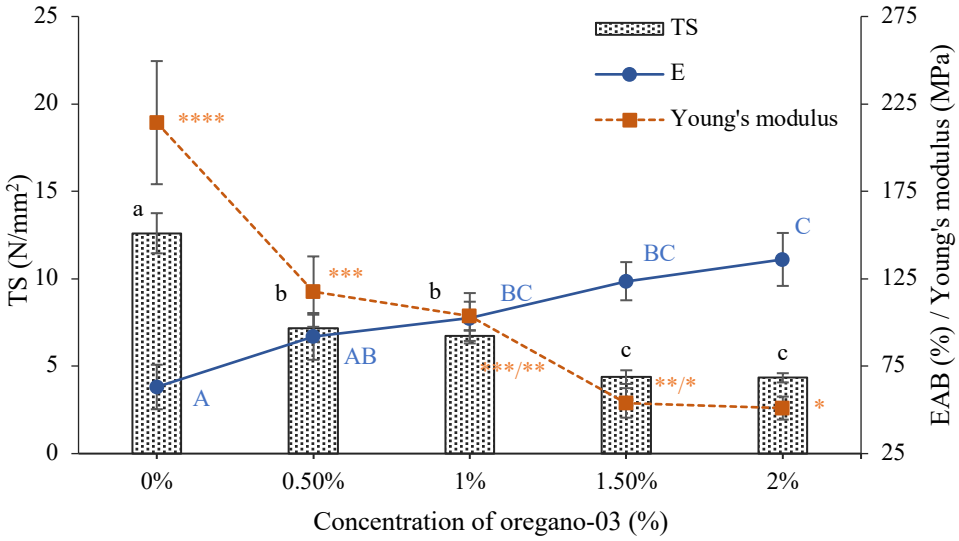


Figure 4.20 Tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus of SPI films prepared with various concentrations of oregano-03 essential oil and 1.5% glycerol in the film-forming solution. (Different letters and symbols represent significant differences, $P \leq 0.05$).

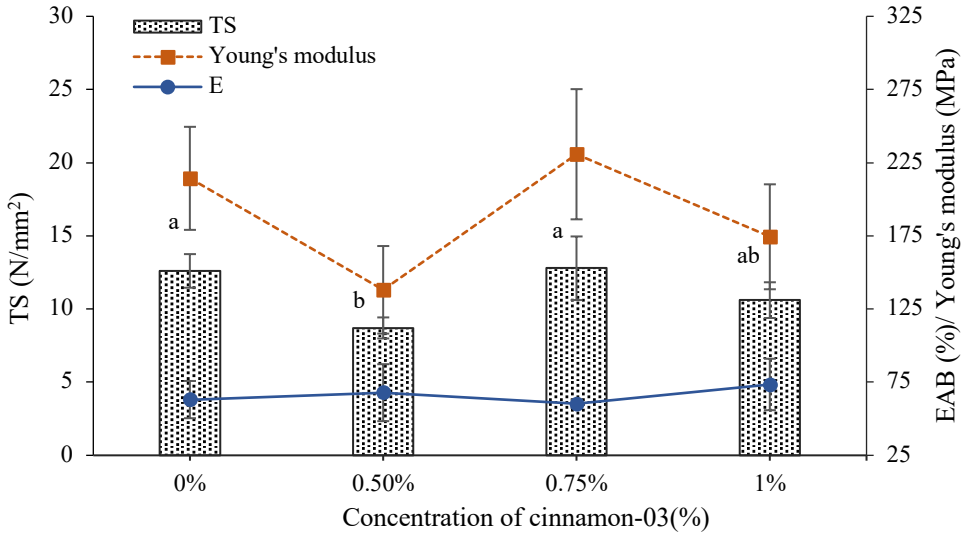


Figure 4.21 Tensile strength (TS), percentage elongation at break (EAB%) and Young's modulus of SPI films prepared with various concentrations of cinnamon-03 essential oil and 1.5% glycerol in the film-forming solution. (Different letters and symbols represent significant differences, $P \leq 0.05$)

In the case of cinnamon-03 essential oil in our study (**Fig. 4.21**), the TS of SPI films prepared with 0.75% cinnamon essential oil was higher than that of the films prepared with 0.5% cinnamon essential oil ($P < 0.05$) but was not significantly different from that of the control films (prepared without addition of essential oil). In addition, there were no significant differences in EAB% and Young's modulus among all the groups in **Fig. 4.21** ($P > 0.05$), although slight increases of EAB% with increasing concentration of cinnamon essential oil were observed. The TS of films incorporating cinnamon essential oil ranged between 8.7 and 12.8 N/mm^2, which is higher than that of the films incorporating oregano essential oil, while their EAB% values were lower. Thereby, cinnamon essential oil may have opposite effects on the mechanical properties of SPI films from those of oregano essential oil and carvacrol.

4.3.3.3 **Water vapor permeability (WVP) and water solubility of soy protein films incorporating oregano or cinnamon essential oil**

Table 4.4 shows the water vapor permeability (WVP), thickness and rate of water vapor transmission (WVTR) of soy protein films prepared with different concentrations of oregano-03 EO or cinnamon-03 EO. No significant changes in thickness, WVTR and WVP of films were observed with increasing essential oil content ($P > 0.05$). However, the slight reduction of WVTR from 28.0 $g/m^2 \cdot h$ to 27.3 $g/m^2 \cdot h$ with increasing content of oregano essential oil is consistent with the results presented above for the films incorporating carvacrol. In contrast, the WVTR of films incorporating cinnamon essential oil showed the opposite trend, which may indicate that incorporation of cinnamon essential oil had a different effect on the WVP of films and the microstructure of the film matrix compared with incorporation of carvacrol and oregano essential oil. The trend of WVP increase with increase in film thickness previously observed with the films incorporating carvacrol was also followed by all films incorporating oregano and cinnamon essential oils. The largest WVP values for these two sets of films were 2.46±0.55 $mm \cdot g/m^2 \cdot h \cdot kPa$ and 2.71±0.35 $mm \cdot g/m^2 \cdot h \cdot kPa$, respectively, at RH 30% -60%. These values are lower than WVP values reported by Otoni *et al*. (2016) for SPI films incorporating cinnamaldehyde or carvacrol (2.83± 0.09 $mm \cdot g/m^2 \cdot h \cdot kPa$). Similar results were also reported by many researchers (Lee *et al*., 2016; Maizura *et al*.,2007), who found the WVP of red pepper seed meal protein films or partially hydrolyzed sago starch-alginate films increased as the content of oregano or lemongrass essential oils increased, and their WVP values were even higher than our results. The same increasing effect of cinnamon essential oil on WVP was also demonstrated for chitosan and gelatin films by Perdones *et al*. (2014) and Wu *et al*. (2017). Besides the effects of lipid particle size and the ratio of

hydrophobic to hydrophilic components discussed previously, the influence of lipid addition on the microstructure of the protein film matrix may also be a determining factor for water permeability properties of the films. Atarés *et al*. (2010) postulated that the integration of lipid into the protein chain network could impact the moisture barrier of films by causing matrix disruptions and occurrence of void spaces at lipid-protein interfaces. They reported that the presence of a small proportion of ginger essential oil and cinnamon essential oil led to a decrease in the water vapor barrier properties of soy films. This result might be due to the low concentration of EO such that the lipid discontinuities were not sufficient to increase the tortuosity factor for transfer of water molecules, which is responsible for the reduction of WVP (Perez-Gago & Krochta, 2001a). Thus, the impact of lipid addition into a biopolymer matrix on water barrier efficiency is quite complex and may depend on the combined effects of several factors.

Water solubility of soy protein films prepared with different concentrations of oregano-03 or cinnamon-03 essential oil and 1.5% glycerol in the film-forming solution was determined and these results are shown in **Table 4.4**. The water solubility of the films slightly decreased to 25.24 ± 0.07% with increasing concentration of oregano EO, while the water solubility of the films prepared with cinnamon essential oil decreased to 22.38%, There were no significant differences ($P > 0.05$) in water solubility among films prepared with and without addition of EO. The water solubility of films is usually a decisive index of the hydrophilicity of films (Kim and Ustunol, 2001). Decreased solubility of soy films in water might be caused by increased hydrophobicity of the matrix with increasing content of EO, corresponding to our results showing decreasing WVTR of soy films with EO. The water solubilities of all soy films in our study were consistent with the solubilities of soy films (23%-24.6%) reported by Soliman *et al*. (2007) and were slightly lower than that of gelatin-chitosan blended films (30%-32.87%), whose film matrix materials were more soluble than soy protein (Wu *et al*., 2014). Decreasing solubility of whey films and blended protein-polysaccharide films with increasing concentration of oregano EO was reported by Bahram *et al*. (2014) and Wu *et al*. (2014), respectively. Therefore, addition of EO could also impact the water solubility of soy films depending on the extent of interaction between the protein and EO molecules as well as by changing the relative proportions of hydrophobic and hydrophilic constituents in the film matrix.

Table 4.4 Water vapor permeability (WVP), thickness, rate of water vapor transmission (WVTR) and water solubility (%) of soy protein films prepared with different concentrations of oregano-03 or cinnamon-03 essential oil and 1.5% glycerol in the film-forming solution

Essential oil	Concentration (w/v)	Thickness (mm)	WVTR ($g/m^2 \cdot h$)	WVP ($mm \cdot g/m^2 \cdot h \cdot kPa$)	Water solubility (%)
Oregano-03	0%	0.1167 ±0.0094 [a]	28.1986±1.3032 [a]	2.2281±0.2825 [a]	27.1403±0.9483 [ab]
	0.5%	0. 0963±0.0194 [a]	28.2908±1.6587 [a]	1.8270±0.2891 [a]	26.3813±0.7721 [ab]
	1.0%	0.1000±0.0140 [a]	28.1986±0.6384 [a]	1.9033±0.2386 [a]	27.5673±0.0271 [a]
	1.5%	0.1263±0.0159 [a]	27.4614±0.7819 [a]	2.3479±0.3621 [a]	26.2410±0.5962 [ab]
	2.0%	0.1333±0.0306 [a]	27.2771±0.3686 [a]	2.4553±0.5465 [a]	25.2400±0.0716 [b]
Cinnamon-03	0%	0.0963±0.0194 [a]	28.0143±1.0426 [a]	2.3060±0.3815 [a]	27.1403±0.9483 [a]
	0.50%	0.0992±0.0063 [a]	28.1986±0.7819 [a]	2.3282±0.0638 [a]	22.3808±1.9591 [a]
	0.75%	0.1038±0.0088 [a]	28.4751±1.1007 [a]	2.5374±0.0543 [a]	22.6315±0.3458 [a]
	1.00%	0.1108±0.0151 [a]	28.5058±0.2128 [a]	2.7105±0.3515 [a]	22.9189±0.2067 [a]

Different letters represent significant differences, $P \leq 0.05$

4.3.3.4 FTIR spectroscopic study of soy protein films incorporating oregano or cinnamon essential oil

Before examination of the FTIR spectra of the soy films incorporating oregano and cinnamon essential oils, some characteristic peaks in the FTIR spectra of the essential oils will be considered first.

The ATR-FTIR spectrum of oregano-03 EO is shown in **Fig. 4.22**. The sharp peaks in the region between 2800 and 3000 cm^{-1} are associated with C-H stretching vibrations, while the bands between 1600 and 1400 cm^{-1} are assigned to phenolic compounds like carvacrol and thymol as well as aromatic hydrocarbons present in oregano essential oil (Pelissari *et al*., 2009). The band at 1223 cm^{-1} has been assigned to the C-OH stretching vibration of carvacrol, and characteristic peaks associated with C-H bending and out-of-plane C-H (aromatic structure) wagging vibration are at 937 and 810 cm^{-1} (Kwon *et al*., 2017; Topala and Tataru, 2016).

The FTIR spectrum of cinnamon-03 essential oil is shown in **Fig. 4.23**. The peak at 1731 cm^{-1} is associated with the C=O stretching vibration of a non-conjugated aldehyde functional group whereas the peak corresponding to the C=O stretching vibration of cinnamaldehyde, an α, β-unsaturated aldehyde, is observed at 1673 cm^{-1} and the peak at 1626 cm^{-1} may be assigned to the stretching vibration of the conjugated C=C bond. The peak at 1575 cm^{-1} is assigned to the aromatic ring C=C skeleton of an aromatic substance. The peak at 1450 cm^{-1} is very characteristic of alcohols. In addition, some peaks characteristic of esters and eugenol are observed in the spectrum of the cinnamon essential oil, including peaks due to the in-plane bending of =C-H in aromatic rings at 1294 cm^{-1} and the stretching vibrations of the aromatic acid ester C-O-C and the phenolic C-OH group at 1250 cm^{-1}. Peaks attributed to C-O stretching and C-OH deformation are observed at 1120 and 1072 cm^{-1}, and peaks observed at 970 and 745 cm^{-1} have been assigned to C-H bending and benzene ring =CH bending, respectively (Li*et al*., 2013).

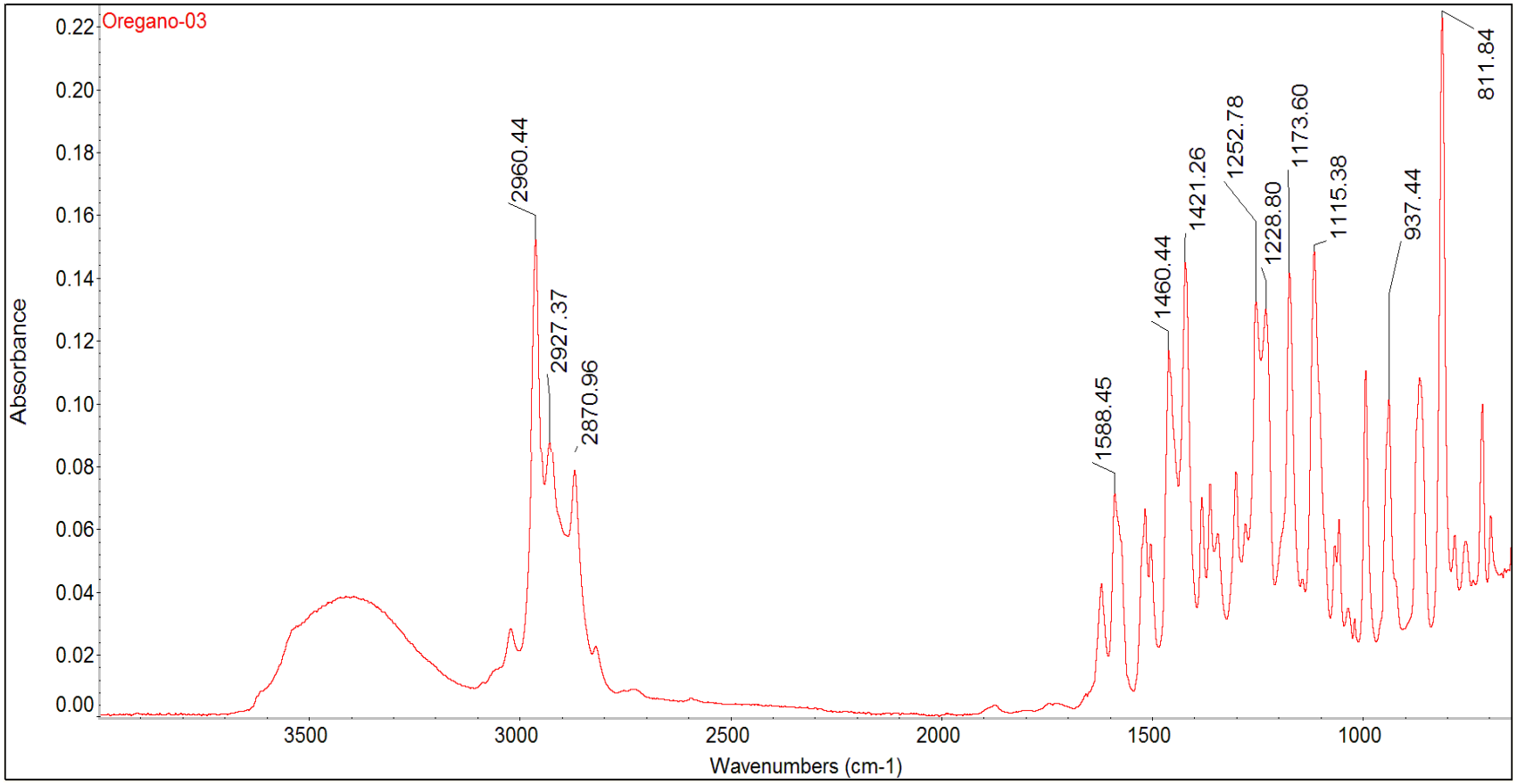


Figure 4.22 FTIR spectrum of oregano-03 essential oil and the wavenumber positions of some characteristic peaks in the 3000-700 cm^{-1} region.

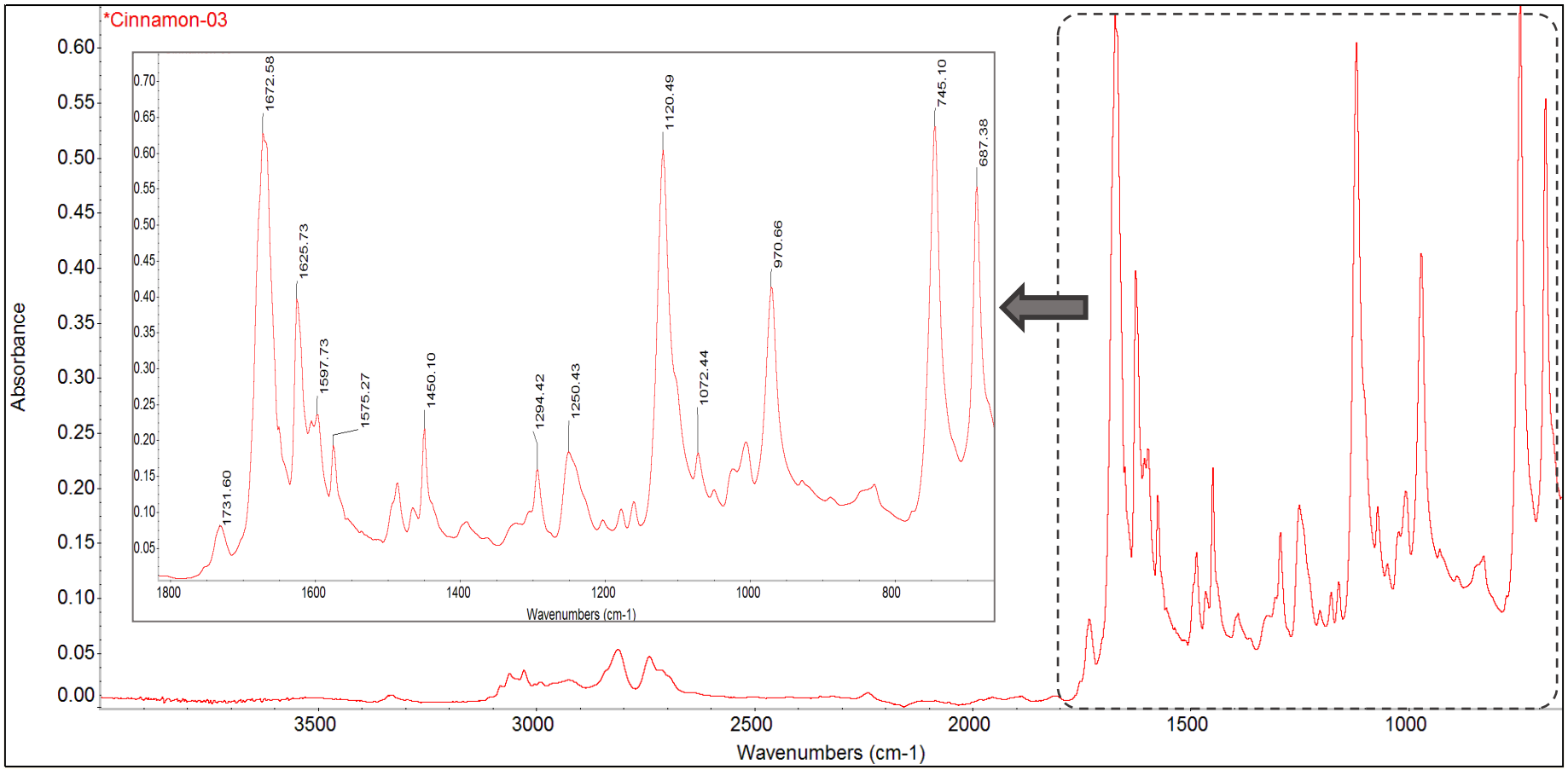


Figure 4.23 FTIR spectrum of cinnamon-03 essential oil and peak positions in the 1800-600 cm^{-1} region

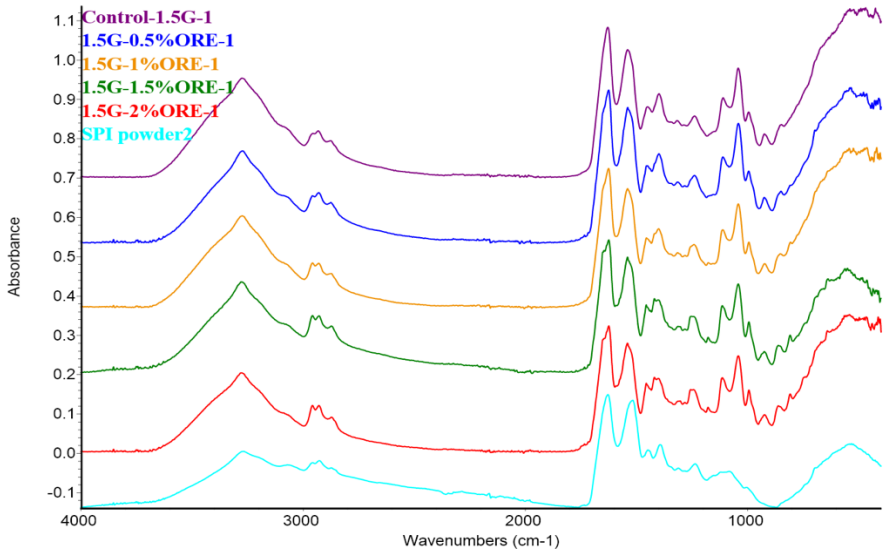


Figure 4.24 FTIR spectra of soy protein-based edible films prepared with different concentrations of oregano-03 EO (0-2% in the film-forming solution) together with the spectrum of pure SPI powder.

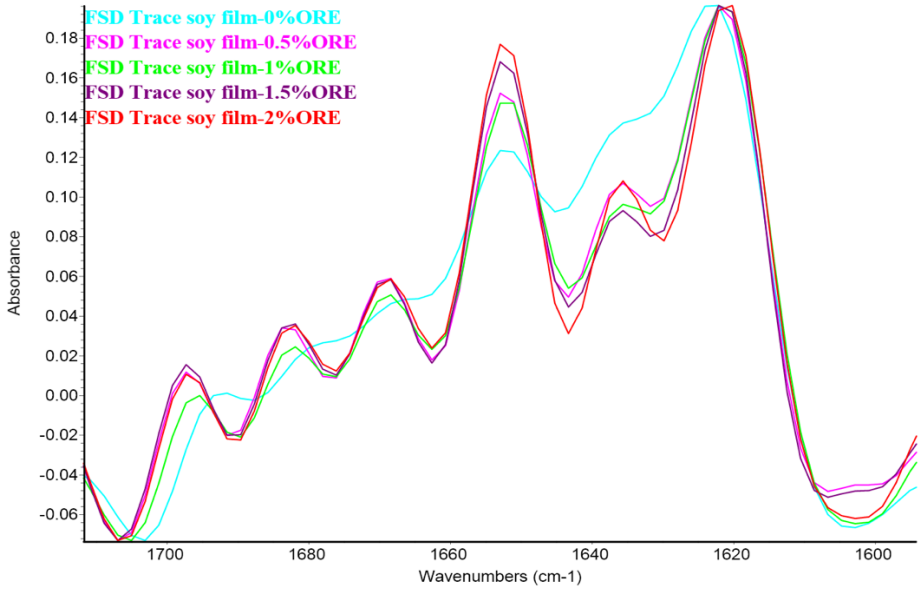


Figure 4.25 Fourier self-deconvoluted (FSD) protein amide I region in the FTIR spectra of soy protein films prepared with different concentrations of oregano essential oil (0-2% in the film-forming solution) after subtraction of the spectrum of oregano essential oil.

Table 4.5 Estimated percentages of protein secondary structure types in SPI films incorporating oregano essential oil

Concentration of oregano EO in film-forming solution	Type of secondary structure			
	Anti-parallel β-sheet (%)	Parallel β-sheet (%)	α-Helix (%)	β-Turn (%)
Control	67.2	4.6	26.4	1.8
0.5%	49.0	4.8	29.5	16.7
1.0%	56.1	3.5	28.4	12.1
1.5%	48.4	3.6	32.3	15.8
2.0%	50.7	5.5	31.4	12.3

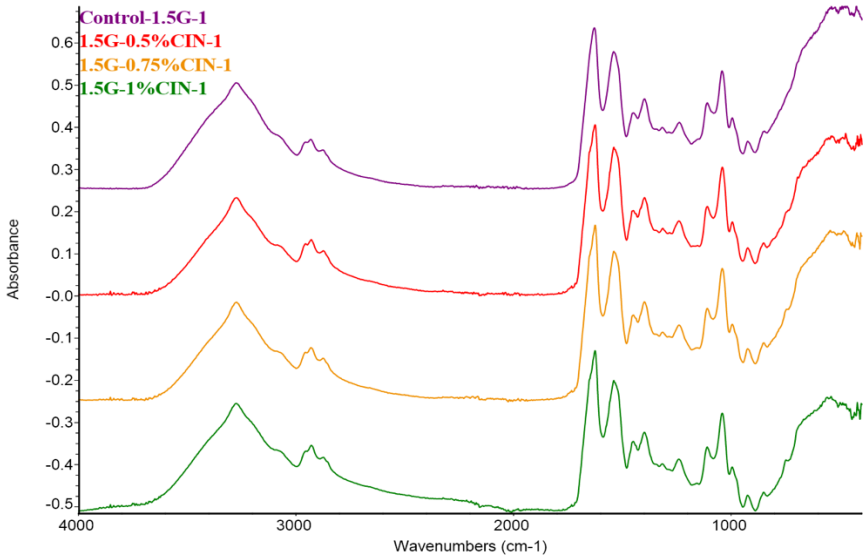


Figure 4.26 FTIR spectra of soy protein-based edible films prepared with different concentrations of cinnamon-03 essential oil (0.5%-1% in the film-forming solution) and control film.

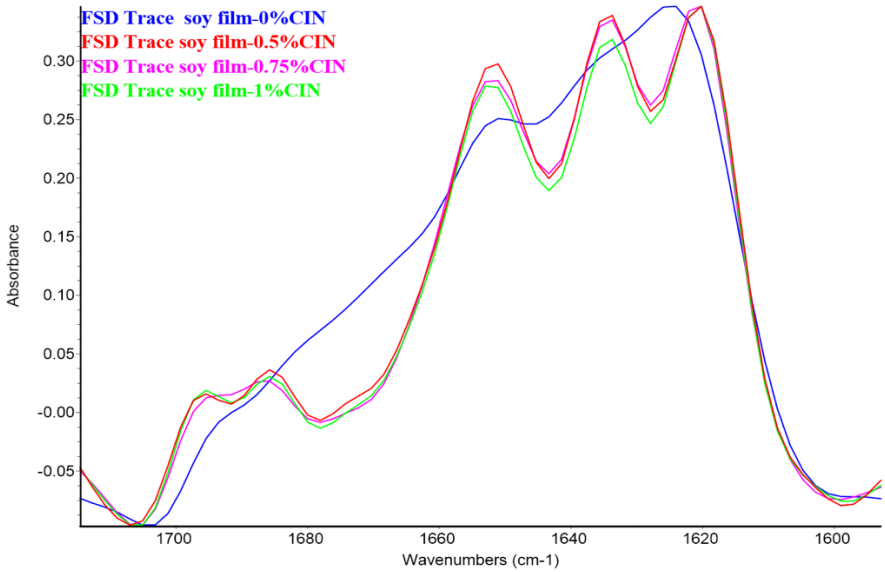


Figure 4.27 Fourier self-deconvoluted (FSD) amide I region in the FTIR spectra of soy protein films prepared with different concentrations of cinnamon essential oil (0-1% in the film-forming solution) after subtraction of the spectrum of cinnamon essential oil.

Table 4.6 Estimated percentages of protein secondary structure types in SPI films incorporating cinnamon essential oil

Concentration of cinnamon essential oil in film-forming solution	Type of secondary structure			
	Anti-parallel β-sheet (%)	Parallel β-sheet (%)	α-Helix (%)	β-Turn (%)
Control	57.4	5.8	37.9	0
0.50%	39.9	16.8	38.6	4.7
0.75%	40.9	15.7	40.2	3.1
1.00%	41.6	15.3	38.7	4.4

The FTIR spectra of soy protein films prepared with different concentrations of oregano and cinnamon essential oil are presented in **Fig. 4.24** and **Fig. 4.26**, respectively, and are quite similar to those of the control films, but small differences can be observed in some bands. In the case of addition of oregano essential oil into soy protein films, slight broadening of the band between 3600 and 3000 cm^{-1} associated with O-H and N-H stretching vibrations indicates more extensive hydrogen bonding in the film matrix, which may be due to hydrogen bonding between the protein and phenolic compounds present in oregano essential oil such as carvacrol and thymol. In addition, a shoulder on the high-wavenumber side of the amide I band in the spectra of films incorporating oregano essential oil is absent in the spectrum of the control film and appears to increase as the content of oregano essential oil in the film increases, The spectrum of oregano essential oil shown in **Fig. 4.21** shows only very minor absorptions in this region, suggesting that the observed changes are due to changes in protein secondary structure in SPI films in the presence of oregano essential oil. Thus, the spectra in **Fig. 4.24** were further analyzed in the same manner as described previously for the spectra of the soy protein films incorporating carvacrol. The results obtained after subtraction of the spectrum of oregano essential oil, vector normalization in the region of 1720-1490 cm^{-1} and FSD of the amide I band, with a bandwidth of 26.4 and enhancement of 2.4, are shown in **Fig. 4.25.** Six peaks within the amide I region are observed in **Fig. 4.25** and may be assigned as follows, based on established wavenumber ranges for secondary structure components in the amide I region of FTIR spectra of proteins in H_2O aqueous environment (Yang *et al*., 2015; Wang *et al*., 2016; Liu *et al*., 2011; Alvarez *et al*., 2008): the peaks at 1622, 1635 and 1696 cm^{-1} are associated with β-sheet structure, the peak at 1652 cm^{-1} is assigned to α-helical structure and the peaks at 1668 and 1682 cm^{-1} belong to β-turns. The areas of these peaks were calculated to estimate the percentages of each type of secondary structure in each of the films and these estimates are shown in **Table 4.5**. With addition of oregano essential oil into the soy film matrix, the intensities of the peaks at 1696 and 1622 cm^{-1} associated with antiparallel β-sheet structure gradually decreased, resulting in a decrease in the estimated percentage of anti-parallel β-sheet structure in the protein structure from 57.4% in the control film to 50.7% in the film with the highest content of oregano EO. Meanwhile, the area of the peak at 1635 cm^{-1} attributed to parallel β-sheet structure decreased slightly except in the case of the film prepared with 2% oregano EO. On the other hand, the area percentage of the α-helix component increased from 26.4% to as high as 32.3% with addition of the essential oil into the protein film matrix. These results revealed that the decreasing proportions of β-sheet structure were likely due to transformation of some β-sheets to α-helices, corresponding to

the transformation of some β-sheet structure to a new α-helical component that occurred with addition of carvacrol into soy films, as described above. Owing to the complex composition of oregano essential oil and the larger size of some of its components relative to carvacrol, some differences between the effects of oregano essential oil and carvacrol on protein structure in SPI films would be anticipated. Thus, the formation of a new α-helical band in the FSD spectra of the films incorporating carvacrol was not observed in the FSD spectra in **Fig. 4.27**; instead, the increasing intensity of the pre-existing α-helical band was observed with increasing content of oregano essential oil in the SPI films. It may be noted that addition of chestnut bur extracts (CBE) in SPI films was reported to induce the transformation of β-sheets to both α-helical and unordered (random coil) structure (Wang *et al*., 2016; Yan *et al.*, 2017).

Analysis of the amide I region in the FTIR spectra of the SPI films incorporating cinnamon essential oil in **Fig. 4.26** is complicated by the spectral interferences from cinnamon essential oil. In particular, the spectrum of cinnamaldehyde, the major compound in cinnamon essential oil, exhibits two peaks (1672 and 1625 cm^{-1}) in the protein amide I region that would interfere with the estimation of protein secondary structure in the film matrix Consequently, the subtraction of cinnamon essential oil peaks in the amide I region was necessary for further analysis of the amide I band. Thus, the spectrum of cinnamon essential oil was subtracted from the spectra in **Fig. 4.26** by scaling the subtraction on the band of cinnamon essential oil at 745 cm^{-1}. The resulting spectra were processed by vector normalization in the region of 1720-1490 cm^{-1} followed by Fourier self-deconvolution in the amide I region, with a bandwidth of 17.5 and enhancement factor of 1.8, As seen in **Fig. 4.27**, five peaks were observed in the FSD spectra: the peaks at 1620, 1634 and 1695 cm^{-1} can be assigned to β-sheet structure, the peak at 1652 cm^{-1} to α-helical structure and the peak at 1685 $cm^{-1}$to β-turns (Yang *et al*., 2015; Wang *et al*., 2016; Liu *et al*., 2011; Alvarez *et al*., 2008), The areas of these peaks were calculated to estimate the percentage of each type of secondary structure in the films (**Table 4.6**). **Fig. 4.27** shows similar peak locations but different relative peak intensities in the FSD spectra of the films incorporating different concentrations of cinnamon essential oil. Unlike the transformation of secondary structure of soy proteins in films by oregano essential oil and carvacrol, there were no obvious changes in the percentages of different types of secondary structure with increasing content of cinnamon essential oil in the films. However, the content of incorporated essential oil in these films was much lower than in the cases of oregano essential oil and carvacrol. On the other hand, **Table**

4.6 shows slightly decreased percentage of parallel β-sheets and increasing percentages of anti-parallel β-sheets and α-helical structure with increasing content of cinnamon essential oil in the films. It is likely that the high dipole moment (D = 4.9 or -1.5) (Zinn *et al.*, 2015) and low dielectric constant of cinnamon essential oil could induce an α-helix conformation as we discussed above (Nitthiyah *et al.*, 2016; Jackson & Mantsch, 1992). Therefore, the addition of cinnamon essential oil to soy films may slightly change the secondary structure of the proteins in the film matrix.

4.3.4 **The antimicrobial activities of soy protein films incorporating carvacrol, oregano essential oil, and cinnamon essential oil**

The antimicrobial activities of soy protein films incorporating different concentrations of oregano and cinnamon EOs and carvacrol are shown in **Table 4.7**. The films incorporating carvacrol and oregano EO exhibited strong antimicrobial activities against *L. grayi* and *E. coli* K12 in direct contact with the bacteria, and the EO vapor released from these films also was effective in inhibiting the growth of *L. grayi*. The films prepared with 2-3% carvacrol and 1.5-2% oregano EO produced significantly larger inhibition zones ($P \leq 0.05$) than the other films in the disk diffusion and vapor diffusion tests with *L. grayi* and in the disk diffusion tests with *E. coli* K12, indicating that the films incorporating carvacrol and oregano EO in high concentration had higher antimicrobial activities. The lack of effect of the EO vapor from the soy films in the vapor diffusion tests with *E. coli* K12 revealed the higher resistance of *E. coli* K12 in comparison with *L. grayi* to EOs, as observed in our previous studies. Unfortunately, in the case of the films incorporating cinnamon EO, inhibition zones were only observed in the disk diffusion tests with *L. grayi,* where only films prepared with the higher concentrations of cinnamon EO produced small inhibition zones.

Table 4.7 The diameter of the inhibition zone for soy protein films prepared with different concentrations of carvacrol, oregano EO, and cinnamon EO against the growth of *L. grayi* and *E. coli* K12 *in vitro*.

Essential oil	Concentration in film-forming solution	Disk diffusion (mm)		Vapor diffusion(mm)	
		L. grayi	*E. coli* K12	*L. grayi*	*E. coli* K12
Carvacrol	0.00%	-	-	-	-
	1.00%	15.49±1.34 [a]	12.11±0.43[a]	7.28±0.97 [a]	-
	2.00%	23.68±3.69 [b]	15.98±0.32[b]	19.32±2.73 [b]	-
	3.00%	23.83±1.00 [b]	17.62±0.49[c]	19.37±1.38 [b]	-
Oregano	0.00%	-	-	-	-
	0.50%	10.63±0.42 [a]	9.78±0.27 [a]	-	-
	1.00%	12.65±0.46 [b]	12.87±0.58 [b]	-	-
	1.50%	16.81±1.02 [c]	12.60±0.31 [b]	8.72±1.96 [a]	-
	2.00%	20.94±0.94 [d]	15.81±1.03 [c]	11.76±1.67 [a]	8.53±3.75
Cinnamon	0.00%	-	-	-	-
	0.50%	-	-	-	-
	0.75%	8.44±0.03 [a]	-	-	-
	1.00%	8.41±0.23 [a]	-	-	-

- Indicates that no inhibition zone was observed.
Different letters represent significant differences, $P \leq 0.05$

Although the films with cinnamon EO had almost no antimicrobial effects, their yellow color indicated the presence of cinnamon EO inside the films, and the peak at 745 cm^{-1} in their FTIR spectra, which is observed in the spectrum of cinnamaldehyde, indicated that this phenomenon was not caused by the evaporation of cinnamaldehyde during the drying of the films. Instead, formation of a Schiff base between the aldehyde group in cinnamaldehyde and side-chain -NH_2 groups in the soy protein could account for the very low antimicrobial activity of these films. In a study by Higueras *et al*. (2015) in which Schiff base formation between cinnamaldehyde and -NH_2 groups in chitosan films was investigated, the antimicrobial activity of the films was reported to be related to the rate of release of cinnamaldehyde by hydrolysis of the Schiff base and was low under conditions of slow hydrolysis (pH 7, 4°C). FTIR spectra of these polysaccharide films exhibited a band at 1637 cm^{-1} that was assigned to the stretching vibration of the imino bond in the Schiff base. However, the intense protein amide I band would preclude observation of this band in the spectra of protein-based films, and accordingly we could not obtain FTIR spectroscopic evidence of Schiff base formation in the SPI films incorporating cinnamon essential oil.

4.4 **Conclusion**

Based on the conditions for the preparation of soy protein films as carriers for essential oils in active-packaging applications, the conditions selected for film preparation were 2% glycerol, pH 10 for the film-forming solution and heating of this solution at 85 ºC for 10 minutes. These conditions were used to prepare the soy films incorporating carvacrol, oregano essential oil and cinnamon essential oil. The mechanical properties, water vapor permeabilities, and opacity of SPI films were influenced by the type of essential oil, the concentration of glycerol and essential oil, and emulsification treatments. The emulsified carvacrol and oregano essential oil acted as plasticizers like glycerol to reduce resistance and rigidity of SPI films and increase their extensibility, resulting in increases in elongation at break (EAB) coupled with decreases in tensile strength (TS) and Young's modulus of SPI films. In contrast, incorporation of cinnamon essential oil in SPI films had the opposite effect on the mechanical properties of the films. The addition of glycerol and essential oils was found to increase the WVP of SPI films, whereas the effect of the essential oils on the rate of water vapor transmission through the films depended on the type and concentration of essential oil. High concentrations of glycerol and essential oils improved the transparency of SPI films, which was also affected by the emulsification treatment employed. Fourier transform infrared (FTIR) spectroscopic analysis of protein secondary structure in the films revealed the transformation of β-sheet to α-helical structure with addition of carvacrol and oregano essential oil, which may have been a contributing factor to the increased flexibility of the films with higher content of carvacrol and oregano essential oil. In addition, the effectiveness of the soy films incorporating essential oils in inhibiting microbial growth showed that films prepared with ≥1.5% oregano EO or ≥2% carvacrol in the film-forming solution exhibited high antimicrobial activities against *L. grayi* in the vapor phase and could be employed in active packaging to improve the safety of vegetable products.

CONNECTING STATEMENT 3

The characteristics of incorporation of oregano and cinnamon EOs and carvacrol in soy protein films were presented in Chapter IV. The type of EO, the concentrations of glycerol and EO, and the emulsification treatment employed in preparing the films influenced their mechanical properties, water vapor permeabilities, and opacities. FTIR spectroscopic results also indicated that some transformation of protein secondary structure resulted from interactions between soy proteins and the EOs incorporated in the films, consistent with a plasticizing effect of the EOs. In addition, the vapor of oregano EO and carvacrol released from soy films can effectively inhibit the growth of *L. grayi in vitro*. In order to investigate the diffusion kinetics of active compounds of essential oils from packaging polymers, cellulose stickers impregnated with EOs in Chapter III and soy films incorporating carvacrol in Chapter IV have been evaluated by vapor phase-Fourier transform infrared (FTIR) spectroscopy and a Weibull distribution model was used to fit the results, as described in Chapter V. Furthermore, the rates of evaporation of carvacrol from different packaging matrices were tested and compared in terms of calculated parameters from the Weibull model.

This work will be presented together with results in Chapter IV as oral presentation at the 2020 AOCS Annual Meeting & Food-American Oil Chemists' Society, and subsequently will be submitted together with results in Chapter IV as a manuscript for publication in the journal *Food Control*.

CHAPTER 5 MONITORING EVAPORATION OF CARVACROL FROM PACKAGING MATERIAL BY FOURIER TRANSFORM INFRARED SPECTROSCOPY

Abstract

The antimicrobial properties of carvacrol, a major constituent in oregano and thyme essential oils, make it a good choice for active-packaging applications. As an aroma compound, carvacrol has sufficient vapor pressure to exhibit antimicrobial effects in the headspace of food packaging by evaporation from essential oils in suitable carrier matrices. In designing such types of active packaging, the rate of release of the active compound into the headspace of the packaging material is an important consideration in selecting the carrier matrix. In this chapter, the use of gas-phase Fourier transform infrared (FTIR) spectroscopy to monitor the release of carvacrol from various carrier matrices inside an infrared gas cell was investigated. For this purpose, the integrated intensity of the absorption band of carvacrol at 810 cm^{-1} in the vapor-phase spectra was employed to track the increasing concentration of carvacrol as a function of time, and the results were fitted to a Weibull model by non-linear regression analysis. This methodology was tested by monitoring the evaporation of a 1-mL volume of pure carvacrol inside the gas cell, and fitting of the Weibull model to the results yielded a coefficient of determination of 0.988 and a root-mean-square error of 0.001. Comparison of the derived Weibull model parameters with those obtained for vapor release from carvacrol stickers and from thyme essential oil encapsulated in alginate beads indicated the potential suitability of both these delivery methods for the rapid release of carvacrol into the headspace of packaging. The FTIR methodology was also applied in the study of soy protein films incorporating carvacrol and different concentrations of glycerol as plasticizer and revealed that the plasticizing effect of glycerol may promote carvacrol diffusion and release. Finally, monitoring of carvacrol release from four common matrix materials showed that differences in the microstructure of the matrix greatly affected the release kinetics, with the evaporation rate decreasing in the order fumed silica > cotton > clay > chitosan. The simplicity and versatility of the FTIR methodology make it a valuable tool for studying the release kinetics of essential oil active compounds for active-packaging applications.

Keyworks: Fourier transform infrared (FTIR) spectroscopy, Weibull model, carvacrol.

5.1 **Introduction**

Active packaging is an innovative concept in food packaging that aims to provide better protection to the food product than conventional packaging by actively interacting with the inner environment of the package (Yam *et al*., 2005). It is commonly known that food spoilage is mostly caused by microbial contamination. Antimicrobial packaging systems are a version of active packaging with an emphasis on controlling the growth of microorganisms (Floros *et al*., 1997). In active-packaging systems using an antimicrobial agent to control microbial growth, the aim is to release the antimicrobial agent in a controlled manner. By comparison with conventional packaging in which an excess amount of the active agent is applied at once, this controlled release is advantageous in terms of maintaining optimum antimicrobial effect for a longer time. On the other hand, the release rate should not be so slow that inhibitory effects are not achieved during the lag phase of microbial growth.

Among the antimicrobial agents that may be considered for active-packaging applications, essential oils and their constituents have attracted a lot of attention. The strong antimicrobial effects of carvacrol, a constituent of oregano and thyme essential oils, make it a good choice for active-packaging applications (López *et al*., 2007). Two approaches can be used to introduce carvacrol into packages: One is by incorporating it inside the package in components such as sachets and absorbent pads, and the other is by encapsulation or surface coating (Suppakul *et al*., 2002). In antimicrobial packaging, carvacrol can be released by diffusion through direct contact with the food or by evaporation in the headspace of the package. The latter is more advantageous in packaging of fresh products such as meat, cheese, fruits, vegetable or dry products. Controlled release of carvacrol in the packaging system is very important for several reasons. First, carvacrol has a pleasant and distinct odor at low concentration, but the odor will be bitter and pungent at higher concentration (Giatrakou *et al*., 2008). If the evaporation rate is too high, the organoleptic attributes of the food will be compromised, and the antimicrobial effect will also not be sustainable. Second, if the evaporation rate is too low to reach the minimum inhibitory concentration (MIC) before the end of the lag phase, the microbial growth will be out of control (Kurek *et al*., 2014).

Several factors affect the rate of evaporation of carvacrol into the headspace of a food package, including the type and microstructure of the matrix in which it is incorporated and the environment inside the package. In a matrix system, the evaporation process can be divided into three stages: (i) diffusion of the active agent to the surface of the matrix; (ii)

partition of the active agent between the matrix and the surrounding medium (air surrounding the matrix); and (iii) transportation of the active agent from the matrix surface. In the first step, the diffusion rate within the matrix depends on its microstructure and the specific molecular interactions of the carvacrol with the matrix particles. These interactions include hydrogen bonding, adsorption, entrapment and encapsulation (Godshall, 1997; Kinsella, 1989). In the second step, the carvacrol molecules partition between the matrix and the surrounding atmosphere in a ratio that depends on temperature and pressure. Other parameters also can greatly affect the evaporation rate. For example, the release of carvacrol from a chitosan matrix was found to be dependent on relative humidity (RH), and this dependence was attributed to swelling of the chitosan microstructure with increasing RH, resulting in a higher diffusion rate of carvacrol molecules in the matrix and consequently a higher evaporation rate (Kurek *et al*., 2014). As the concentration of carvacrol in the gas phase increases with time, less carvacrol will be distributed from the matrix to the gas phase, and the evaporation rate will decrease.

Four materials are commonly used in food packaging as a carrier matrix. (i) Clay (GRAS) is a fine-grained natural rock or soil material and consists of small platelets that are less than 2 μm in diameter. Many studies have shown that the interaction of clay with the plastic polymers in biopolymer films can accelerate the evaporation of aroma compounds from the clay matrix (Mascheroni *et al*., 2011; Kurek *et al*., 2014; Kurek *et al*., 2012). In addition, clay is a commonly used matrix in controlled-release systems, not only because of its low cost but also because of its biocompatibility, stability and high absorption capacity (Murray, 2000). (ii) Chitosan is a linear polysaccharide that is extracted from the shells of shrimp and other crustaceans. It has been widely studied as a carrier matrix for volatile compounds in food packaging (Kurek *et al*., 2012). The combined effects of relative humidity and temperature can cause plasticization of its microstructure, increasing the mobility of entrapped aroma compounds, resulting in increased evaporation rate of the aroma compounds in a high-RH environment (Chalier *et al*., 2009). (iii) Cotton is a natural polymer that has been used in food packaging since early times. Cotton is normally used in absorbent pads as the carrier material. Cotton absorbent pads with antimicrobial agents can offer an extension of shelf life not only by releasing antimicrobial vapor but also by absorbing bacteria-containing extruded liquid from the food product (Otoni *et al*., 2016). The fabric microstructure of cotton is also used as a frame to combine with other materials to form controlled-release systems (Abdel-Mohdy *et al*., 2008; Liu *et al*., 2001). (iv) Silica is a

potential inorganic matrix material that can be used as an alternative to polymeric materials. As an inorganic matrix, silica retains aroma compounds in its porous microstructure without affecting their antimicrobial activities. Silica mesoporous supports (SMPS) are widely used in controlled-release systems because of their large surface area and high load capacity for volatile compounds (Janatova *et al*., 2015). Fumed silica consists of branched chainlike particles with very low density and high surface area.

In this study, all these materials were tested as carrier matrix materials for carvacrol. Fourier transform infrared (FTIR) spectroscopy is an analytical technique that is applicable to samples in the solid, liquid, or vapor phase. This technique can obtain a spectrum over the full mid-infrared range of wavelengths at high resolution in a scan time of less than one minute. With the use of an infrared gas cell, FTIR spectroscopy can be used to monitor the release of aroma compounds into the vapor phase and track their concentrations as a function of time. Compared with the conventional ways of studying aroma compound release from polymers by extraction of the residues (Mascheroni *et al*., 2011; Requena *et al*., 2017) or measuring the amounts released into water as a model system (da Costa *et al*., 2012; Yin, & Yates, 2009), the speed, sensitivity, and high throughput of FTIR spectroscopic analysis make this approach advantageous (Reichenbacher *et al.*, 2014). However, to date, few researchers have used FTIR spectroscopy as a method to monitor the kinetics of release of vapor from polymers or powders (Wang & Lim, 2014). Thus, in the research presented in this chapter, we investigated the capability of gas-phase FTIR spectroscopy to monitor the release of carvacrol from various matrices into the confined space of an infrared gas cell.

As part of this investigation, we explored the application of the Weibull distribution to model the FTIR data we acquired in order to gain additional information about the release kinetics of carvacrol from different matrices. Weibull models are empirical models that are established based on the Weibull distribution, which has been widely used because of well-fitting of a variety of events, like time to completion or time to failure. In an evaporation process, the probability density of vaporized molecules at a particular time follows the Weibull distribution. The cumulative distribution equation reflects the probability of a particular molecule to evaporate at any time. On a macro-level, the probability reflects the percentage of the molecules released into the air:

$$\ln\left(\frac{C_t - C_\infty}{C_0 - C_\infty}\right) = -(t/\alpha)^\beta \quad (4)$$

where. C_∞, C_t and C_0 are the concentrations of these molecules in the air at different times (infinity, time t and time 0). In equation (4), the scale parameter α (in units of time), the shape factor β (dimensionless) and the theoretical C_∞ are the calculated Weibull model parameters that are regressed to fit the data. Weibull models have been used to describe the kinetics of various processes in food, including the moisture transmission during air drying and dehydration processes and microbial killing kinetics under heat treatment, as well as the diffusion of drugs from sustained-release polymeric suspensions (Meisamisal *et al.,* 2010; Menges & Ertekin, 2006; Morales *et al*., 2004; Poças *et al.*, 2012). Compared with the conventional model of Fick's 2nd law, the simple mathematical calculation of the Weibull model can also quantify the additive migration from plastic packaging to food with equal precision. Furthermore, it can better describe some atypical curve patterns, for instance, the migration of certain substances from paper packaging with an observed initial lag (β > 1) (Poças *et al.*, 2012). Thus, despite the empirical nature of Weibull models, it is possible to identify relationships between the Weibull model parameters and underlying phenomena. In this chapter, this possibility will be investigated for Weibull models fitted to FTIR data acquired by monitoring the release of carvacrol vapor from various matrices by gas-phase FTIR spectroscopy.

5.2 **Materials and Methods**

5.2.1 **Materials**

Carvacrol (food grade, 98% purity) was obtained from Sigma-Aldrich (Milwaukee, WI, USA). The oregano and cinnamon essential oils employed in this study were supplied by BSA Frutarom (Saint-Leonard, QC, Canada) and Novotaste Inc. (Montreal, QC, Canada), respectively. They are the essential oils designated herein and in Chapter III as ORE-03 EO and CIN-03 EO, respectively. Alginate beads containing essential oils (2.2% w/w; 0.2% sage and 2% thyme essential oils) were made at Agriculture and Agri-Food Canada's Centre of Research and Development in Saint-Hyacinthe, Quebec (CRD St-Hyacinthe) and the size of the beads was 510 ±150 µm. Low-molecular-weight (55-60% deacetylated) chitosan, fumed silica and organic clay were provided by Thermal Lube Inc. (Pointe-Claire, QC, Canada), and cotton pads were purchased from a local retail outlet. Celloluse paper impregnated with EOs and soy protein films incorporating carvacrol were prepared as described in Section **3.2.2.6** and **4.2.2.2**, respectively, and were cut into circular pieces 98 mm in diameter.

5.2.2 **Development of FTIR methodology for monitoring release kinetics of carvacrol and essential oils from various carrier matrices**

Evaporation of carvacrol and essential oils was monitored by gas-phase FTIR spectroscopy using an experimental setup similar to that described by Reichenbacher *et al.* (2014), illustrated in **Fig. 5.1**. An Excalibur FTIR spectrometer (Agilent Technologies, Santa Clara, CA, USA) was employed, and a plastic tube sealed by polyethylene film at both ends served as an infrared gas cell. Samples were placed tin this gas cell in a plastic cap, and the gas cell was then sealed and placed in the sample compartment of the spectrometer, with the sample situated below the beam path so that the infrared beam passing through the gas cell to the detector would be attenuated only by infrared absorptions of molecules released into the vapor phase. FTIR spectra were collected every 15 min for the first 4-5 h and then every 2 h for the next 35-40 h. The spectra were recorded between 4000 and 600 cm^{-1} at a resolution of 4 cm^{-1} by co-addition of 64 scans. The integrated intensities of selected absorption bands in the vapor-phase spectra were measured by spectral analysis software (Omnic 3.8, Thermo Fisher Scientific, Waltham, MA, USA and SpectrAnalyze, (CogniSolve Inc., Montreal, QC, Canada). These FTIR data were analyzed and fitted to a Weibull model as described in Section **5.2.4**.

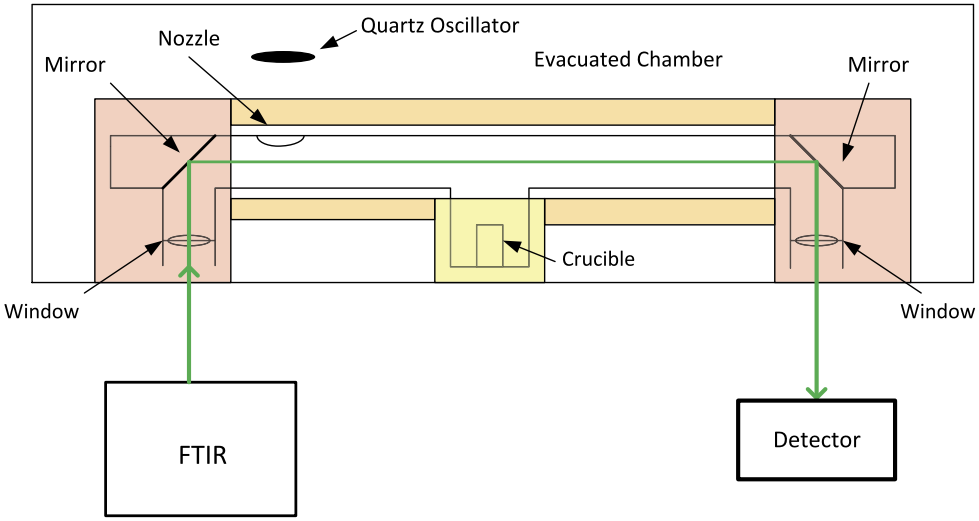


Figure 5.1 Schematic diagram of the experimental setup employed by Reichenbacher *et al.* (2014).

5.2.3 **Study of carvacrol release kinetics from different matrices**

5.2.3.1 **Sample preparation**

Samples were prepared by mixing between 0.5 and 1 g of different matrix materials with 1 or 2 g of carvacrol. The mixtures were stirred with a glass rod for 30 min to allow for better mixing. Four matrix materials were employed: clay, chitosan, fumed silica, and cotton. The exact amounts of carvacrol and matrix material that were mixed together are shown in **Table 5.1**.

Table 5.1 Combination of different amounts of carvacrol with different matrix materials.

Sample	Matrix	Weight of matrix (g)	Weight of carvacrol (g)	Concentration of carvacrol (wt %)
CR-CL	Clay	1.0027±0.0035	1.0015±0.0007	49.97±0.09
CR-CH	Chitosan	1.0006±0.0004	1.0136±0.0112	50.32±0. 27
CR-FS	Fumed silica	0.5044±0.0032	1.0113±0.0124	66.71±0.32
CR-CT	Cotton	0.7822±0.0048	2.0403±0.0101	72.28±0.02

5.2.3.2 **Microscopic examination of matrix microstructure**

An optical microscope (was used to examine the microstructure of the matrix materials in the clay, chitosan, silica and cotton samples prepared as described above. Control samples without carvacrol were also prepared for comparison purposes. Samples were put on a glass

slide coated with a gold layer and examined under the microscope. The microscope was set to reflection mode and 160x magnification.

5.2.3.3 **Monitoring release of carvacrol vapor from common matrix materials by FTIR spectroscopy**

Release of carvacrol vapor was monitored as a function of time by FTIR spectroscopy as described in Section **5.2.2**. Samples prepared as described in Section **5.2.3.1** were put on a plastic plate, which was then placed in the infrared gas cell. The absorption band at 810 cm^{-1} was employed to monitor the concentration of carvacrol in the vapor phase.

5.2.4 **Statistical analysis**

Non-linear regression analysis was performed with SPSS statistics software (IBM, USA) and MATLAB (MathWorks Inc., Natick, MA, USA.). Weibull models were fit to FTIR spectroscopic data by estimation method of sequential quadratic programming and the estimated values of the scale parameter α, shape factor β, and concentration at infinity C_∞ were put in before running the non-linear regression analysis. The exact values of the model parameters (α, β, and C_∞) were calculated from the analysis and the coefficient of determination (R^2) and the root mean square error (RMSE) were used to estimate how well the model fit the FTIR data. All experiments were performed in duplicate. The data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine statistical significance of differences ($P < 0.05$) between means. SPSS statistics software (IBM, USA) was employed for the statistical analysis.

5.3 **Results and discussion**

5.3.1 **Monitoring evaporation of carvacrol and essential oils by gas-phase FTIR spectroscopy**

To establish an FTIR methodology for the study of the release kinetics of essential oils or their components from a variety of matrices, several sets of experiments were conducted. The first set of experiments entailed placing 1-mL volumes of carvacrol, oregano essential oil (ORE-03 EO) and cinnamon essential oil (CIN-03 EO) in a plastic cap inside an infrared gas cell and monitoring the concentration of key components in the vapor phase as a function of time by FTIR spectroscopy. In the case of carvacrol and oregano essential oil, the integrated intensity of the band at 810 cm^{-1}, assigned to the out-of-plane -CH wagging vibration of the phenolic ring (Topala & Tataru, 2016), served as the measure of the vapor-phase concentration of carvacrol or other phenolic monoterpenes that may be present in oregano essential oil, In the case of cinnamon essential oil, the integrated intensities of absorption bands of cinnamaldehyde at 1118 and 970 cm^{-1} were employed as measures of vapor-phase concentration. It may be noted that quantitative determination of concentrations in the vapor phase was not the intended purpose of the FTIR methodology, and accordingly calibrations relating the integrated intensities to molar concentrations of carvacrol or cinnamaldehyde in the vapor phase were not developed. However, a linear Beer's law relationship between the band intensities in these vapor-phase spectra and concentration in the vapor phase can be assumed. Furthermore, when one applies the Weibull model to fit the FTIR data, the units of concentration [in the present study, absorbance units (a.u.)] cancel out, as seen from the following equation (5) for the Weibull distribution:

$$C_t = C_\infty \cdot \left[1 - e^{-\left(\frac{t}{\alpha}\right)^\beta}\right] \tag{5}$$

where C_t is the vapor-phase concentration at time t and C_∞ is the concentration in the vapor phase at infinity. As the initial concentration in the vapor phase is equal to 0 in our experiments, the above equation for the Weibull model is in the form obtained when C_0 is set to 0. In the Weibull model, α is termed the scale parameter and represents the time to accomplish approximately 63.8% of the process; for a process that proceeds at a constant rate, α is related to the reciprocal of the rate (Poça *et al.*, 2012). β is termed the shape factor and is

related to the velocity of the vapor mass transfer at the beginning of the process (Cunha *et al*., 2001). When $\beta = 1$, the equation for the Weibull model becomes the equation for first-order kinetics. A lower value of β (<1) means that the rate of the process is faster at the beginning, while a larger β value (>1) reveals the existence of a lag phase during the process.

Fig. 5.2 shows the results obtained in this first set of experiments and the dashed lines in **Fig. 5.2** are the trendlines of the Weibull model fitted to the experimental data. In plotting the curves for carvacrol and oregano essential oil, the integrated intensity of the phenolic absorption band at 810 cm^{-1} at each time point was divided by its integrated intensity at infinity, predicted from the Weibull model. Accordingly, owing to the linear relationship between absorbance and concentration in these vapor-phase spectra, the plotted values correspond to C_t/C_∞ and are expected to fall in the range between 0 and 1. The two curves for cinnamon essential oil in **Fig. 5.2** were plotted in a similar manner based on the absorption band indicated in the figure legend.

Integrated intensities of different absorption bands cannot be directly compared owing to inherent differences in molar absorptivities. On the other hand, the curves in **Fig. 5.2** can be directly compared because each set of data has been normalized against the integrated intensity of the selected band at infinity (Requena *et al.*, 2017). The differences in slope of the fairly linear parts of these curves and the differences in the time at which the plateau is reached are indicative of differences in evaporation rate, such that the equilibrium vapor-phase concentration was reached in <12 h in the case of oregano essential oil as compared to >20 h in the case of pure carvacrol. Both curves for cinnamon essential oil show that cinnamaldehyde evaporated at such a slow rate that the equilibrium vapor-phase concentration was not reached by the end of the monitoring period. These curves also show that the two absorption bands of cinnamaldehyde that were monitored yielded fairly consistent data.

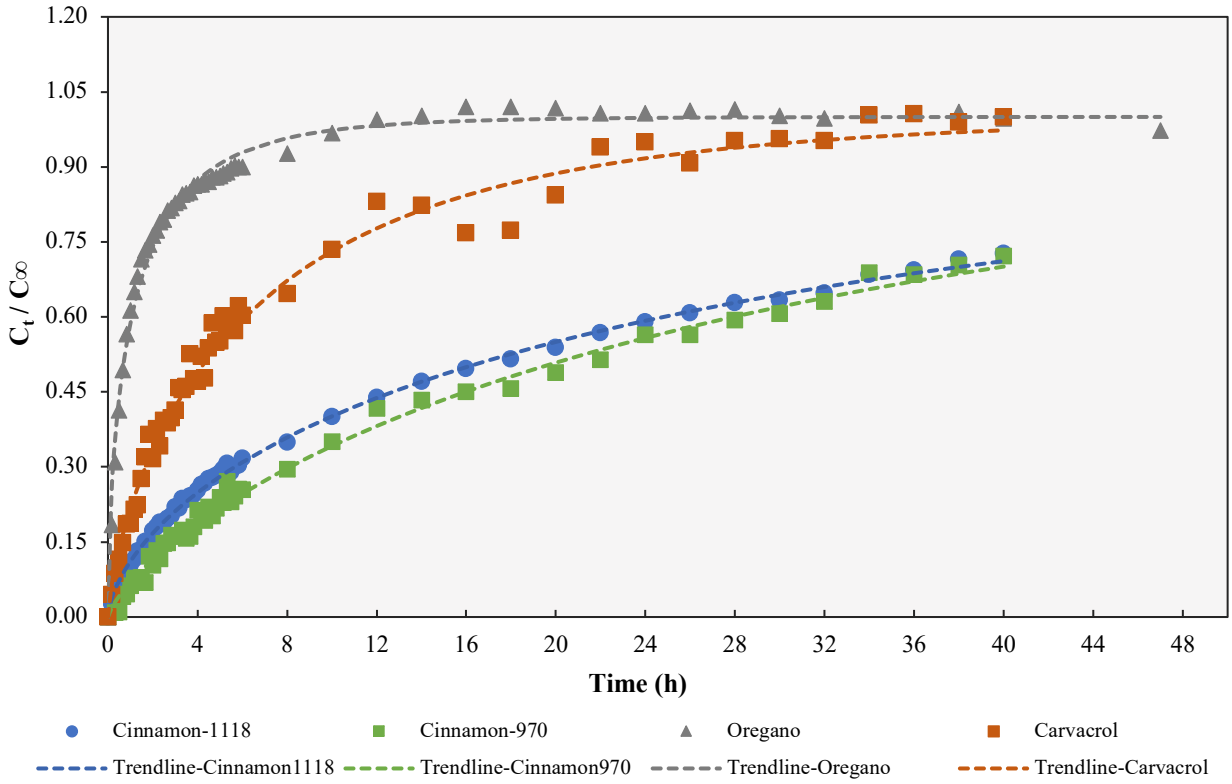


Figure 5.2 Rates of evaporation of carvacrol, oregano essential oil and cinnamon essential oil monitored by Fourier transform infrared spectroscopy.

In all cases, a high coefficient of determination ($R^2 \geq 0.988$) and low root mean square error (RMSE ≤ 0.0090) were obtained, showing the good fit of the Weibull model to our FTIR data. It may be noted that the calculated C_∞ in the Weibull model for oregano EO in **Table 5.2** is relatively high. This arises because the values of C_t (in absorbance units) were large, which might be attributed to some compounds in oregano EO, including thymol and *p*-cymene, which have peaks at similar wavenumbers as carvacrol (807 cm^{-1} for thymol; 813 cm^{-1} for *p*-cymene) (Valderrama & De, 2017). Combination of these peaks in the range of 840-800 cm^{-1} resulted in a large peak area and thus C_t values for oregano EO as well as the large calculated value of C_∞.

In the Weibull model equation, the scale parameter α represents the time to accomplish approximately 63.8% of the process and the shape factor β, also referred to as the behavior index, is related to the initial process rate (Cunha *et al.*, 2001). The α value for oregano essential oil was somewhat lower than that obtained for the same volume of liquid carvacrol and much lower than the values obtained for cinnamon essential oil, reflecting the differences among the curves in **Fig. 5.2** described above. The β value for oregano essential oil was also lower, indicating a fast-initial rate of evaporation, as seen in **Fig. 5.2**. The different vapor

pressures of components other than carvacrol in oregano essential oil might account for the differences between the Weibull model parameters for oregano essential oil and pure carvacrol. Other major components in oregano essential oils include α-pinene, thymol, *p*-cymene, and γ-terpinene, all of which may contribute to the integrated intensity of the absorption band at 810 cm^{-1} (Ozkan *et al.*, 2010; Li *et al.*, 2013; Gotmare*et al*., 2019). The vapor pressure of carvacrol is 0.03 mm Hg at 25 ^{o}C while the vapor pressures of some of the compounds in oregano essential oil listed above are in the range of 0.016-4.75 mm Hg (Philis, 2005; Martucci *et al.*, 2015). On the other hand, the vapor pressures of the major components in cinnamon essential oil are in the range of 0.007-0.038 mm Hg (Gotmare *et al.*, 2019; Lee *et al.*, 2008), accounting for its much slower evaporation rate. The fast evaporation rate of the oregano essential oil tested in this experiment (ORE-03 EO) is consistent with its high antimicrobial activity in the vapor diffusion tests reported in Chapter III. However, in relation to active-packaging applications, this fast evaporation rate might lead to rapid loss of the essential oil in a semi-open packaging environment, so cinnamon essential oil could be a better choice in this situation. On the other hand, in a sealed packaging system, oregano essential oil would be favored owing to its high antimicrobial activity in the vapor phase.

Table 5.2 Derived Weibull distribution model parameters (α, β, C_∞), coefficient of determination (R^2) and root mean square error (RMSE) for the fitted trendlines in **Fig. 5.2**.

Liquid sample	Absorption band monitored (cm^{-1})	α (h)	β	C_∞ (a.u.)[a]	R^2	RMSE
Carvacrol	810	6.8867±0.4657	0.7305±0.0301	0.0313±0.0006	0.9881	0.0010
Oregano-03 EO	810	1.2262±0.0377	0.6128±0.0182	0.4012±0.0024	0.9893	0.0090
Cinnamon-03 EO	1118	28.4347±3.6509	0.6387±0.0139	0.1522±0.0073	0.9983	0.0013
	970	31.2940±6.9512	0.7637±0.0310	0.0494±0.0050	0.9937	0.0008

[a]a.u., Absorbance units;

All Weibull model parameters (α, β, C∞), the coefficient of determination (R^2) and the root mean square error (RMSE) obtained by fitting the Weibull model to the experimental data plotted in **Fig. 5.2** are listed in **Table 5.2**.

In a second set of experiments, the FTIR methodology was employed to monitor evaporation of carvacrol applied onto cellulose filter papers, corresponding to the carvacrol stickers employed in storage tests described in Chapter III. In addition, release of carvacrol and the isomeric compound thymol from thyme essential oil encapsulated in alginate beads was examined as a potential delivery system for these compounds in the headspace of packaging. In both cases, the samples were placed inside the infrared gas cell on a plastic plate below the beam path.

Fig. 5.3 shows the results of these experiments together with those for evaporation of liquid carvacrol. In all three cases, the integrated intensity of the absorption band at 810 cm^{-1} was again employed as the measure of vapor-phase concentration. The fitting of the Weibull distribution to the FTIR data is represented by the dashed lines in **Fig. 5.3** and the corresponding Weibull model parameters are presented in **Table 5.3.** The coefficient of determination (R^2) was higher than 0.985 with low RMSE (<0.0015), demonstrating again that the Weibull model could be used to fit the FTIR data.

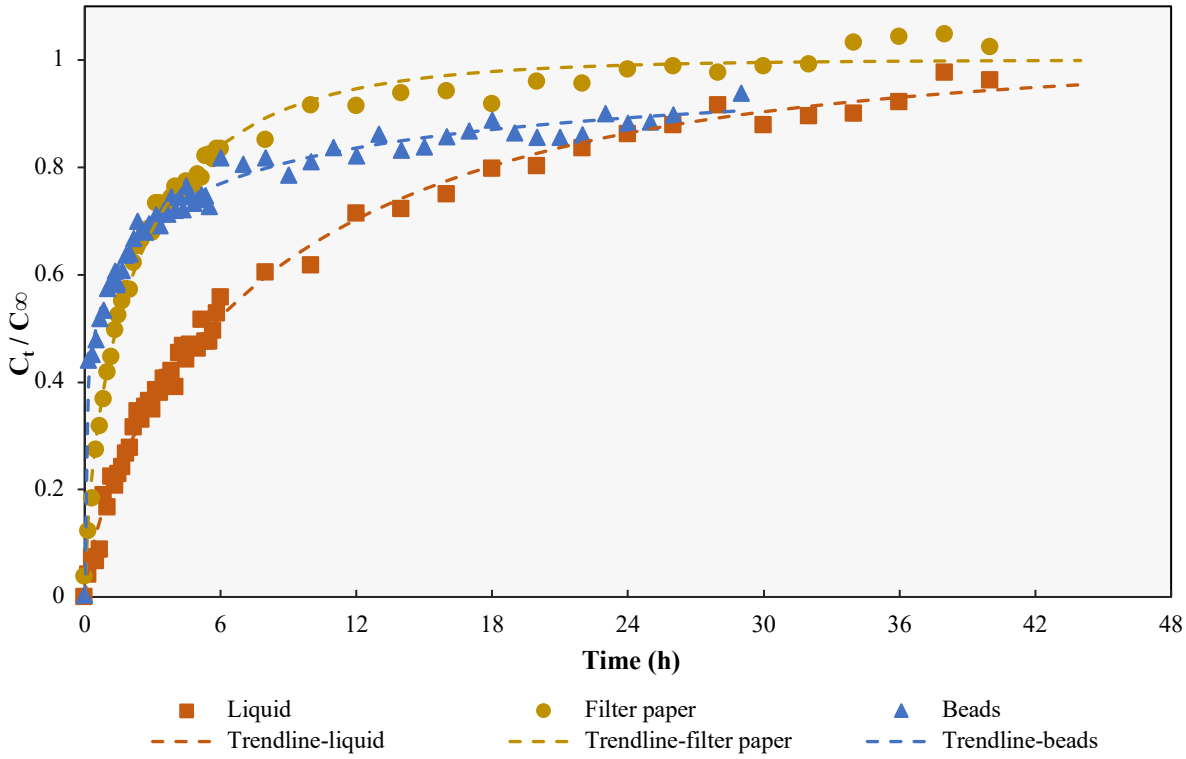


Figure 5.3 Monitoring release of carvacrol vapor from carvacrol stickers and from alginate beads by Fourier transform infrared spectroscopy.

Table 5.3 Derived Weibull distribution model parameters (α, β, C_∞), coefficient of determination (R^2) and root mean square error (RMSE) for the fitted trendlines in **Fig. 5.3**.

Matrix	α (h)	β	C_∞ (a.u.)[a]	R^2	RMSE
Essential oil beads	1.6739±0.3292	0.3007±0.0191	0.0797±0.0026	0.9858	0.0015
Cellulose filter paper	2.4213±0.0717	0.6709±0.0200	0.0825±0.0006	0.9902	0.0020
Carvacrol (liquid)	9.1502±0.6225	0.7163±0.0225	0.0398±0.0009	0.9940	0.0009

[a]a.u., Absorbance units.

The values of the scale parameter α in the Weibull models for release of vapor from carvacrol stickers and from alginate beads (2.42 and 1.67 h, respectively) were much lower than the value in the model for evaporation of pure liquid carvacrol (9.15 h). In accounting for these differences in the α value, differences in the surface area of the three types of samples have to be taken into consideration. The lowest β value is 0.302 in the Weibull model for release of vapor from beads, indicative of a high release rate in the initial stage of the process, as can also be seen from the curve for the beads in **Fig. 5.3.** With regard to the C_∞ values, the low C_∞ value for evaporation of liquid carvacrol revealed that the amount of carvacrol molecules in the vapor phase at equilibrium would be lower than the amounts released from the carvacrol stickers and the beads. Therefore, both these delivery methods could prove suitable for the rapid release of carvacrol into the headspace of packaging.

5.3.2 **Monitoring vapor-phase release of carvacrol incorporated into soy protein films by gas-phase FTIR spectroscopy**

In another set of experiments, the FTIR methodology described above was applied to study the release of carvacrol vapor from a soy protein film matrix. The results obtained for soy protein films prepared with different concentrations of glycerol (0.5%, 1.5%, and 3%) and 1.5% carvacrol in the film-forming solution are shown in **Fig. 5.4**, and the values of the derived Weibull distribution model parameters (α, β, C_∞), R^2 and RMSE are presented in **Table 5.4**. The R^2 and RMSE values obtained in the case of the 3% glycerol films indicate good fit of the Weibull model to the experimental data. The poorer fit in the other two cases can largely be attributed to the random fluctuations caused by low C_∞ of carvacrol in the headspace from the FTIR data seen in **Fig. 5.4.**

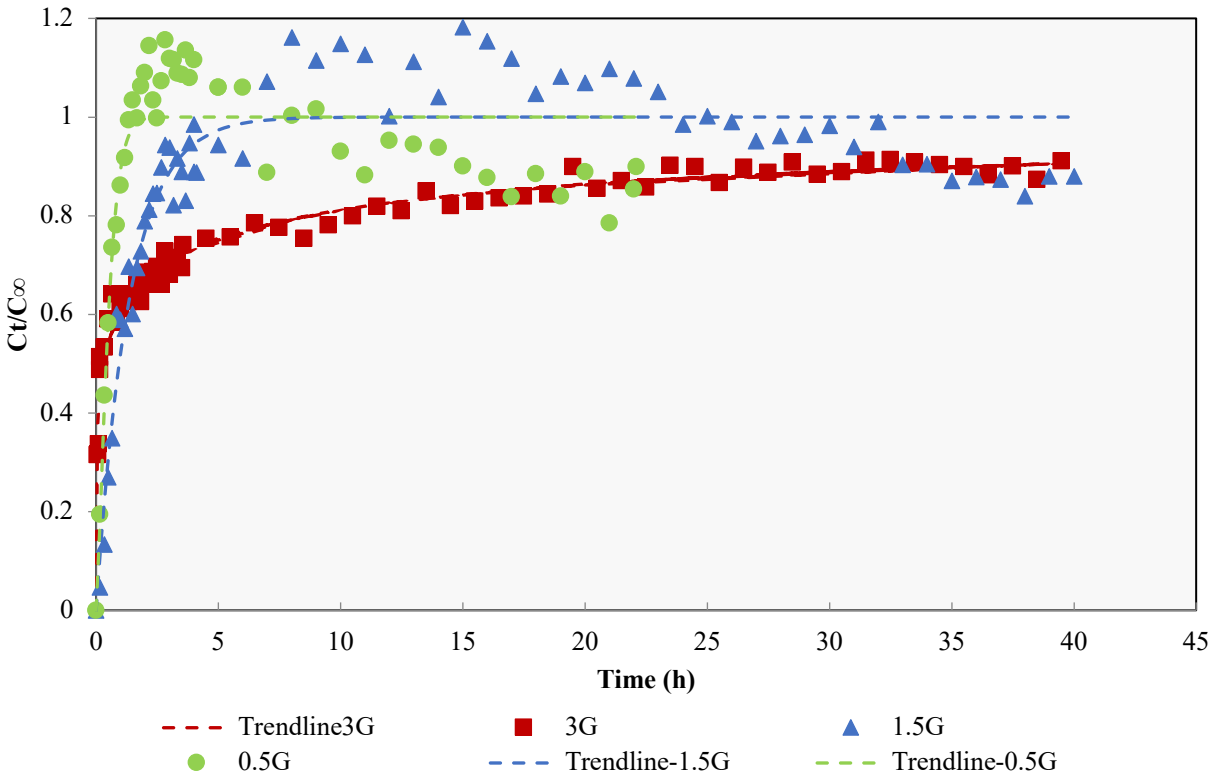


Figure 5.4 Release of carvacrol vapor from soy protein films prepared with different concentrations of glycerol, as monitored by Fourier transform infrared spectroscopy.

Table 5.4 Derived Weibull distribution model parameters (α, β, C_∞), coefficient of determination (R^2) and root mean square error (RMSE) for the fitted trendlines in **Fig. 5.4**.

Concentration of glycerol in film-forming solution	α (h)	β	C_∞ (a.u.)[a]	R^2	RMSE
0.5%	0.5445±0.0570	1.3201±0.2672	0.0190±0.0003	0.8233	0.0020
1.5%	1.3648±0.0949	0.9953±0.1026	0.0191±0.0003	0.8962	0.0017
3.0%	1.3237±0.3738	0.2534±0.0180	0.0559±0.0021	0.9764	0.0014

[a]a.u., Absorbance units.

Fig. 5.4 shows that the equilibrium vapor-phase concentration of carvacrol was reached after around 5 h in all three cases. In comparison with the experiments described above, the rate of release of carvacrol vapor from the soy protein films was very fast and the α values were correspondingly low, ranging between 0.54 and 1.36 h. In addition, the β value noticeably decreased from 1.32 to 0.25 with increasing glycerol concentration in the films, indicating the faster release of carvacrol in the initial stages. This suggests that the glycerol incorporated in the films may favor the release of carvacrol from the matrix. Furthermore, the films with the highest concentration of glycerol had the highest C_∞ value. Kurek *et al.* (2014) ascribed the impact of relative humidity on carvacrol release from chitosan films to the plasticization of these hydrophilic films by water, leading to increased carvacrol diffusivity within the films (Whorton & Reineccius, 1995). Similarly, the plasticizing effect of glycerol in our soy protein films may promote the diffusion and release of carvacrol. These experiments illustrate the potential utility of this FTIR methodology in comparing the rates of release of active compounds from films prepared under different conditions, with the aim of identifying means of achieving optimal release from a particular active-packaging system designed for a particular type of food product.

5.3.3 **Study of release kinetics of carvacrol from common matrix materials by gas-phase FTIR spectroscopy**

5.3.3.1 **Microscopic examination of samples**

5.3.3.1.1 Carvacrol in clay

Dry clay powder consists of small opaque particles that are scattered. When the powder was mixed with the same weight of carvacrol with mechanical stirring, aggregation of these small particles was observed. It was also found the particles become translucent after absorbing carvacrol (**Fig. 5.5**).

Clay particles form small montmorillonite platelets, which have a typical size of 1 nm in thickness and range in diameter from 10 to 100 nm (Starodoubtsev *et al.,* 2006). Due to their sandwich shape structure (two layers of silica containing an octahedral sheet of alumina), the platelets are always negatively charged, and the platelet surface charge is heterogeneous (Cadene *et al*., 2005). The van der Waals forces between the sheets lead to a face-to-face disordered aggregation (Cadene *et al*., 2005). The weakness of the link between the micro-platelets makes this structure subject to many external changes. The mechanical stirring and the cohesion of carvacrol droplets disrupted the aggregation of montmorillonite platelets and caused them to re-aggregate in the oil phase. Larger particles can be seen in the photomicrograph, with carvacrol dispersed in the small spaces between the particles, which are hydrophilic. The size of the clay pores is around 10 to 100 nm, allowing light to pass through

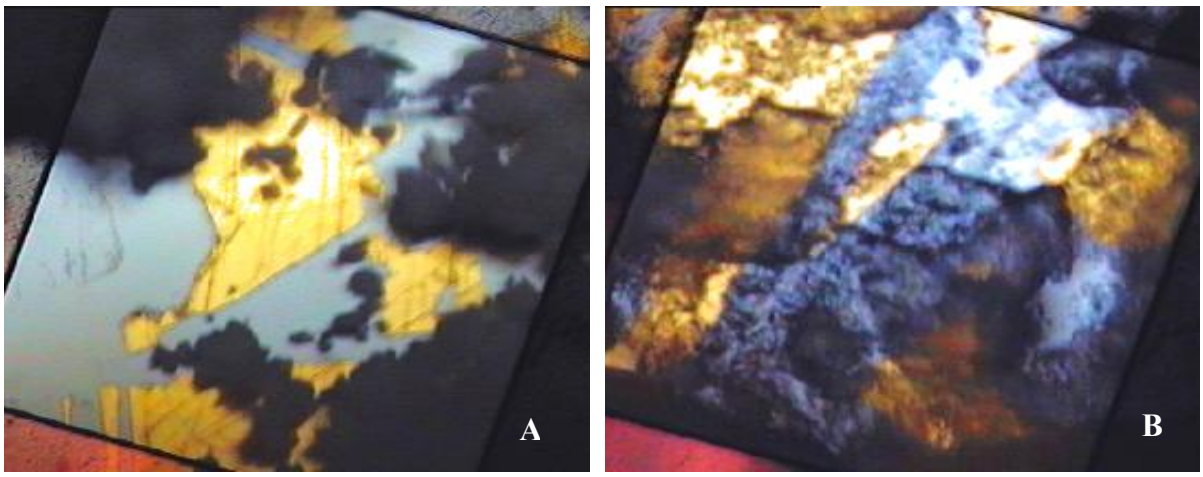


Figure 5.5 Photomicrographs of clay samples. (A) Dry clay powder; (B) clay powder mixed with carvacrol (50%, w/w).

5.3.3.1.2 Carvacrol in fumed silica

Dry silica is a fine white powder under the microscope. When mixed with carvacrol, fumed silica becomes translucent gel (**Fig. 5.6**). Fumed silica is produced by burning volatile silanes in an oxygen-hydrogen flame. When first generated in the flame, the primary particles are formed. These particles made of silica and oxygen have a typical size of 10 nm. When the temperature gets lower, the primary particles hit each other and link with each other to form a clear branched linear structure called an aggregate, which has a mean size of 100 to 200 nm (Barthel *et al*., 1995). When the temperature further drops to near room temperature, aggregates crosslink and form agglomerates. The agglomerates are clustered linear aggregates, which have an average size of 10 μm. The pore size of silica varies from 1 to 19 nm depending on the type. The larger the aggregate is, the larger the pore size becomes. When carvacrol is added, it fills in the pores of agglomerates. On the macroscopic level, the volume of fumed silica powder decreased greatly and formed a white dense gel with a smooth surface. The carvacrol liquid fills in the spaces between the agglomerates, and the cohesion of carvacrol binds the agglomerates together to form gel-like clusters.

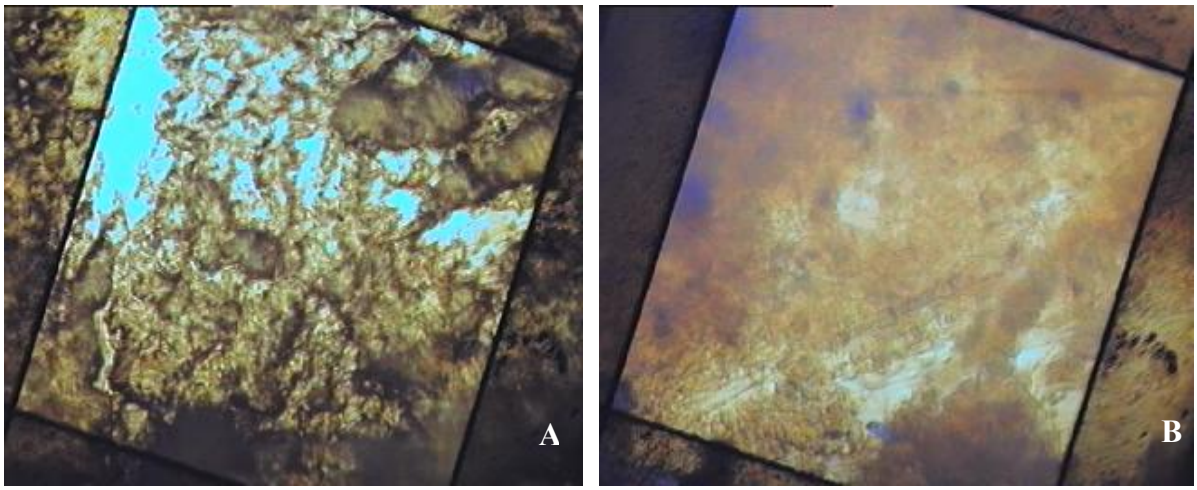


Figure 5.6 Photomicrographs of silica samples. (A) Dry fumed silica; (B) fumed silica mixed with carvacrol (66%, w/w).

5.3.3.1.3 Carvacrol in chitosan

Dry chitosan consists of white, opaque crystals as seen under the microscope. When carvacrol is added, the crystals are surrounded by the oil phase and become transparent (**Fig. 5.7**). Chitosan is a polysaccharide derived from the exoskeleton of shrimp and crab shells. The chitosan molecule consists of a variable number of β-(1-4)-linked units of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) (Cunha *et al.*, 2012). As a result, the molecular weight is variable. As a hydrophilic molecule, chitosan does not have a strong interaction with carvacrol. The chitosan crystals consist of multiple layers of chitosan polymer chains, with a small distance of 0.2-0.3 nm between the layers. Unlike the surface charge on clay platelets, the distribution of charge on the surface of the sheet is homogeneous. As a result, the crystals are compact with a clear surface. Because the distance between the sheets of chitosan layers is very small, carvacrol is only present in the space between the chitosan crystals. The gap between the chitosan crystals is much larger than the pores in clay particles and silica agglomerates. When carvacrol is added to chitosan powder, the fine powder aggregates to form larger sand-like particles. In the microstructure, the pits on the surface of chitosan crystals are filled by carvacrol, making the crystals become brighter when carvacrol is added.

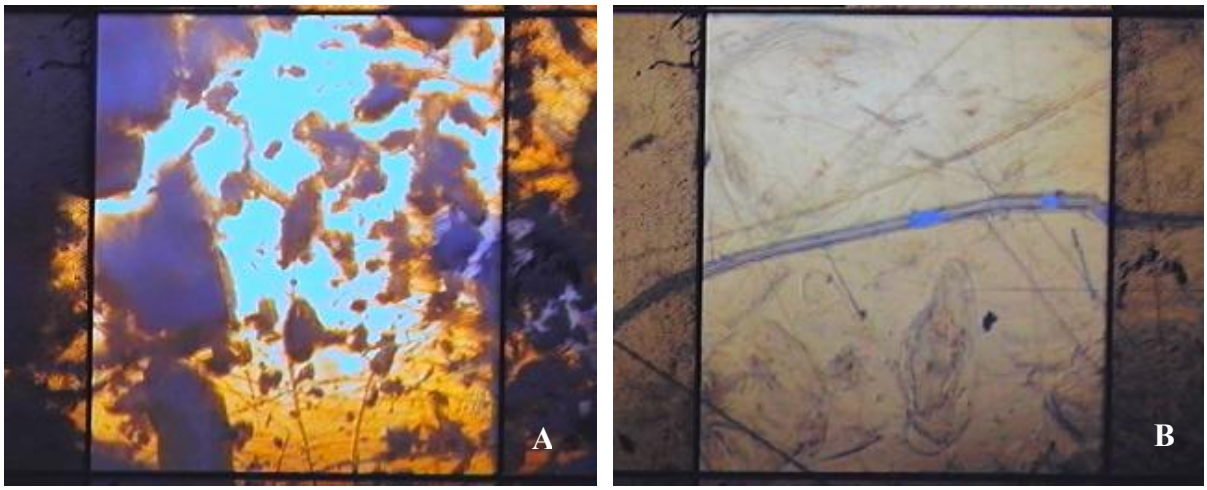


Figure 5.7 Photomicrographs of chitosan samples. (A) Dry chitosan crystals; (B) chitosan crystals with 50% (w/w) carvacrol.

5.3.3.1.4 Carvacrol in cotton

White translucent fibers of cotton can be observed under the microscope. When carvacrol is added, the fibers are soaked, and small droplets of carvacrol can be seen at the intersection of overlapped cotton fibers (**Fig. 5.8**). Cotton is a plant fiber consisting of cellulose. The microstructure responsible for carvacrol retention is mainly the distance between cotton fibers, which is much larger as compared with the pores in the microstructure of clay and fumed silica.

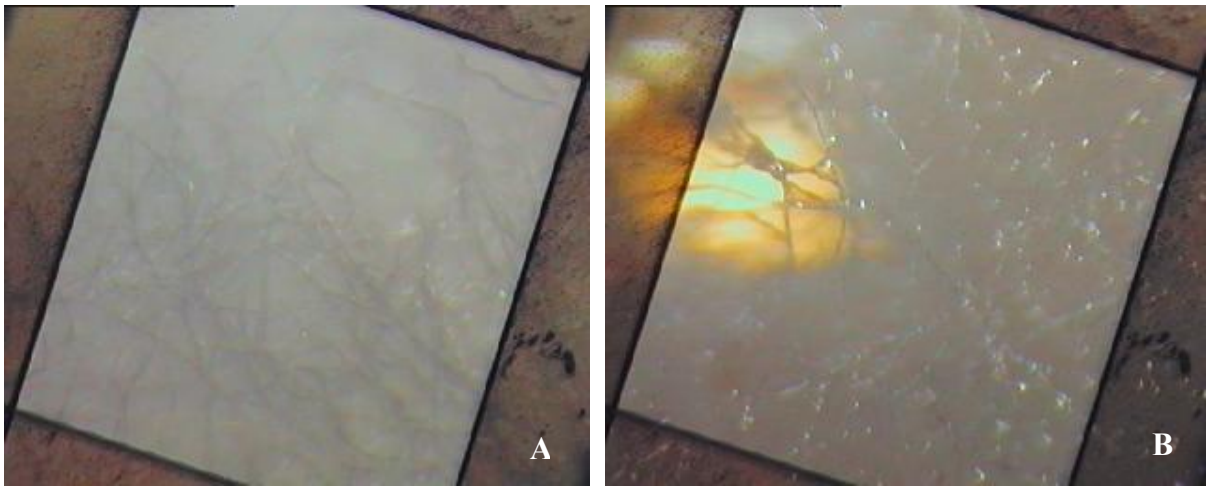


Figure 5.8 Photomicrographs of cotton samples. (A) Dry cotton fiber; (B) cotton fiber with 50% (w/w) carvacrol.

5.3.3.2 **Release kinetics of carvacrol from different matrices**

The release of carvacrol vapor from the four matrices described above as a function of time was monitored by the FTIR methodology employed in the experiments discussed previously. As shown in **Fig. 5.9**, the time at which the concentration of released carvacrol vapor in the infrared gas cell reached a plateau depended on the type of matrix. This plateau was reached within 7-8 h when carvacrol was released from the fumed silica and cotton matrices, and after 20 h when it was released from the clay matrix. Release of carvacrol from the chitosan matrix was much slower, and the plateau was not reached after 40 h.

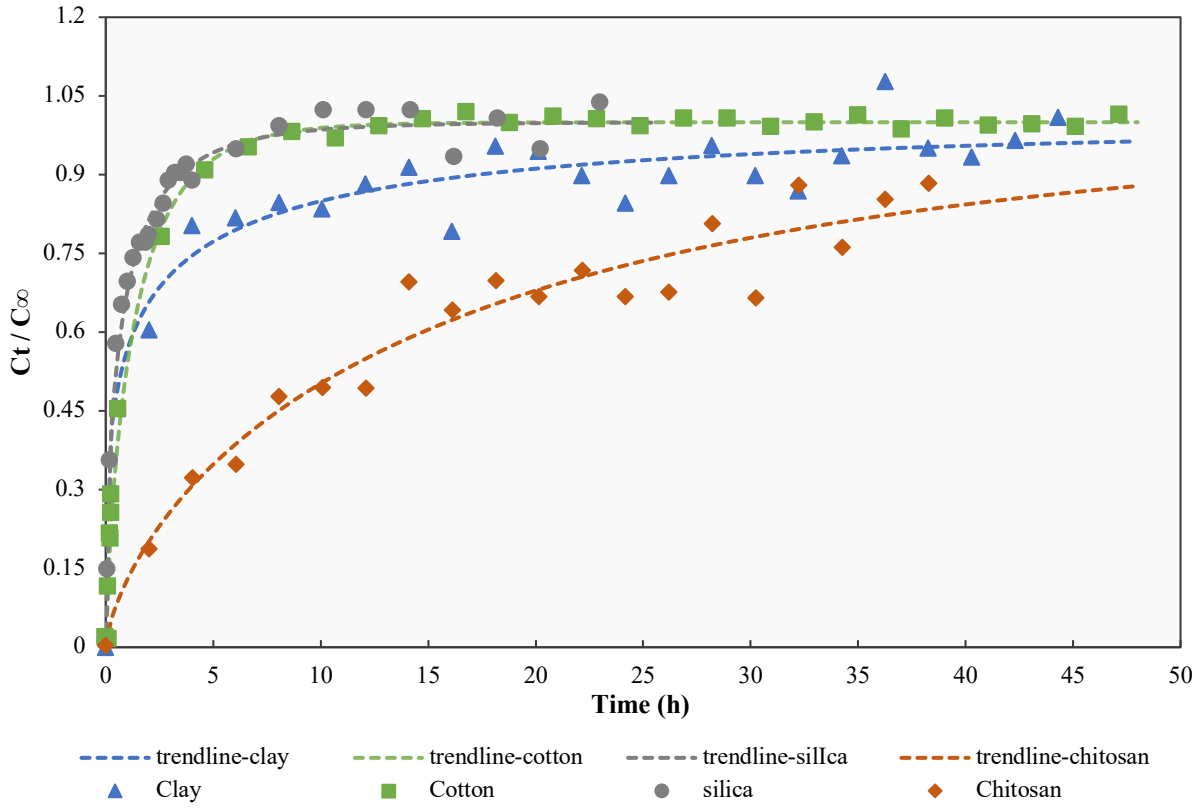


Figure 5.9 Release of carvacrol vapor from different matrices, as monitored by Fourier transform infrared spectroscopy. The dashed lines are the trendlines of the Weibull mathematical model.

Table 5.5 Derived Weibull distribution model parameters (α, β, C_∞), coefficient of determination (R^2) and root mean square error (RMSE) for the fitted trendlines in **Fig. 5.9**.

Matrix	α (h)	β	C_∞ (a.u.)[a]	R^2	RMSE
Fumed silica	0.8110±0.0589	0.5758±0.0348	0.0675±0.0009	0.9780	0.0022
Cotton	1.4019±0.1111	0.7679±0.0401	0.0793±0.0006	0.9920	0.0026
Clay	1.6572±0.6096	0.3557±0.1469	0.0544±0.0048	0.9397	0.0029
Chitosan	16.6623±10.1725	0.7048±0.1654	0.0517±0.0118	0.9461	0.0030

[a]a.u., Absorbance units.

The dashed lines in **Fig. 5.9** are typical fits of Weibull distributions to data derived from monitoring the release of carvacrol vapor by FTIR spectroscopy, and the parameters of the Weibull model are listed in **Table 5.5.** The modeling of the FTIR data for carvacrol release from the fumed silica and cotton matrices yielded coefficients of determination (R^2) larger than 0.978 with low RMSE (<0.003), whereas slightly poorer fits were obtained in the other two cases (clay and chitosan matrices), which can be seen to be largely attributable to random fluctuations in the FTIR data. Because the gas cell is not airtight, a slow air exchange with the surrounding environment could be a possible source of error. In all cases the R^2 value is in line with values reported in various studies in which the Weibull distribution was successfully employed to model rehydration, salting and migration processes in food (Poças *et al*, 2012; Corzo *et al*., 2013; Marabi *et al.*, 2003; Cai *et al.*, 2017).

Examination of the values of the Weibull distribution model parameters in **Table 5.4** provided further information about the release kinetics. The scale factor α varied greatly among the models for carvacrol release from the four matrices, ranging from 0.81 h to 16.64 h. The faster the vapor-phase concentration of carvacrol reaches equilibrium, the smaller the α value is. The experimental data indicate that the interactions between carvacrol and different matrices might not be the most important factor affecting the release kinetics, contrary to our original assumption. According to our results, the rate of diffusion follows the order fumed silica > cotton > clay > chitosan. Our current assumptions are as follows: (i) The smaller the pore size is, the slower the evaporation process; (ii) the pore size of the matrices decreases in the order fumed silica > cotton > clay > chitosan. One explanation for this phenomenon is that the carvacrol doesn't disperse into the clay aggregates, the silica agglomerates or the chitosan crystals. The C_∞ value in our research is the calculated maximum intensity of the characteristic peak of carvacrol vapor at equilibrium. These values vary from 0.051 to 0.079 for the different matrices, but the variance is not very large and may be slightly related to the pore size or the distribution rate of materials.

The shape factor β has been utilized by researchers to elucidate diffusion mechanisms in a number of food systems. For instance, in studies of rehydration processes of dried particulate foods, a β value between 0.67 and 0.81 indicated that the mechanism was internal mass transfer by diffusion, whereas a β value in the range of 0.97-1 indicated that external resistance to mass transfer (for example, resulting from case hardening during the initial drying process) was the controlling factor (Marabi *et al*., 2003). As the β value is associated with the shape of the distribution, it has been found to be independent of temperature (Poças

et al, 2012; Cunha *et al*., 2001). In our experiments, the β values for the different matrices are in the range of 0.35–0.76. This fairly wide range suggests that different mechanisms of diffusion might be involved. In particular, a β value as low as 0.35 may indicate that the process includes a very fast initial component (Marabi *et al*., 2003), possibly suggesting that some of the carvacrol molecules in the clay matrix evaporate rapidly via a non-diffusion-controlled process. However, due to the complex microstructure of the matrices and many possible different mechanisms involved in the carvacrol evaporation process, the shape factor β alone is not sufficient to explain the detailed mechanisms involved (Wang & Lim, 2014).

It may be noted that when the release of carvacrol from the fumed silica matrix was tested, a white layer of silica powder was found on the inner surface of the gas cell. This suggested that the evaporation of carvacrol from the fumed silica matrix caused the release of a small amount of fumed silica particles at the same time. This potentially reduced the distance that the carvacrol molecules had to diffuse through. Moreover, this release might affect the potential utility of fumed silica as a carrier matrix in a food packaging system.

In active packaging, the rate of release of carvacrol vapor into the headspace of the food product is very important to the antimicrobial effects on the food product. For instance, the concentration of carvacrol in the headspace needed to avoid spoilage should exceed the minimum inhibitory doses (MID) within 24 h at 100% relative humidity (Mascheroni *et al*., 2011), otherwise, the microbial growth would pass the lag phase, and the MID might not effectively inhibit microbial growth owing to the increased initial microbial load. The speed of the evaporation process and the sequence of reaching equilibrium followed the order fumed silica > cotton > clay > chitosan. Thus, the chitosan-based matrix might not be suitable in an antimicrobial packaging system owing to the slow release (Mascheroni *et al.,* 2011). Therefore, differences in the microstructure could greatly influence the evaporation rate of carvacrol from the matrix.

5.4 **Conclusion**

In conclusion, a new rapid, versatile and simple methodology for studying the release kinetics of essential oils or individual active compounds from potential carrier matrices and delivery systems for active-packaging applications was demonstrated in this research. This methodology is based on the application of gas-phase FTIR spectroscopy coupled with fitting of the FTIR data to a Weibull distribution model to aid in the comparison of the results obtained from different experiments. By applying this methodology, we examined the large differences in the relative rates of evaporation of active compounds from oregano and cinnamon essential oils as well as the relatively small differences in the release kinetics of carvacrol from soy protein films prepared with different concentrations of glycerol, In addition, modelling of the evaporation of carvacrol from different matrices by fitting the Weibull distribution to FTIR data showed that differences in the microstructure were greatly associated with differences in the release kinetics.

CHAPTER 6 GENERAL DISCUSSION AND RECOMMENDATIONS FOR FUTURE WORK

6.1 **General discussion**

Over the past decades, consumers' concerns about the safety of synthetic food additives have led to high demand for "clean label products" in the market. Recently, a number of essential oils that have potential utility as green-label preservatives in food because of their antimicrobial and antioxidant properties have been investigated as possible replacements for traditional preservatives, such as benzoic acid, sorbate, and nitrate (Han *et al.*, 2000; Jideani &Vogt, 2016). The antimicrobial active compounds that certain essential oils contain, such as carvacrol (the major component of thyme and oregano essential oils) and cinnamaldehyde (present in cinnamon bark essential oil), have been demonstrated to have activity against bacteria, molds and yeasts and thus have potential to extend the shelf life and ensure safety of perishable foods, including meat, fruits, and vegetables. Several attempts have been made to develop active packaging systems incorporating essential oils into polymeric materials that can slowly release the volatile active compounds onto the food surface to inhibit the growth of spoilage and pathogenic microorganisms. In addition, edible films and coatings based on soy protein, which is a by-product of soybean oil extraction, have been used in several studies as the carrier of essential oil in active packaging. The effects of essential oils incorporated in these and other types of protein films on the physical and antimicrobial properties of the films have also been reported (Ojagh *et al*., 2010; Pires *et al.,* 2013; Atarés *et al.,* 2010b; Zinoviadou *et al.*, 2009; Atarés and Chiralt, 2016).

Building on the research reported in the literature, as reviewed in Chapter II, the main objective of the research presented in this thesis was to lay the scientific foundation for the development of active packaging incorporating essential oils to inhibit the growth of *Listeria* on vegetables, which is a major food safety concern owing to the serious health risks of listeriosis in vulnerable populations. The first aspect of this research, presented in Chapter III, entailed screening a panel of 25 locally sourced essential oils for antilisterial activity *in vitro* by disk diffusion (direct contact) and vapor diffusion tests. For purposes of comparison, tests were conducted in parallel with a Gram-negative bacterium (*E. coli* K12). Among the 25 essential oils, which included four cinnamon and three oregano essential oils from different suppliers, cinnamon-03 and oregano-03 essential oils proved to be effective in inhibiting the growth of *Listeria grayi* and, to a lesser extent, *E. coli* K12 both by direct contact and in the vapor phase. Most of the other oregano and cinnamon essential oils also showed antimicrobial activity in these *in vitro* tests. These findings were not unexpected given that

oregano and cinnamon essential oil generally contain high levels of phenolic monoterpenoids (carvacrol and thymol) and cinnamaldehyde, respectively, which have well-known antimicrobial properties. Furthermore, the susceptibilities of *L. grayi* to oregano and cinnamon essential oils were similar whereas *E. coli* K12 was more susceptible to cinnamon essential oil than to oregano essential oil. As noted in Chapter III, these differences in relative susceptibilities are consistent with findings reported in the literature and may be indicative of differences in the mechanisms of action of carvacrol and cinnamaldehyde in the inhibition of Gram-negative bacteria. More specifically, the mechanism of action of carvacrol was reported to involve disintegration of the outer membrane of Gram-negative bacteria, leading to increased permeability of the cytoplasmic membrane to ATP and release of lipopolysaccharides (LPS) (Burt, 2004). At similar concentrations, cinnamaldehyde also inhibited the growth of the Gram-negative bacteria *S. typhimurium* and *E. coli* O157:H7, but it did not disintegrate the outer membrane of the bacterial cells or deplete the intracellular ATP pool (Burt, 2004).

The development of active-packaging systems employing a selected type of essential oil such as, for instance, a natural antimicrobial is complicated by the requirement of ensuring that the desired properties can be reproducibly delivered. The natural variability among essential oils of the same type is very high owing to the dependence of their chemical composition on the plant species, geographic locale and growing season, among other factors. For example, among the essential oils extracted from nine "carvacrol-rich" oregano species and analyzed by Figuérédo *et al*. (2006) using gas chromatography-mass spectrometry, the carvacrol level ranged from 55.9% to 86.1%. In another study, the amount of carvacrol in wild oregano plants was reported to fluctuate between 51.6 and 564.3 mg/100 g depending on the season in which the plants were collected (Jerković *et al*., 2001). In addition to this natural variability in the chemical composition of essential oils, differences in extraction methods and processing conditions are also important sources of variability. In the case of cinnamon essential oil extracted from the bark of *Cinnamomum cassia* Blume, the concentration of cinnamaldehyde was reported to range from 90% to 62%–73%, depending on whether the essential oil was extracted by steam distillation or Soxhlet extraction (Nabavi *et al*., 2015). The effects of variability in chemical composition and particularly in the concentrations of active compounds on antimicrobial properties were evident in the results of the *in vitro* tests in our study. Most notably, unlike the other oregano and cinnamon essential oils we tested, one of the oregano essential oils and one of the cinnamon essential oils lacked antimicrobial

activity against both *L. grayi* and *E. coli* K12. To account for the source of this variability, we employed ATR-FTIR spectroscopy to acquire spectral fingerprints of the neat essential oils in the information-rich mid-infrared range (4000-600 cm^{-1}). Unsupervised classification of these spectra by hierarchical cluster analysis clearly showed that the cinnamon and the oregano essential oil that lacked antimicrobial activity were spectrally dissimilar to their respective counterparts in our panel of essential oils. More detailed examination of the spectra revealed that the oregano and the cinnamon essential oil did not exhibit absorption bands characteristic of carvacrol or thymol and cinnamaldehyde, respectively, accounting for the low antimicrobial activities of these two essential oils in the disk diffusion tests. From a more general perspective, this limited study demonstrated the strong potential of ATR-FTIR spectroscopy to serve as a rapid screening technique for incoming batches of essential oils destined for use as antimicrobials in active packaging. With the use of a dedicated reference spectral database for each type of essential oil that adequately represents the spectral variability among samples meeting a targeted level of antimicrobial activity, incoming batches can be accepted or rejected on the basis of criteria of spectral similarity rather than chemical composition.

The susceptibility of *Listeria grayi* to various essential oils was investigated for the first time in these studies. As such, the relative susceptibilities of *L. grayi* and *L. monocytogenes* to essential oils were unknown. Accordingly, in additional *in vitro* tests, the susceptibilities of *L. monocytogenes* to oregano (ORE-03) and cinnamon (CIN-03) essential oils were compared with those of *L. grayi*. The results of these tests showed that *L. grayi* was slightly less susceptible to these two essential oils than *L. monocytogenes*, making it a suitable non-pathogenic surrogate for *L. monocytogenes* in this study.

Further evidence of the effectiveness of CIN-03 and ORE-03 EOs as well as pure carvacrol in inhibiting the growth of *L. grayi* and *E. coli* K12 included their low minimum inhibitory concentrations measured by the broth microdilution method and their low minimum inhibitory doses in the vapor phase. Based on the results of these *in vitro* tests, CIN-03 and ORE-03 were the essential oils selected for trials with vegetables inoculated with *L. grayi*. In storage tests, fresh-cut green peppers packaged with cellulose stickers impregnated with ORE-03 or carvacrol showed the reduction of *L. grayi* compared with the controls. Furthermore, stickers impregnated with CIN-03 reduced the *Listeria* count to 10 CFU/g after 2 days of storage as compared to 8.9 log CFU/g for the controls. These results showed that the active compounds released by the stickers into the headspace of the packaging were

effective in inhibiting the growth of *L. grayi* on the vegetables during storage. Given the slightly higher susceptibility of *L. monocytogenes* to the two essential oils, noted above. The extremely large surface area these results lay the foundation for further development of an active packaging system for pre-cut ready-to-eat or frozen vegetable products to inhibit the growth of this pathogenic psychrotrophic microorganism. On the other hand, growth of *L. grayi* on broccoli florets was not inhibited by packaging with the stickers in the manner described above of broccoli florets was considered to be the most likely reason for this result. For this type of product, an alternative approach based on treatment with aerosolized essential oil in a sealed container was demonstrated. Aerosolization has been previously investigated as a method for the delivery of aqueous sanitizers onto fresh produce to reduce microbial load (Oh *et al*., 2005). However, there do not appear to have been any studies dealing with the antimicrobial activities of aerosolized essential oils against foodborne pathogens. In our experiments, aerosolized ORE-03 was highly effective against both *L. grayi* and *E. coli* K12, such that there was almost no survival of these bacteria on agar plates after 15-min exposure to the aerosolized essential oil. In contrast, aerosolized CIN-03 was almost ineffective under the same test conditions. In view of the effectiveness of this essential oil in the disk diffusion and vapor diffusion tests, this finding remains to be explained but would appear to indicate that the composition of the aerosol differed substantially from that of the liquid and from that of the vapor in equilibrium with the liquid. Treatment of broccoli florets with aerosolized ORE-03 for 45 min prior to storage at 4ºC proved highly effective in inhibiting the growth of *L. grayi* during the first 12 days of storage and had some inhibitory effect for up to 3 weeks. These results could be of particular interest to frozen vegetable processors, as treatment of vegetables with aerosolized oregano essential oil immediately prior to freezing may inhibit the growth of *L. monocytogenes* that may otherwise occur if consumers allow the product to thaw and may have serious consequences if the product is then consumed without being cooked in accordance with the instructions on the product label.

Sensory evaluation of frozen broccoli and french fries after storage in packaging with the two selected EOs in the headspace revealed that the products exposed to CIN-03 vapor at the higher level tested were less acceptable to panelists than those exposed ORE-03 vapor.

Chapter IV addressed the conditions for the preparation of soy protein films as carriers for oregano and cinnamon essential oils as well as carvacrol in active-packaging applications. The mechanical properties of films cast from solutions of soy protein isolate (SPI) depended on the concentration of glycerol added to the film-forming solution as a plasticizer, the pH of

the solution, and the heating temperature/time applied. These experiments resulted in the selection of the following conditions for film preparation: 5% soy protein isolate (SPI), 1.5% glycerol, and pH 10 for the film-forming solution and heating of this solution at 85 ºC for 10 minutes. These conditions were employed in the preparation of the soy protein films incorporating carvacrol and oregano and cinnamon essential oils. The mechanical properties, water vapor permeability (WVP), opacity and water solubility of these films were influenced by the type of essential oil, the concentrations of glycerol and carvacrol or essential oil in the film-forming solution, and the emulsification treatment applied. An increase in the elongation at break of the SPI films coupled with decreases of tensile strength and Young's modulus were found with increasing concentration of glycerol, carvacrol and oregano essential oil, indicating that emulsified carvacrol and oregano essential oil acted as plasticizers like glycerol to reduce resistance and rigidity of SPI films and increase their extensibility. Contrastingly, incorporation of cinnamon essential oil was found to have the opposite effects on the mechanical properties of the SPI films. In addition, incorporation of glycerol and essential oils tended to increase the WVP of SPI films but had variable effects on the rate of water vapor transmission through the films, depending on the type and the concentration of essential oil, indicating that the factors influencing the water vapor barrier properties of the films were quite complex. It was also found that high concentrations of glycerol and emulsified essential oils improved the transparency of SPI films and that incorporation of essential oils also impacted the water solubility of SPI films. Finally, analysis of the ATR-FTIR spectra of the SPI films prepared in this study revealed that interactions between the soy proteins and oregano essential oil (or carvacrol) incorporated in the films resulted in transformation of some β-sheet structure to α-helical structure, consistent with a plasticizing effect of the essential oil. The effects of cinnamon essential oil on the protein secondary structure were not as clearly discerned owing to the relatively low amount of cinnamon essential oil incorporated as well as its absorption bands in the protein amide I region that may interfere with the analysis of protein secondary structure.

Our main interest in incorporating essential oils in SPI films was their potential utility as antimicrobial packaging films, with a particular focus on inhibiting the growth of pathogenic *Listeria* on vegetables. In both disk diffusion and vapor diffusion tests, SPI films prepared with ≥2% carvacrol or ≥1.5% oregano essential oil were effective in inhibiting the growth of *L. grayi*. Unfortunately, SPI films incorporating cinnamon essential oil had little antimicrobial activity, which could be due to the reaction of the aldehyde group of

cinnamaldehyde with NH_2 groups of lysine side chains in the soy proteins. Overall, the results presented in Chapter IV demonstrated that the incorporation of essential oil could improve the physical properties of soy protein films for food packaging applications and that the antimicrobial activity of certain of these films could also result in extended shelf life and enhanced microbiological safety of the food system.

When active packaging systems are based on the release of volatile active compounds from essential oils incorporated in a carrier matrix, their efficacy in controlling microbial growth will be affected by the rate of release of the active compounds into the headspace of the packaging. In the present study, we developed an FTIR-based methodology that provides a simple and convenient means of monitoring and comparing the rates of release of active compounds from different matrices into the vapor phase. Characteristic absorption bands of active compounds in the vapor phase are measured with the use of an infrared gas cell and their intensities as a function of time are fitted to a Weibull distribution model by non-linear regression analysis to characterize the release kinetics in terms of the scale parameter and shape factor of the Weibull distribution. In Chapter V, as an extension of the studies in Chapter III and Chapter IV, we employed this methodology to evaluate the release kinetics of carvacrol from different matrices. The methodology was initially tested by monitoring the evaporation of liquid carvacrol, and the FTIR data were well fitted to the Weibull model, with a high correlation coefficient (>0.995) and low RMSE (<0.001). Comparison of the release kinetics of carvacrol vapor from soy protein films prepared with different concentrations of glycerol revealed that the glycerol in soy protein films could favor the evaporation of carvacrol, as evidenced by the high value of the Weibull model estimate of the concentration at infinity (C_∞). Monitoring the evaporation of carvacrol from different matrix materials by this methodology showed that differences in their microstructure greatly influenced the rate of evaporation of carvacrol from the matrix, decreasing in the order silica > cotton > clay > chitosan.

6.2 **Conclusion and recommendations for future work**

While most of the experiments in this study were conducted with *L. grayi*, *in vitro* tests showed it to be a suitable surrogate for *L. monocytogenes*. Thus, the research summarized above has laid the foundation for the application of oregano and cinnamon essential oils in food packaging to inhibit the growth of *Listeria monocytogenes* on frozen and ready-to-eat vegetable products and thereby improve the safety of these types of products. In addition, spectral fingerprinting of individual lots of essential oils by the rapid, non-destructive ATR-FTIR spectroscopic method used in this research was shown to facilitate the screening or quality control of essential oils for a given active-packaging application. Furthermore, a new FTIR-based methodology was established to monitor and model the release kinetics of active compounds from carrier matrices into the headspace of packaging. Extension of this methodology to quantitate the concentrations of active compounds released could be achieved by calibrating the absorbance data against concentration data obtained by headspace analysis using gas chromatography and would then provide a rapid and simple alternative to the latter technique. Besides, the changes in microstructures of various carrier materials on the essential oil release kinetics could be the subject of further study in the future and may provide a better understanding of mechanism of essential oil release from packaging material.

Finally, in relation to further development of antimicrobial soy protein packaging films through the incorporation of essential oils, triggering of the release of active compounds from packaging films as a result of interactions with components of the packaged food may be required for controlled release. In this context, further studies of the soy protein films incorporating cinnamon essential oil, which were found to lack antimicrobial activity in the present study, may be of interest. We postulated that this lack of activity may be due to reaction of cinnamaldehyde, the major active compound in cinnamon essential oil, with a lysine side chain in the protein to form a Schiff base. In such a case, conditions favoring hydrolysis of the Schiff base could provide a triggering mechanism for controlled release of the active compound. On a more fundamental level, infrared imaging of biopolymer-based packaging films by focal-plane-array (FPA) FTIR spectroscopy may provide a more detailed understanding of film structure and diffusion processes, which may in turn aid in the design of films for active-packaging applications.

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