MICROENCAPSULATION TECHNOLOGY USING ESSENTIAL OILS TO PRODUCE ACARICIDES AGAINST HOUSE DUST MITES

by

JOO RAN KIM

(Under the Direction of Suraj Sharma)

ABSTRACT

The purpose of this study was to produce a safer microcapsules loaded with clove and thyme oils to reduce the population of house dust mites (HDM). In the study, gelatin-based microcapsules with sizes of 4 to 85 μm were created with agitation speed and type of oil playing critical role in governing the size of microcapsules. Microcapsules made up of single spherical units of less than 30 μm stayed individually on the fiber, whereas larger microcapsules of over 30 μm ruptured or aggregated. Through AATCC mortality tests against *Dermatophagoides farinae* on the fabric, clove oil containing more phenolic monoterpenoids (eugenol) was more effective in reducing the number of live HDM (94% mortality). Clove bud oils from safe and natural sources can be alternative materials for controlling the population of HDM.

INDEX WORDS: microencapsulation, house dust mites, essential oils, clove, thyme, acaricides, and factorial design.

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B.S., Sook-Myung Woman's University, Seoul, Korea, 2004

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DEDICATION

This thesis is dedicated to my parents and brother Yong-Hyong Kim.

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CHAPTER 1

INTRODUCTION

House dust mites (HDM) threaten human health causing asthma, allergy rhinitis, and atopic dermatitis (Colloff et al., 1992; Korsgaards, 1983). They are also significant as an asthma-triggering allergen in children (Arshad et al., 1992; James and Banks, 2004). House dust mites have a tendency to be present in the highest concentrations in non-washable bedding, carpets, and pillows. Specifically, it has been reported that HDM levels in non-washable bedding mattresses in the U.S. homes comprised over half of HDM content (decomposed body and dropping) in the total weight of allergens in the house. It included minimum 2 μg of allergens per 1 g of dust (Nadchatram, 2005). Currently used synthetic acaricides, such as the pyrethroids, pose a risk of neurotoxicity to humans and other mammals (Loucif-Ayad et al., 2008). This problem has guided research efforts to develop safer and capable alternatives to control HDM indoors.

Many studies have indicated that certain monoterpenoids in essential oils could reduce the population of HDM (Isman. 1999; 2000; Holley and Patel, 2005). Specifically, volatile monoterpenoids of clove bud (Kim et al., 2004; El-zemity et al., 2006) and thyme red oils (El-zemity et al., 2006; Deans and Ritchie., 1995) have been found to posses efficient acaricidal activities against house dust mites (HDM). However, most essential oils are volatile or easily oxidized and have a strong smell.

The microencapsulation technique can make up for the weak points in the properties of essential oils. The technique is used in the packing of solids, liquids, or gasses for controlled release in pharmaceutical, cosmetic, or food applications. The coacervation process of encapsulation has been widely used for both the controlled release and protection of core materials. Gelatin (Maji et al., 2007; Passino et al., 2004), polyvinyl alcohol (Bachtsi and Kipparissides, 1996) and other polymers have been employed for encapsulating essential oils. Among them, gelatin as a shell material has been popularly investigated for its advantageous emulsifying properties to form superior films and exhibit good sealing and controlled release when desired (Moretti et al., 2002). Factorial designs are popularly used in microencapsulation process research associated with multiple factors because factorial design is useful to investigate the most significant factor when the data are insufficient for investigation among many variables. This analysis is defined as 'changing a factor at a time', that is, one factor acts additively when other factors are constant (Box et al, 1978).

Microencapsulation coupled with vacuum drying is widely used for dehydrating products containing heat sensitive ingredients such as gelatin protein (Desai and Park, 2005). It can be an effective procedure for preventing the aggregation of microcapsules, which often exhibit this problem. Additionally, through this process, microcapsules can be attached to the fabric without a binder because of the natural properties of gelatin.

The overall objective of this study was to develop sustainable and safe acaricides against house dust mites using natural essential oils. The first step was to produce microcapsules and investigate the significant process factors on the size of microcapsules using factorial design. The second step was to investigate the acaricidal activity against HDM (*Dermatophagoides farinae*)

of clove bud and thyme red oil through direct contact bioassays and AATCC mortality tests using fabric attached with microcapsules.

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CHAPTER 2

LITERATURE REVIEW

2.1 The ecology of house dust mites

The size of a typical house dust mite (HDM) is known to be 250-330 μm long with a weight of approximately 0.2 μg (Tongu et al., 1986). Worldwide, there are two prevailing species of HDM. One is *Dermatophagoides pteronyssinus* (*Der. p*), known as the European house dust mite, and the other is *Dermatophagoides farinae* (*Der. f*), known as the American house dust mite (Platts-Mills et al., 1986), shown in Figure 2.1. Although *Der. f* is found worldwide, it is more abundant in North America than in Europe. This might suggest that *Der. f* can survive in more continental and barren climates than *Der. p.* These species can be morphologically distinguished, as *Der. p* is lacking the four long hairs on the end of its abdomen (Thomas, 2004; Nadchatram, 2005), as shown in Figure 2.1. The optimal conditions for their growth are temperature from 22 °C to 26 °C and humidity level of over 55% (Bessette, 2005; Colloff, 1993). The average life cycle for a male HDM is 10 to 19 days. A mated female HDM survives for approximately 70 days and lays about 60 to 100 eggs (Walter, 1999). Humans generally shed 0.5 to 1.0 g of skin dander per day. HDM can survive for a few months with 250 mg on exfoliated human dander (Klingman, 1964; Walter, 1999). However, stripped off skin dander cannot be served as a diet to HDM directly because it is usually too dry and includes much fat. Therefore, HDM feed on human dander only after it has been decomposed by fungi under optimal carbon and oxygen-rich environments (Hay et al., 1992). HDM excrement, which is tiny pieces 10 to 40 μm in size and contains antigens,

directly causes allergies. HDM produces approximately 2000 excrement particles during its relatively short lifespan (Thomas and Smith, 1999). These pieces easily stick to diverse upholstered furniture, carpets, and bedclothes, leading to the accumulation of allergens in the indoor environment.

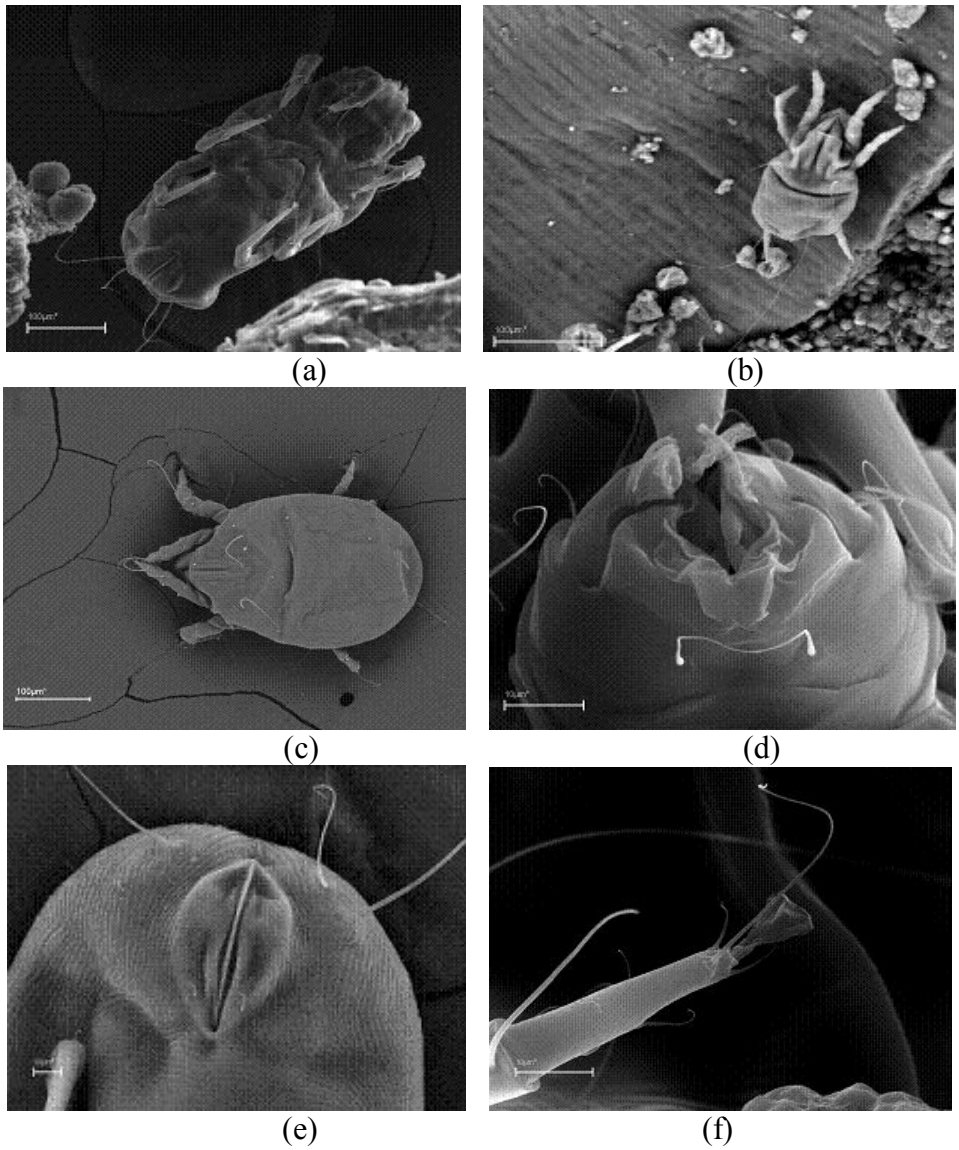


Figure 2.1 SEM images of house dust mite (*Dermatophagoides farinae*) (a) underside with four hairs (b) upside (c) size of the adult (d) mouth without eyes. (e) the genital organ (f) a leg with hair

2.1.1 The influence of HDM on humans in the U.S. households

Inhalant allergens, such as HDM, threaten human health by inducing asthma, allergic rhinitis and atopic dermatitis (Korgaard, 1998; Kraft et al., 1998). Although their allergen-containing excrement causes a direct health threat through inhalation, the increased chance of contact with their excrement under indoor circumstances results in an increased frequency of sensitization and the incidence of asthma in the people. Worldwide, about 100 million people suffer from HDM atopic dermatitis (Cameron. 1997). HDM present in the highest concentrations in non-washable bedding, carpets, and pillows. Specifically, it has been reported that HDM levels in non-washable bedding mattresses in the U.S. homes comprised over half of HDM content (decomposed body and dropping) in the total weight of allergens in the house. It included minimum 2 μg of allergens per 1 g of dust (Nadchatram, 2005). Furthermore, approximately 23.2 million (10% of population) U.S. households linked to asthma and allergies showed high levels of HDM allergens, according to the survey conducted by the National Institute of Environmental Health Services (Zeldin and Vojta, 2001).

2.2 U.S. market volume for related categories of dust mite allergy treatment

The U.S market volume related to HDM allergy is increasing. According to Global Information, Inc. (2004), the U.S. market volume of available HDM containment products comprised over 5.5 billion U.S. dollars in 2004, as shown in Figure 2.2. Within this market volume, 65% is attributed to drugs related to HDM allergy; 17% is for hygienic products, which account for 1 billion dollars; and 8% is for encasings, such as specially treated mattress covers.

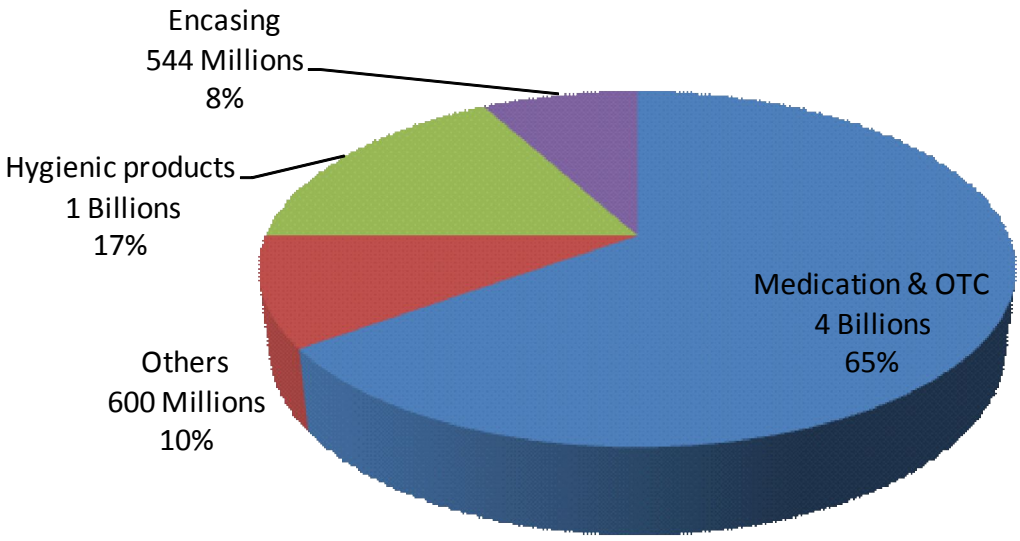


Figure 2.2 The market share of products related to HDM allergy in the U.S. (Global Information Inc., 2004)

2.2.1 Currently available treatments against dust mite allergy

Currently available treatments related to HDM can be categorized as allergy immunotherapy, prescription and over the counter drugs, encasing products and hygienic products (Global Information Inc. 2004). Firstly, allergy immunotherapy is accomplished through vaccinations for persons with serious allergy disease. This treatment is effective, but expensive. Secondly, prescription and OTC drugs, such as Claritin®, account for a major share of the market, but this treatment presents only temporary relief and cannot get rid of the source causing the HDM allergy. Thirdly, encasing products, such as finished mattress covers, are less permeable to air and moisture. But it may interfere with the intrinsic quality of the fabric. These products have to be washed in the laundry, resulting in less effect as time goes by. Lastly, hygienic products, such as sanitizing wipes and insecticide sprays, have short-term effects and may contain synthetic

toxic chemicals. Thus, some consumers would not want to directly apply them to their bedding for safety reasons.

2.3 Synthetic acaricides

Acaricides used in medical and agricultural applications are defined as pesticides against mite and tick species (Mullen et al., 2009).

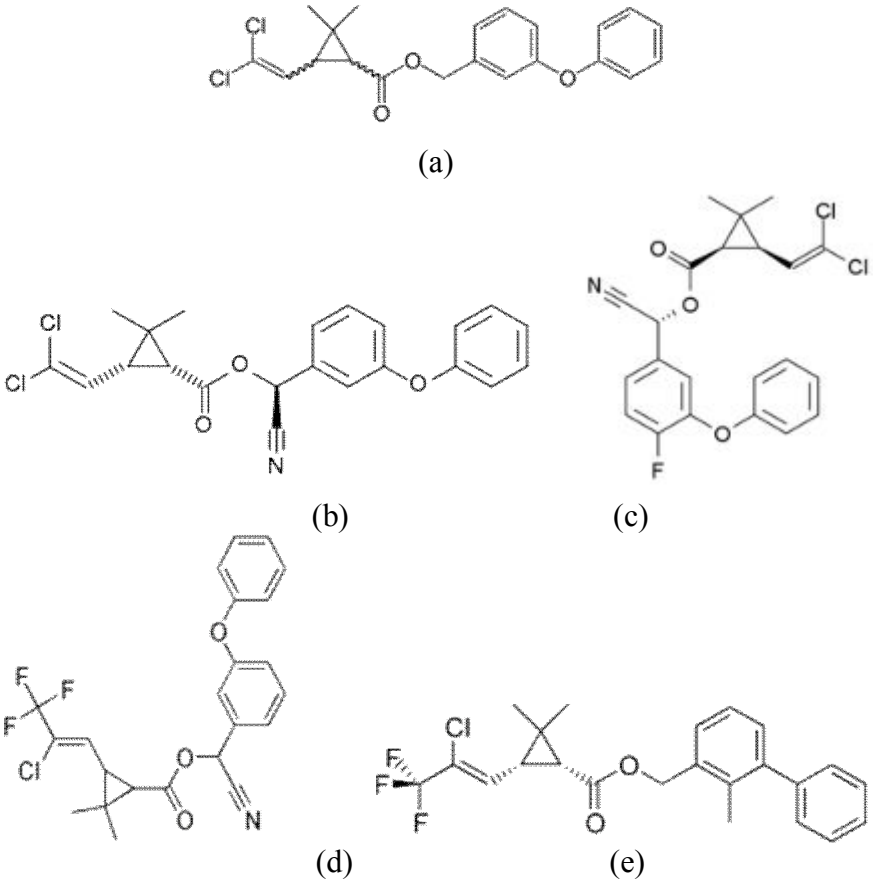


Figure 2.3 Chemical structures of synthetic pyrethroids (Gan et al., 2008) (a) permethrin (b) cypermethrin (c) cyfluthrin (d) λ–cyhalothrin (e) bifenthrin

In non-agricultural environments, such as houses, various synthetic pyrethroids, such as permethrin, cypermethrin, cyfluthrin, λ- cyhalothrin and bifenthrin, are used, as shown in Figure

2.3 (Gan et al., 2008). The pyrethroids exhibit extreme hydrophobic properties, which influence environmental toxicity, human health and reduce bioavailability (Loucif-Ayad et al., 2008). Moreover, these chemicals cause dysfunction of neurotransmitters, delivering a signal through the calcium (Ca^{2+}) and sodium (Na^{+}) channels of cell membranes. These pyrethroids are also highly toxic when exposed in the environments, by rapidly accumulating to toxic levels that may cause human health risks (Gan et al., 2008). Therefore, the use of many conventional pyrethrum-based insecticides is restricted by recent government action through U.S. the Food Quality Protection Act. This action stimulates a market for the production of synthetic acaricides and has led to research efforts to develop safer and more efficient alternatives for controlling HDM under indoor circumstances.

2.4 The function of essential oils as natural acaricides

An essential oil is defined as hydrophobic and concentrated aroma liquid with volatile compounds from plants. Many studies have indicated that specific phenolic monoterpenoids in essential oils reduced HDM populations (Isman. 1999; 2000; Holley and Patel, 2005) with relatively low toxicity to humans (Isman, 1999; 2001). Acaricidal compounds occuring in nature included butylidenephthalide from *Cnidium officinale Makino* bulbs (Kwon and Ahn, 2002); perilla oil (Watanabe et al., 1989); the essential oil from *Lauraceae* trees (Furuno et al., 1994); and pisiferic acid from *Chamaecyparis pisiferta Sieb* leaves. Natural plant essential oils can be a powerful source for controlling HDM because they are composed of effective phenolic monoterpenoids containing bioactive principles. They are generally safe to use as cosmetic fragrances and food flavoring additives. Through previous research, clove, thyme, horseradish, coriander oils (Kim, et al, 2004); clove bud oil (Km et al., 2004; El-zemity et al., 2006); thymol,

cinnamaldehyde (El-zemity et al., 2006; Deans et al., 1995); and *Cnidium officinale* rhizome extracts (Kwon and Ahn, 2002) were found to be most effective with up to 100% HDM mortality. Natural plant essential oils were also studied for their antimicrobial activity against Gram positive and Gram negative bacteria. Volatile phenolic oils with a large amount of eugenol from allspice or clove, cinnamamic aldehyde from cinnamon bark, and thymol and *para-cymene* from thyme oil are responsible for the antimicrobial activities (Lis-Balchin et al., 1998; Davidson and Naidu, 2000). In general, the phenolic monoterpenoids in the essential oils exhibit antimicrobial and acaricidal activities (Deans et al., 1995). These monoterpenoids in the essential oils are non-persistent in the water and soils with breaking down (Misra and Pavlostathis, 1997). Specifically, eugenol is approximately 1500 time less toxic than the synthetic pyrethoids with complete degradation (Stroh et al., 1998). This information demonstrated certain monoterpenoids-based acaricides can be effective against HDM and safe to health and environments.

2.4.1 Monoterpenoids derived allyl or alkyl group in the essential oils.

Terpenes, also called isoprenoids, compose of five carbon units, are found in diverse class of natural living things. Specifically, monoterpene, a sub-category of the terpene, consists of two isoprene units ($C_{10}H_{16}$). The oxidized or rearranged compounds from monoterpene are called monoterpenoids. Monoterpenoids with alkyl groups ($-CH_3$), such as thymol and *para-cymene* from thyme oil, have ten carbons and two missing hydrogens from the monoterpene structure. On the other hands, monoterpenoids with allyl group, such as eugenol from clove oil, have a hydrocarbon group with the chemical structure $H_2C=CH-CH_2$ consisting of a methylene (CH_2) and a vinyl group ($CH=CH_2$) (Gan et al., 2008; Dev and Koul, 1997), as shown in Figure 2.4.

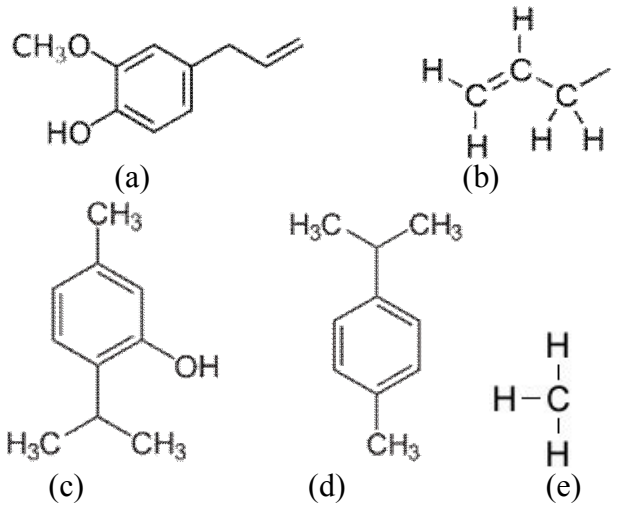


Figure 2.4 Chemical structures of major monoterpenoids of clove bud and thyme red oils (Ashurst, 1995) (a) eugenol in clove bud oil (b) allyl-structure (c) thymol (d) *para-cymene* in thyme red oil (e) methyl group (alkyl structure)

2.4.2 Clove oil

Clove buds have been found to have the most powerful acaricidal activity among the essential oils derived from plants. Eugenol from clove bud oil has been reported to be the most effective agent against Japanese mites (Park and Shin, 2005) and *Der. p* (El-Zemity et al., 2006). Oils with a large amount of eugenol (4-Allyl-2-methoxyphenol derivatives or methoxyphenol derivatives, $C_{10}H_{12}O_2$) as a phenolic monoterpenoid are found in clove oil. There have been known to have antiseptic and antimicrobial properties since ancient times (Bensky, 2004). Among the three kinds of clove oils from buds, leaves, and stems, the main monoterpenoids of clove oil are generally 82 to 93% eugenol, exhibiting a strong and warm fragrance as a phenolic compound, and are 3 to 7% caryophyllene, with a spicy smell and woody properties (Ashurst, 1995).

Clove oils are used not only in food flavor blends, but also in fragrances of banana, blackberry and cherry flavors. There is no legal limitation or associated health hazards in using all three types (bud, stem, and leaf) of clove oils. They are safe and generally regarded as safe

(GRAS) by the Flavor and Extracts Manufacturers Association (FEMA) (Ashurst, 1995; Miller et al., 1983).

2.4.3 Thyme red oil

Thyme red oil (*Thymus vulgaris*) commonly consists of 20 to 54% thymol and 15 to 35% *para-cymene*. Thymol has been used as the major active principle in the commercial Listerine mouthwash and in bandaging as medication before the introduction of current antibiotics (Pierce, 1999). Besides, thymol has powerful antifungal activity against mold and mildew (Ramsewak, 2003). The major phenolic monoterpenoids of thyme red oils are thymol (2-isopropyl-5-methylphenol, $C_{10}H_{14}O$), comprising approximately 50% of thyme red oil, with a sweet fragrance; approximately 15% *para-cymene*, possessing a light color, citrus smell, and resinous properties; and approximately 11% γ-terpinene, having a light color and citrus and herbal fragrance properties. Thyme oil is often used in seasoning mixtures for cooking. According to FEMA, there is no limitation on the use of thyme red oil (Ashurt, 1995).

2.4.4 Viscosity of oil

Viscosity is the restraining of the flow of the liquid particles due to the forces induced by intrinsic molecular attractive forces, such as ionic dipoles, dipole-dipole interaction, or hydrogen bonding in long chain molecules. Surface tension is caused by the cohesive forces of the liquid molecules among themselves rather than adhering to the other liquid. Surface tension influences the surface area of the emulsion droplets. For example, a decrease in viscosity results in an increased surface tension causing larger capsules (Gallo et al., 1984; Burger et al., 1985).

2.4.5 Mechanism of antimicrobial and acaricidal action

The phenolic terpenoids of essential oils exhibit their antimicrobial activity by interrupting the structure of the cytoplasm membrane resulting from extended membrane permeability causing swelling and reducing membrane functions (Sikkema et al, 1995). The swelling of the cell membrane brings on an effluence of potassium ions (K^+) that regulates the body's pH control and stimulates osmotic shock in the cell to damage the cell and adenosine triphosphate (ATP) system. Sustaining a loss of cell membrane permeability is usually defined as a major factor of cell death (Brul and Coote, 1999).

2.5 The need for microencapsulation

Microencapsulation techniques are widely used in various fields such as in food ingredients, pharmaceuticals and drugs, and cosmetics. The major advantages are as follows: (1) the encapsulated core materials can be released under control when desired at the right time; (2) volatile or labile ingredients as core materials, such as essential oils or vitamins, can be protected from oxidation; (3) undesired smells or flavors are prevented; (4) the functional or bioactive aspects of drugs or diet, are enhanced (Shefer and Shefer, 2003). Microencapsulation in the textile industry continues to grow as a means of imparting finishes and properties by encapsulating repellents, vitamins, fragrances, enzymes, or flame retardants. Microencapsulation techniques are cost-effective and easy to perform. Besides, they do not influence the existing textile properties (Nelson, 2002).

2.6 Different methods of microencapsulation

There are several methods for producing microcapsules. For example, spray drying is the major commercial technique for large scale production of microcapsules. In this method, the shell material has hydrophilic property. However, this method has limitations due to the high temperatures that may break the shell through cracking (Barbosa-Canovas, 2005). Another method, namely coacervation, is widely used in pharmaceuticals, cosmetics, and fragrances. The simple coacervation method develops a single layer, whereas the multi coacervation method makes multi layers. This technique requires that the encapsulated core materials, such as liquids or solids, be hydrophobic, similar to the spray drying method (Dziezak, 1998). However, vacuum drying is more advantageous in that it has tendency to conserve the shape of the microcapsules under the diverse formation processes. Another method is the extrusion technique, which can also be used to produce microcapsules with a long shelf life, protecting the core material from oxidation. However, this method is expensive and requires a high process temperature and low solubility of shell materials in the water at low temperatures (Reineccius, 1994). Another alternative, namely the use of the ultrasonic atomizer, has an advantage of low cost and consistent atomization, but has the restriction of appropriating only low ultrasonic vibration power. This process has not been examined well and little research has reported producing microcapsules by this technique (Cole-Parmer Instrument Company, 2005).

2.7 Microencapsulation using coacervation

2.7.1 Formation of emulsion using coacervation

The simple coacervation process is related to the use of only one colloid and the elimination of the water surrounding the dispersed colloids induced by ethanol or sodium sulfate which exhibits

stronger hydrophilic property than the shell materials (Deasy, 1984). Encapsulated core materials in the coacervation process can be either liquid or solid, and have to be hydrophobic. The oil phase as core material is encapsulated through high agitation and dispersing in droplets during the aqueous stage, resulting in a colloid under the condition of an oil-in-water (O/W) emulsion.

An important factor in the development of microcapsules using the simple coacervation method is the selecting of the suitable three-phase materials shown in Figure 2.5 for the shell and core materials. The selecting of hydrophilic chemicals induces the colloid rich phase according to the Hofmeister series. This shows the order of the salts exhibiting high hydrophilic property, resulting in protein precipitates. To explain the diagram shown in Figure 2.5, when 25% aqueous Na_2SO_4 is inserted in a 5% w/w gelatin solution, point X will move to point Y. The shaded region (Figure 2.5) indicates visible coacervation phase due to the addition of large quantity of sodium sulfate (Deasy, 1984).

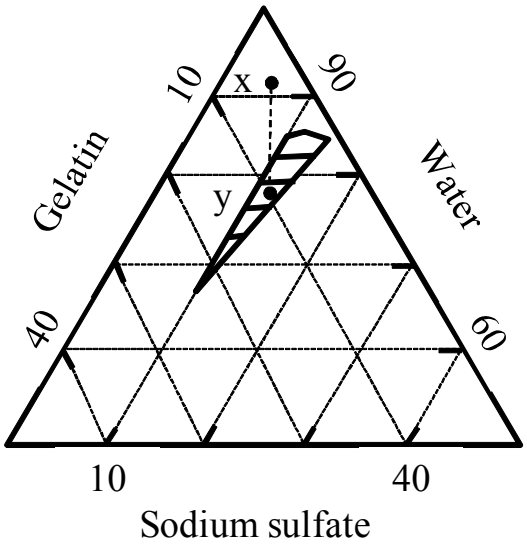


Figure 2.5 Phase diagram for three immiscible phases (Desey, 1984)

2.7.2 Hardening of the coating material

Glutaraldehyde is broadly used as a protein cross linking agents in structure settlement. In this study, the shell in the gelled stage was crystallized by the addition of a cross linking agent such as glutaraldehyde. Quiocho and Richards (1964) reported that glutaraldehyde for the gelatin cross-linking agent is valuable on account of the great stability and the few changes in the protein during the process. Cross linked gelatin with glutaraldehyde displayed a loss of only lysine, one of the amino acids, in the gelatin. The cross linking reaction of glutaraldehyde is shown in Figure 2.6 (Richards and Knowles, 1968). Crosslinked gelatin with glutaraldehyde does not influence the natural property of gelatin as a carrier; therefore glutaraldehyde is used in many applications such as pharmaceutics and cosmetics.

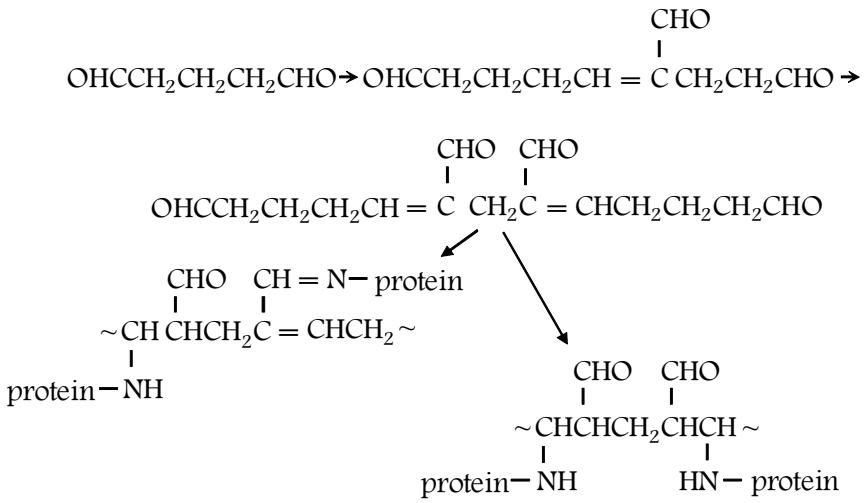
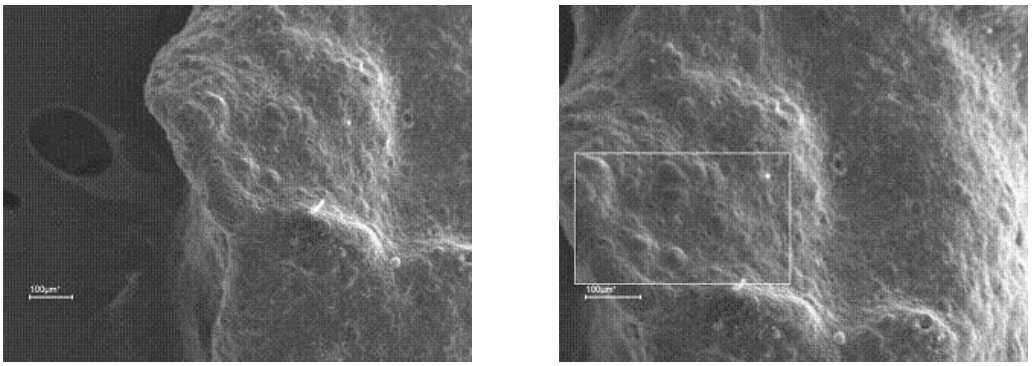


Figure 2.6 Crosslinked protein with glutaraldehyde (Richards and Knowles, 1968)

2.8 Vacuum drying

Freeze-drying and vacuum drying are popular processes for dehydrating the microcapsules and for drying heat sensitive shell materials (Cox, 1991). Freeze-drying has the advantage of developing a longer shelf life than normal drying since this process is used in the storage of

perishable material by lowering the temperature and reducing the enclosed pressure (Barbosa-Canovas et al., 2005). After hardening, the crosslinked microcapsules require the removal of water and uncapsulated oils by drying methods. However, freeze drying may not completely remove the surrounding oil from the microcapsules. Some research reported that the microcapsules using freeze drying would have a tendency to have residual oils on the surface, leading to problems of reflection and non-conduction during an investigation using scanning electron microscopy (Klaypradit, 2006; Martins et al., 2009) as shown in Figure 2.7.



(a) (b)

Figure 2.7 Invisible microcapsules because of residual oil on the surface by SEM

Vacuum drying can be applicable on a wide scale in several industries such as chemical, pharmaceutical, food, and metal powders. Vacuum drying can be a good process for the gelatin that would otherwise be decomposed if exposed to high temperatures in the oven drying. The vacuum eliminates water while inhibiting the oxidation that can take place when certain materials unite with air. Furthermore, vacuum drying has environmentally friendly advantages inasmuch as the material can be desiccated to low levels of water and energy, and used solvents can be recycled afterwards (Cox, 1991).

2.9 Characterization of microcapsules

2.9.1 Gas chromatography- Mass spectrometry (GC-MS)

GC-MS systems are used for the identification of particular substances in a sample, such as drugs or fire hazards in the environment. Gas chromatography (GC) can separate the different molecules of the sample by different retention times using columns. A mass spectrometer (MS) will break or ionize each molecule from the GC and detect the broken fragments by using their mass to charge ratio (Robert & Adams, 2007). This instrument can be used to determine the chemical composition of the essential oils in order to evaluate their role toward acaricidal activity to HDM.

2.9.2 Optical microscopy

A simple microscope is the type that uses visible light to magnify a small sample, whereas scanning electron microscopy does not use visible light. Normal microscopes use converging lens for magnification on the sample plate. The magnification of the device is obtained by changing the lens, distances between the sample and the lens, and the focus of the image (Stephen et al., 1995). Light microscopes are used for investigating the size of emulsion droplets by taking micro photographic images and counting the number of dead HDM. It was used for measuring size and dispersing droplets in the emulsion state under normal conditions.

2.9.3 Scanning electron microscope (SEM)

SEM is the system for three dimensional visualization of surfaces at the cellular and sub cellular levels by scanning with high energy electrons. The morphology of various gelatin based-microcapsules has been widely described by using SEM (Madan et al., 2006). The images display a great depth of focus with relatively easy identification (Kalab et al, 1995). SEM is

capable of 100,000 fold magnification, with a resolution limit of 5 nm. The SEM uses a delicately centered x-ray electron beam to scan across the surface of structures at a narrow angle, and back-scattered electrons reflected by scattering from the sample result in the secondary electron image. Before investigating with SEM, the samples are coated with a thin metal coating in order to achieve a good image by increasing electrical conductivity and back scattered electron emission (Hoppert, 2003).

2.9.4 Thermogravimetric Analysis (TGA)

TGA measures the changes in the weight of microcapsule powder under thermal heating by placing the sample powder in a platinum container. The temperature can be set from 20 °C to 1000 °C depending upon the sample's degradation point. The computer connected with the thermogravimetric machinery measures the percentage of weight loss of the sample in the plot in accordance with the raised temperature. This analysis can be used to determine the weight of shell to core materials in the study.

2.10 Experimental factorial design

Factorial designs are popularly used in experiments associated with multiple factors where it is required to investigate the correlation effect of these factors on a response. Factorial design is useful to investigate the most significant factor when the data are insufficient for investigation among many variables. This method involves 'changing a factor at a time', that is, one factor acts additively when other factors are constant (Box et al, 1978). In manufacturing of the microcapsules, the factors that may influence the size of microcapsules are so many that it is very difficult to create an impeccable investigation. Common factors are protein concentration, type of shell or core materials, denaturation temperature and process, species and concentrations

of surfactant, with or without baffles, agitation speed, and ratio of a shell to a core. In order to investigate a factorial design, two kinds of levels were fixed for each variable (factor) in the experiments, and all possible combinations of tests were performed using an oil-in-water coacervation method (Torrado et al., 1988).

2.10.1 Data Analysis

Factorial design will be analyzed using the table of contrast coefficients (Daniel, 1976; Hibbert, 2007). The weighted sum of the treatment means refers to a contrast, whereas the pattern to be examined is implicated by the coefficients. The weighted sum is plotted on a cumulative probability plot, and the significance of the factors is indicated by the distribution of the plot. Finally, through a student t- test, the results are verified to determine statistical error.

Table 2.1 Effects in the 2 x 2 factorial design

Factors	A	B	AB	Response, Y (mm)
Test 1	(-)	(-)	(+)	10
Test 2	(-)	(+)	(-)	20
Test 3	(+)	(-)	(-)	50
Test 4	(+)	(+)	(+)	150
(-) sum	-10-20	-10-50	-20-50	
(+) sum	+50+150	+20+150	+10+150	
Sum	170	110	90	
Effect	85	55	45	

For example, let's consider two different factors A and B in the microencapsulation process. We can calculate 'Effect' shown in Table 2.1 by adding a column "AB" to consider the interaction of A and B for outcome Y. The summation of each column is calculated by the

corresponding ‘plus’ and ‘minus’ signs. Then, ‘Effect’ is calculated by dividing the summation by half of the number of test cases. By arranging factors by the corresponding ‘Effect’ in the ascending order (Table 2.2), the cumulative probability, p can be calculated by following equation:

$$p = \frac{m}{N+1} \tag{2.1}$$

where, N is the number of the considered factors and their combinations, and m the rank.

Table 2.2 Cumulative probability of main effects A and B and interaction effect AB

m	Factors	Effect	Cumulative Probability
1	AB	45	25%
2	B	55	50%
3	A	85	75%

The mean and the standard deviation of ‘Effect’ are calculated as 61.7 and 20.8. The ‘Effect’ A, B and AB were plotted, as shown in Figure 2.8. These results indicate that the change of A from (-) to (+) has more effect on increasing microsphere size than the average effect, and the change of B and AB has less effect on increasing microsphere size than the average effect. If there are no significant effects, a straight line should be obtained. The distribution of the effects indicates that, if they are on the right side of the line, then the change of the level in each factor indicates an increase of size, and, if the effects are on the left side, they indicate a decrease of size.

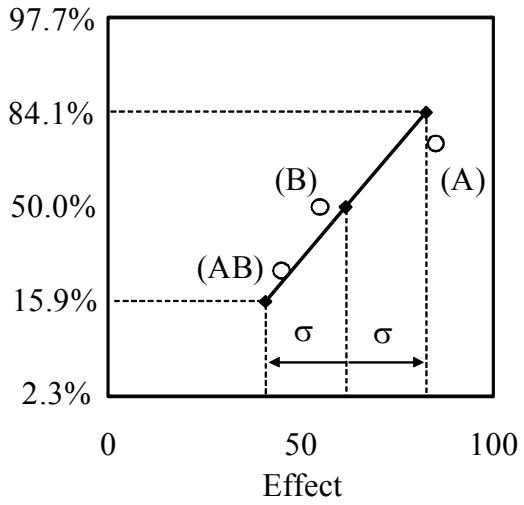


Figure 2.8 Effect of interaction AB and main effects A and B on the cumulative probability plot

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CHAPTER 3

GELATIN-BASED CLOVE OR THYME OIL ENCAPSULATED PARTICLES AND THEIR CHARACTERIZATION

3.1 Introduction

Essential oils have been studied as an excellent source of acaricides (Isman, 1999; 2001; Kim et al., 2004; El-zemity et al., 2006). The use of essential oils is the subject of many investigations in recent decades due to their eco-friendly and biodegradable properties. Specifically, volatile monoterpenoids of clove bud (Kim et al., 2004; El-zemity et al., 2006) and thyme red oils (El-zemity et al., 2006; Deans and Ritchie., 1995) have been found to possess efficient acaricidal activities against house dust mites (HDM). However, essential oils are mostly volatile or easily oxidized and have a strong smell.

The microencapsulation technique can make up for the weak points in the properties of essential oils. The technique is used in the packing of solids, liquids, or gasses for controlled release in pharmaceutical, cosmetic, or food applications. Microcapsules consist of a core and a shell. Compared to *in situ*, interfacial polymerization, and spray drying, the coacervation process of encapsulation has been widely used for both the controlled release and protection of core materials. Gelatin (Maji et al., 2007; Passino et al., 2004), polyvinyl alcohol (Bachtsi and Kipparissides, 1996) and other polymers have been employed for encapsulating essential oils. Suitable shell materials, or carriers, for the encapsulation of core materials, such as essential oils, are necessary. Ideally, a coating material must possess the following properties: solubility in a solvent like water, ability to hold or seal core material and emulsify the core during procedure

and storage, no reactivity to the core during high temperatures, non-toxic and generally regarded as safe (GRAS), ability to release the core under desired conditions, and economic feasibility and stability of supply (Shahidi and Han, 1993). Gelatin as a shell material has been popularly investigated for its advantageous emulsifying properties to form superior films and exhibit good sealing and controlled release when desired (Moretti et al., 2002). Together with water, it forms a semi-solid colloid gel. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling. Moreover, Passion et al. (2004) have reported that, when they added gelatin produced microcapsules loaded with thyme red oil to the diet of certain household pests, there was a high mortality rate due to ingestion of the active monoterpenoids released from the microcapsules.

The essential oil formulations in the study were prepared by a phase separation process (coacervation) according to factorial experimental design under 16 different treatments. A number of studies have evaluated the key factors determining the size of microcapsules, including type of oil (oil viscosity), ratio of gelatin to oil (RGO), agitation speed, and presence of surfactant (Torrado et al., 1988; Maji et al., 2007). In these studies, where many factors are involved, factorial design can be applied to identify those factors with large effects on the size of microcapsules.

Microencapsulation coupled with vacuum drying is widely used for dehydrating products containing heat sensitive ingredients such as gelatin protein (Desai and Park, 2005). It can be an effective procedure for preventing the aggregation of microcapsules, which exhibited the problem shown in much of the research. Additionally, through this process, microcapsules can attach to the fabric without a binder because of the natural properties of gelatin.

One objective of this study was to develop microcapsules containing essential oils using a factorial design to investigate the significant process factors. A second objective was to investigate the characteristics of microcapsules loaded with essential oils.

3.2 Materials and methods

3.2.1 Materials

Gelatin (Type A, mol. wt. around 50,000) from porcine skin, Span®85(surfactant), 25% w/v glutaraldehyde (grade II) as cross-linking agent, and anhydrous sodium sulfate powder were purchased from the Sigma and Aldrich Chemical Co. USA. Clove bud and thyme red oil were purchased from the Sigma and Aldrich Co.USA. Tangletrap sticky coating was purchased from the Tanglefoot® Co. USA. Nylon mesh with a pore size under 50 μm was purchased from Smallparts®. USA. House dust mites were donated from the Insect Control Research Inc., MD, USA.

3.2.2 Preparation of Emulsion

The preparation of microcapsules employed the coacervation phase method, using protein gelatin as an external polymer. A 50 ml aqueous solution of gelatin (4% w/v) was prepared using a glass vessel. The 1:2 or 1:5 (w/w) of gelatin to essential oils (thyme or clove) was added to an aqueous solution above 40 °C under an agitation speed of 500 or 1000 rpm to produce an oil-in-water emulsion. The emulsion was stirred with or without 10% v/v of surfactant, Span® 85 (Park et al., 2007) for 5hrs.

In the experiments, the following four factors, as shown in Table 3.1, were selected as the most significant variables in producing microcapsules: the two types of essential oils, the ratio of gelatin to oil (1:2 or 1:5), an agitation speed of 500 rpm or 1000 rpm, and the presence of

surfactant. Each factor had two levels coded (+) at a high level and (-) at a low level, and the two replications of each experiment provided the standard errors. This study required thirty two experiments because of the four factors at two levels and two replications. We conducted each experiment to find the significance of the factors on the size of microcapsules. A, B, C and D represented each single effect while AB, AC, AD, BC, BD, CD, ABC, ABD, ACD, BCD, and ABCD showed the interaction between the factors, shown in Table 3.1. The total number of tests that were conducted amount to 2^4 x 2 (replication) = 32 experiments.

Table 3.1 Factorial design for four single factors and eleven possible interactions

Test	Single factors				Possible interactions										
	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	ABCD
1	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	-	+	+	-	-	-	+	+	-	-	-	+	+	-	+
4	+	+	-	-	+	-	-	-	-	+	-	-	+	+	+
5	-	+	-	+	-	+	-	-	+	-	+	-	+	-	+
6	+	-	+	-	-	+	-	-	+	-	-	+	-	+	+
7	-	-	+	+	+	-	-	-	-	+	+	+	-	-	+
8	+	-	-	+	-	-	+	+	-	-	+	-	-	+	+
9	-	-	-	+	+	+	-	+	-	-	-	+	+	+	-
10	-	-	+	-	+	-	+	-	+	-	+	-	+	+	-
11	-	+	-	-	-	+	+	-	-	+	+	+	-	+	-
12	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-
13	+	+	+	-	+	+	-	+	-	-	+	-	-	-	-
14	-	+	+	+	-	-	-	+	+	+	-	-	-	+	-
15	+	+	-	+	+	-	+	-	+	-	-	+	-	-	-
16	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-

NOTE: A :(+) Thyme, (-) Clove
B: (+) 1:2 (w/w) ratio of protein to oil, (-) 1:5
C: (+) Stirring speed at 1000 rpm, (-) 500 rpm
D: (+) With surfactant span 85, (-) without surfactant

3.2.3 Induced Coacervation Phase

The coacervation phase was delivered by the gradual addition of 20% w/v Na_2SO_4 solution over 2 hours. It has been reported that the minimum temperature and ratio of gelatin to sodium sulfate was required to be 40 °C and 1:10 w/w, respectively (Maji et al. 2007). In this step, coacervation was visible in the formation of colloid-rich droplets. The aqueous solution containing coacervate droplets was maintained in a cold water bath at 5 °C with agitation for 30 mins.

3.2.4 Cross-linking with Glutaraldehyde and the Removal of Surrounding Oil

In this step, it was necessary to harden the shell. Glutaraldehyde solution (2% w/v) was added and stirred for 90 mins under same agitation as the initial speed. The microcapsules were filtered and washed with Na_2SO_4 (5% w/v) solution and distilled water several times to remove the surrounding oils. Previous studies reported that glutaraldehyde concentrations in the two levels of 1.5 to 5.3% w/v to produce microcapsules did not have a significant change on the size (Torrado et al., 1988; Tomlinson and Burger, 1985; Sheu and Sokoloski, 1986). Additionally, Quiocho and Richards were explained that there was no significant gelatin change in the shell material when cross linked with glutaraldehyde, indicating only a small quantity loss of lysine residues, one of the amino acids in the gelatin (Quiocho and Richards, 1968; Richards and Knowles, 1968).

3.2.5 Vacuum Drying

The microencapsulation process was coupled with a vacuum drying method because vacuum drying is a popular process for drying (Thomasin et al., 1996) heat sensitive materials such as gelatin. The solidified microcapsules were filtered, washed with 5% w/v sodium sulfate solution to remove non-encapsulated oil, and then washed several times with cold water. Then, a low concentration of solidified microcapsules was suspended in the cold distilled water, dehydrated

for 30 mins using vacuum drying with 100% acrylic fabric (blue color, thickness 0.6 mm) instead of filter paper because it prevented the aggregation of microcapsules during drying. It was further completely dried at room temperature for a week.

3.2.6 Emulsion Droplets Size

Particle size was determined by measuring the diameter of droplets under the condition of emulsion by randomly selecting about 50-100 particles per experiment and then calculating the standard deviation (SD) and the mean size in each test. Optical microscopy was used to measure the size of the droplets in emulsion status at 100 x. Many researchers have reported that measuring the size of droplets during emulsion exhibited more precision than examining the size of the final microcapsules (Torrado et al., 1989). An object micrometer (2 mm, 1/100 m/m) was used to control microphotography at 100 x for each test. Then, using a pipette, a 1ml drop from the emulsion was suspended in a 10 cm Petri dish, and photomicrography was performed by random scanning. The diameters and size distribution of the microcapsules were manually measured from the resultant photomicrographs (Gallo et al., 1984; Allen et al., 1981; Vinetsky and Magdassi 1997).

3.2.7 Data Analysis of Emulsion Droplets on Size

The effects on the size of droplets were analyzed using the table of contrast coefficients (Daniel, 1976; Hibbert, 2007). A contrast is the weighted sum of the treatment means, where the coefficients describe the pattern to be tested. The weighted sum was plotted on a cumulative probability plot, and the distribution of the plot indicated if a factor was significant or not. Furthermore, through a student t-test, it was verified to determine the significance of data. The microencapsulation technique is complex and requires the consideration of several variables. The

table of contrast coefficients is an effective method to investigate the most important variables in the production of microcapsules.

3.2.8 Characterization of Microcapsules Loaded with Thyme or Clove Oil

Thermal gravimetric analysis (TGA) was conducted on a Mettler Toledo SDTA851 in the temperature range of 25 to 300 °C at a 10 °C min^{-1} rate under constant nitrogen gas (N_2) flow. This technique was used to determine whether microcapsules contained thyme or clove oil as the core material by measuring the weight and temperature of samples. This test was also used to verify the degradation temperature and residual point. Scanning electron microscopy (SEM) was used to determine morphology and size of microcapsules.

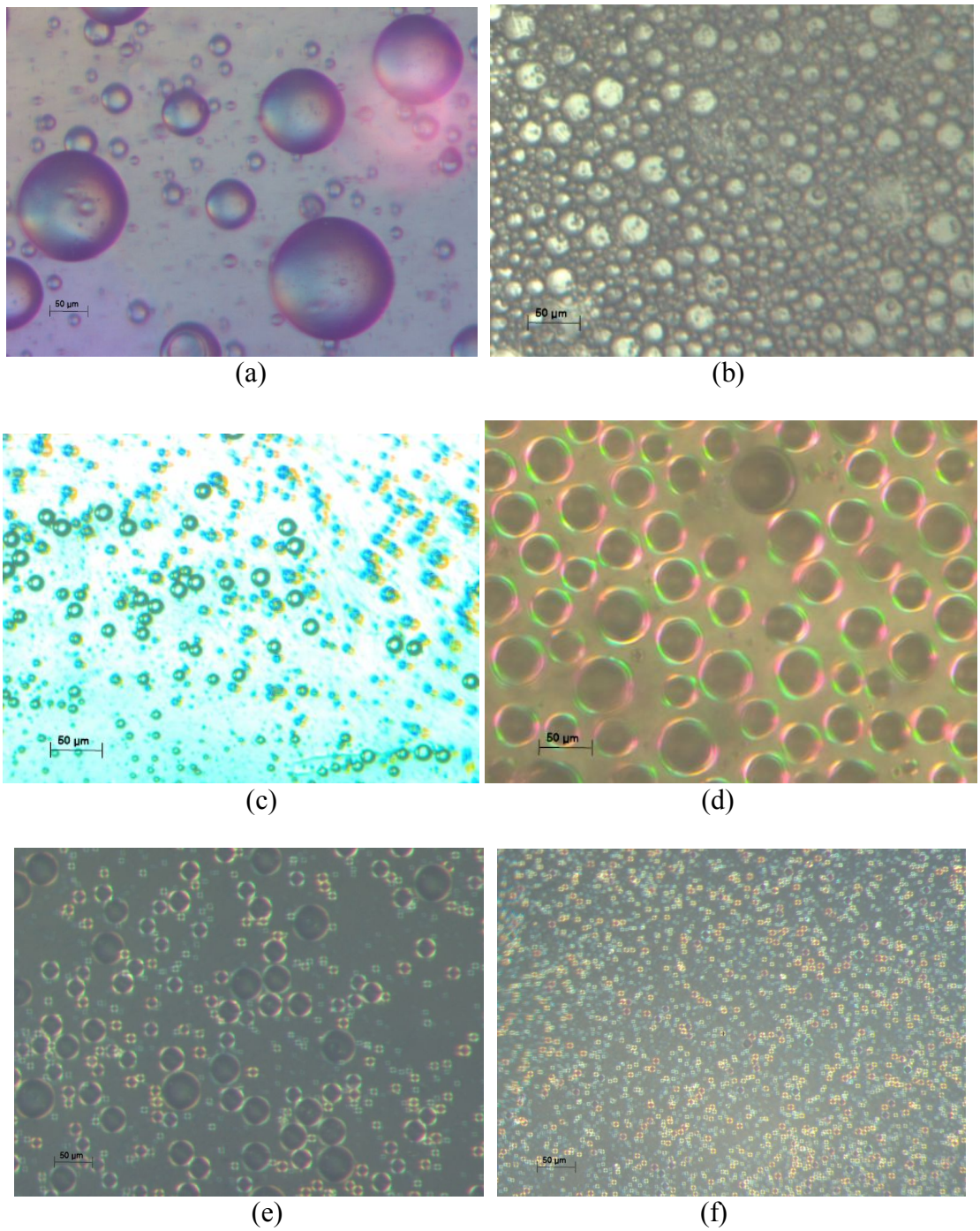
3.2.9 Viscometer

The Brookfield digital viscometer model no. RV was used to investigate the viscosity of clove bud and thyme red oils at both 24 °C and 50 °C using manual no. M/85-160-G. Spindle no. 2 was used at maximum speed of 100 rpm.

3.3 Results and discussion

3.3.1 Emulsion Droplet Size Analysis

The measurements from optical microscopy were used in determining the size of the microcapsules in the emulsion before denaturation. The mean particle size and standard deviation for all experiment conditions are summarized in Table 3.2. The droplets had a spherical shape with different diameters as shown in Figure 3.1.



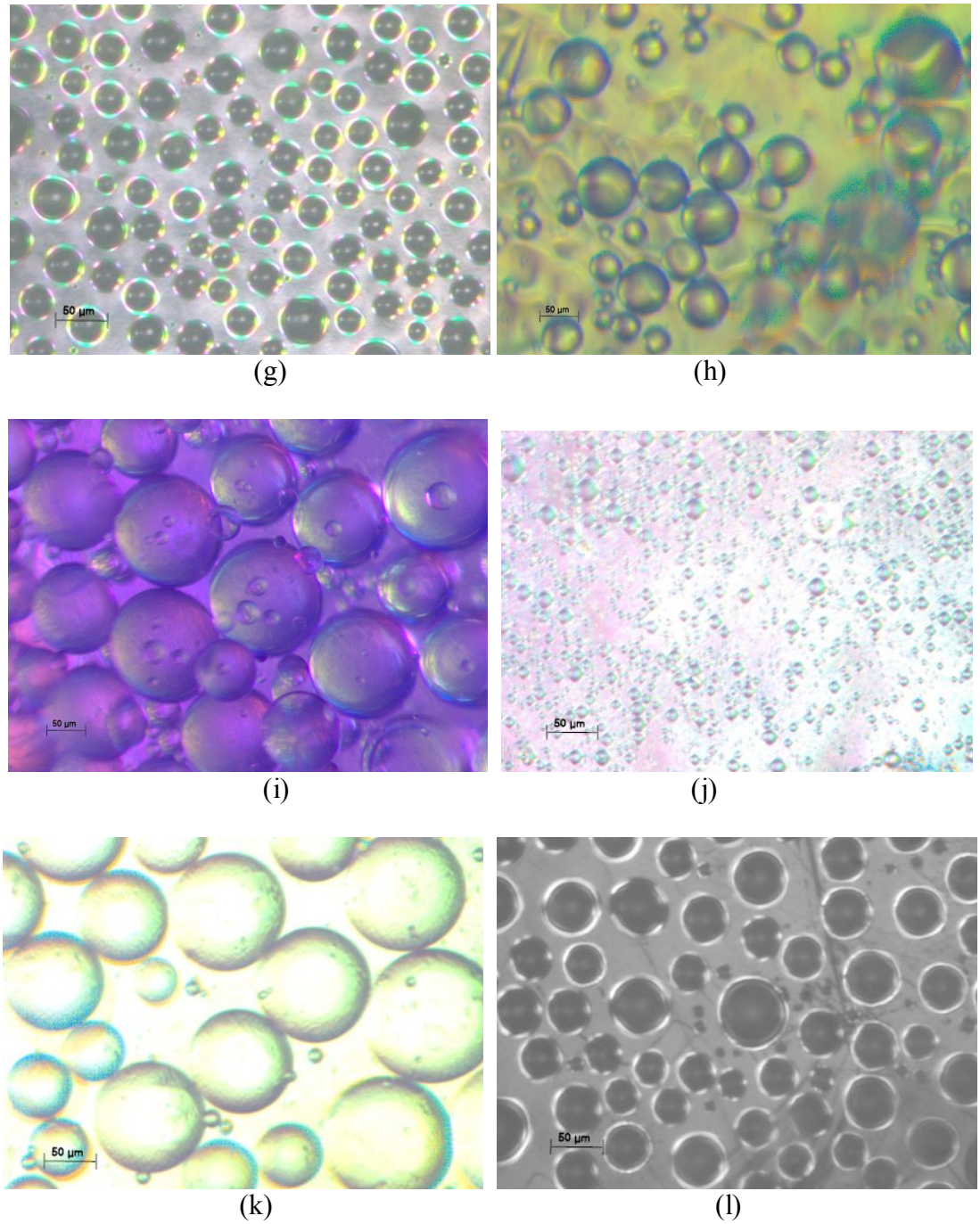
(a) (b)

(c) (d)

(e) (f)

Figure 3.1 Emulsion droplet microscopic pictures under 16 different treatments using optical microscopy at 100× for (a) Test 1 [A(-), B(-), C(-), D(-)], (b) Test 2 [A(+), B(+), C(+), D(+)], (c) Test 3 [A(-), B(+), C(+), D(-)], (d) Test 4 [A(+), B(+), C(-), D(-)], (e) Test 5 [A (-), B (+), C (-), D (+)] and (f) Test 6 [A(+), B(-), C(+), D(-)]

NOTE: A :(+) Thyme, (-) Clove
B: (+) 1:2 (w/w) ratio of protein to oil, (-) 1:5
C: (+) Stirring speed at 1000 rpm, (-) 500 rpm
D: (+) With surfactant span 85, (-) without surfactant



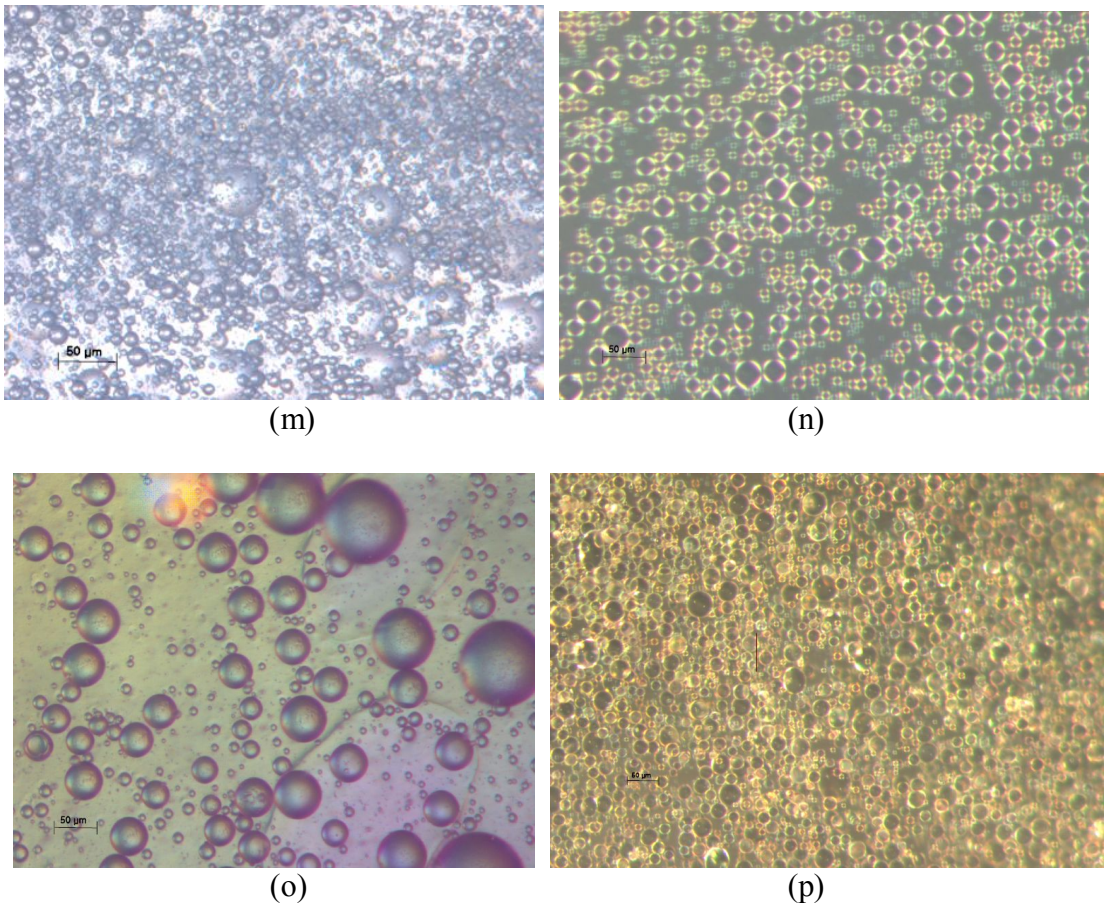
(g) (h)

(i) (j)

(k) (l)

Figure 3.1 Emulsion droplet microscopic pictures under 16 different treatments using optical microscopy at 100× for (g) Test 7 [A(-), B(-), C(+), D(+)], (h) Test 8 [A(+), B(-), C(-), D(+)], (i) Test 9 [A(-), B(-), C(-), D(+)], (j) Test 10 [A(-), B(-), C(+), D(-)], (k) Test 11 [A(-), B(+), C(-), D(-)] and (l) Test 12 [A(+), B(-), C(-), D(-)]

NOTE: A :(+) Thyme, (-) Clove
B: (+) 1:2 (w/w) ratio of protein to oil, (-) 1:5
C: (+) Stirring speed at 1000 rpm, (-) 500 rpm
D: (+) With surfactant span 85, (-) without surfactant



(m) (n)

(o) (p)

Figure 3.1 Emulsion droplet microscopic pictures under 16 different treatments using optical microscopy at 100× for (m) Test 13 [A (+), B (+), C (+), D (-)], (n) Test 14 [A(-), B(+), C(+), D(+)], (o) Test 15 [A(+), B(+), C(-), D(+)] and (p) Test 16 [A(+), B(-), C(+), D(+)]

NOTE: A :(+) Thyme, (-) Clove
B: (+) 1:2 (w/w) ratio of protein to oil, (-) 1:5
C: (+) Stirring speed at 1000 rpm, (-) 500 rpm
D: (+) With surfactant span 85, (-) without surfactant

The coacervation method produced microcapsules from 4 to 85 µm with an average diameter of about 27 µm. We can calculate 'Effect' shown in Table 3.1 by adding a column "AB" to consider the interaction of A and B for outcome Y. The summation of each column is calculated by the corresponding 'plus' and 'minus' signs. Then, 'Effect' is calculated by dividing the summation by half of the number of test cases (8= 16/2).

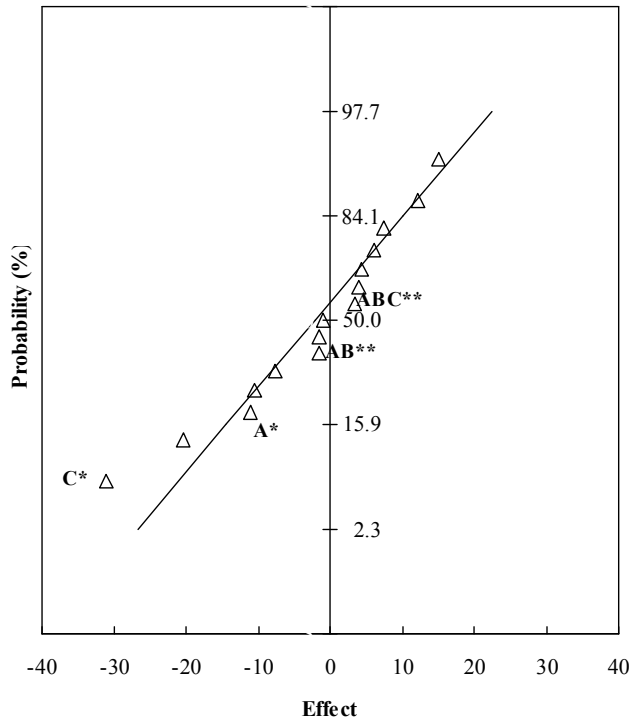


Figure 3.2 Effects of single and interaction between factors on the cumulative probability plot (C* P<0.001, A*P=0.017, AB** P=0.653, ABC** P=0.123)

Factors were arranged by the corresponding in ascending order (Table 3.3). The mean and the standard deviation of 'Effect' were calculated as -2.3 and 12.3. The 'Effect' of each factor and interaction were plotted, as shown in Figure 3.2. The distribution of the effects indicates that, if they are on the right side of the line, then the change of the level in each factor indicates an increase of size, and, if the effects are on the left side, they indicate a decrease of size. The points deviated from the line showed the abnormal effect on the size. The types of oil and agitation speeds played critical roles in governing the size of the microcapsules. Faster stirring speed has been used to produce smaller microcapsules (Ratcliffe et al., 1984; Tomlinson and Burger, 1985).

Table 3.2 Mean and standard deviation of the diameter

Test No.	Size (μm)±1	SD±0.1	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	ABCD
Test 1	24	29.2	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+
Test 2	11	6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Test 3	8	3.9	-	+	+	-	-	-	+	+	-	-	-	+	+	-	+
Test 4	30	12.9	+	+	-	-	+	-	-	-	-	+	-	-	+	+	+
Test 5	20	11.2	-	+	-	+	-	+	-	-	+	-	+	-	+	-	+
Test 6	4	2.2	+	-	+	-	-	+	-	-	+	-	-	+	-	+	+
Test 7	27	11.2	-	-	+	+	+	-	-	-	-	+	+	+	-	-	+
Test 8	51	20.1	+	-	-	+	-	-	+	+	-	-	+	-	-	+	+
Test 9	85	47.9	-	-	-	+	+	+	-	+	-	-	-	+	+	+	-
Test 10	6	3.6	-	-	+	-	+	-	+	-	+	-	+	-	+	+	-
Test 11	75	33.8	-	+	-	-	-	+	+	-	-	+	+	+	-	+	-
Test 12	34	15.1	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-
Test 13	5	3.2	+	+	+	-	+	+	-	+	-	-	+	-	-	-	-
Test 14	15	7.8	-	+	+	+	-	-	-	+	+	+	-	-	-	+	-
Test 15	21	12.1	+	+	-	+	+	-	+	-	+	-	-	+	-	-	-
Test 16	16	6.2	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-
Sum			-88	-62	-248	60	-14	48	-8	34	-162	32	26	98	-12	122	-82
Effect			-11.0	-7.8	-31.0	7.5	-1.8	6.0	-1.0	4.3	-20.3	4.0	3.3	12.3	-1.5	15.3	-10.3

NOTE: A :(+) Thyme, (-) Clove
B: (+) 1:2 (w/w) ratio of protein to oil, (-) 1:5
C: (+) Stirring speed at 1000 rpm, (-) 500 rpm
D: (+) With surfactant span 85, (-) without surfactant

Table 3.3 Cumulative probability of effects

Case	Effect	*m*	CDF (%)	p-value
C	-31	1	6.3%	<0.0001
BD	-20.4	2	12.5%	<0.0001
A	-11	3	18.8%	0.0173
ABCD	-10.4	4	25.0%	0.0255
B	-7.6	5	31.3%	0.1041
AB	-1.6	6	37.5%	0.6526
ACD	-1.5	7	43.8%	0.8570
AD	-1	8	50.0%	0.7463
ABC	3.4	9	56.3%	0.1226
CD	4	10	62.5%	0.0840
BC	4.4	11	68.8%	0.0715
AC	6	12	75.0%	0.0275
D	7.5	13	81.3%	0.0126
ABD	12.1	14	87.5%	0.0006
BCD	15.1	15	93.8%	0.0001
Mean	-2.1			
Std.	12.3			

NOTE: m: the rank by the corresponding by ascending order
CDF: Cumulative Density Probability Function

Table 3.2 showed that a higher agitation speed significantly decreases microcapsule size. Overall, the mean size of the clove droplets was larger than that of the thyme droplets. Most experiments indicated a high SD because of a wide range of size and inconsistent distribution of droplets. Droplets with larger diameters exhibited higher SD because they produced a wider range of distribution, resulting in over 20 SD.

3.3.1.1 The 1st Factor: Type of Oil

Overall, thyme red oil decreased the size of microcapsules, whereas clove bud oil increased the size as shown in Table 3.2. The viscosity of oils can contribute to this size difference. Decreasing viscosity brings out increased interfacial tension, causing larger capsules (Gallo et al., 1984; Burger et al., 1985; Tomlinson and Burger, 1985). The first factor, clove oil (-), contributed to larger sized microcapsules in our study. The viscosity of thyme red oil was 14 cps at 24 °C, but changed to 6.5 cps at 50 °C. For clove bud oil, the viscosity was 9 cps at 24 °C and 5 cps at 50 °C. Under our study, the type of oil was the most important variable affecting the size of the gelatin microcapsules ($p = 0.017$). When the thyme red oil was used, the size decreased, resulting in an average diameter of about 21 μm, whereas clove oil's average diameter was about 33 μm. When clove oil was used instead of thyme oil in the oil phase of the emulsion, the size increased significantly up to 85 μm. The viscosity of the intrinsic and extrinsic phases affected the particle size of the droplets. An increase in the viscosity leads to a smaller particle size because the increase in the internal phase viscosity hinders coalescence among particles. However, there was little difference in the viscosity between the two oils. Thus, it was desirable to investigate both surface tension and viscosity simultaneously prior to, and after, mixing (Sanghvi and Nairn, 1991).

3.3.1.2 The 2nd Factor: The Ratio of Gelatin to Oil at 1:2 or 1:5 (w/w)

The ratio of gelatin to oil (RGO) had less effect on size, but a higher RGO did contribute to a certain increase in the size of the microcapsules. In the present study, the average diameter at 1:5 w/w RGO was about 30μm, compared with a 1:2 RGO showing an average diameter of 24 μm. In other words, an increase in oil concentration to 1:5 RGO gives rise to an increase in both the mean size and the size distribution of the microcapsules. This effect can probably be attributed to a higher relative viscosity of the protein solution (Ishizaka and Koishi, 1981).

Thus, it can be explained that the interactions are more synergetic in Factor A and B because of relative viscosity of the solution. The interaction ABC point showed much deviation from the linear line, as shown in Figure. 3.2. However, the effects of AB and ABC interaction were not significant because of high p-value. Thus more replications are needed to investigate precise results.

3.3.1.3 The 3rd Factor: Agitation Speed

In order to obtain larger gelatin microcapsules, we selected relatively slow speeds (500 or 1000 rpm of agitation). In previous studies, a faster stirring speed has been used to produce smaller gelatin microcapsules (Torrado et al. 1988; Ratciffe et al., 1984; Burger et al., 1985). Table 3.2 showed that a faster agitation speed significantly decreased the size of the microcapsules. The average diameter at 1000 rpm was about 11 μm, while the average diameter at 500 rpm was about 43 μm. Thus, the agitation speed was the most significant factor affecting size and distribution ($p < 0.001$).

3.3.1.4 The 4th Factor: The Presence of Surfactant

The average diameter in the experiment with surfactants was about 31 μm, whereas the experiments without surfactant showed an average of 23 μm. Thus, the presence of surfactant

showed no significant impact on the size of the droplets. However, experiments with surfactant did show a lower SD than tests without surfactant, as shown in Table.3.2. This means that a more uniform diameter could be produced. From the previous research, the emulsion droplet size decreased with an increase in the surfactant concentration (Torrado et al., 1988; Chen et al., 1987; Ishizaka and Koishi, 1981). However, the effect of surfactant in this research increased the size. In order to verify this unforeseen effect of surfactant, there needs to be more replications using different shell materials and concentrations, and species of surfactants.

3.3.2 Morphological Analysis

After vacuum drying the microcapsules, the surface and shape of these solidified microcapsules were analyzed with scanning electron microscopy (Model ZESIS 1450). Figure 3.3 shows that microcapsules are physically attached to the fabric by vacuum drying. The natural bioadhesive property of gelatin makes attachment to the fabric possible without any chemical polymerization (Nelson, 2002). Microencapsulation coupled with vacuum drying has been shown to be an effective process of preventing aggregation of microcapsules, and it directly attaches microcapsules on the fabric, as shown in Figure 3.3. However, this method showed a low quantity of microcapsules on the fabric. When a higher concentration of microcapsules is applied to the fabric, it will have a tendency to aggregate. Microcapsules made up of single spherical units of less than 30 μm in diameter stayed individually on the fiber. The external surface of the microcapsules was quite smooth and demonstrated a regular spherical shape. However, larger microcapsules over 30 μm tends to rupture or aggregate. As a rule, bigger microcapsules contained higher oil loading, but a bursting appearance was observed. Chan et al. (2009) and Maji et al. (2007) have reported similar types of results with SEM photomicrography and

Tomasin et al. (1996) have shown that the condition of vacuum drying influences the morphology of microcapsules loaded with volatile oils.

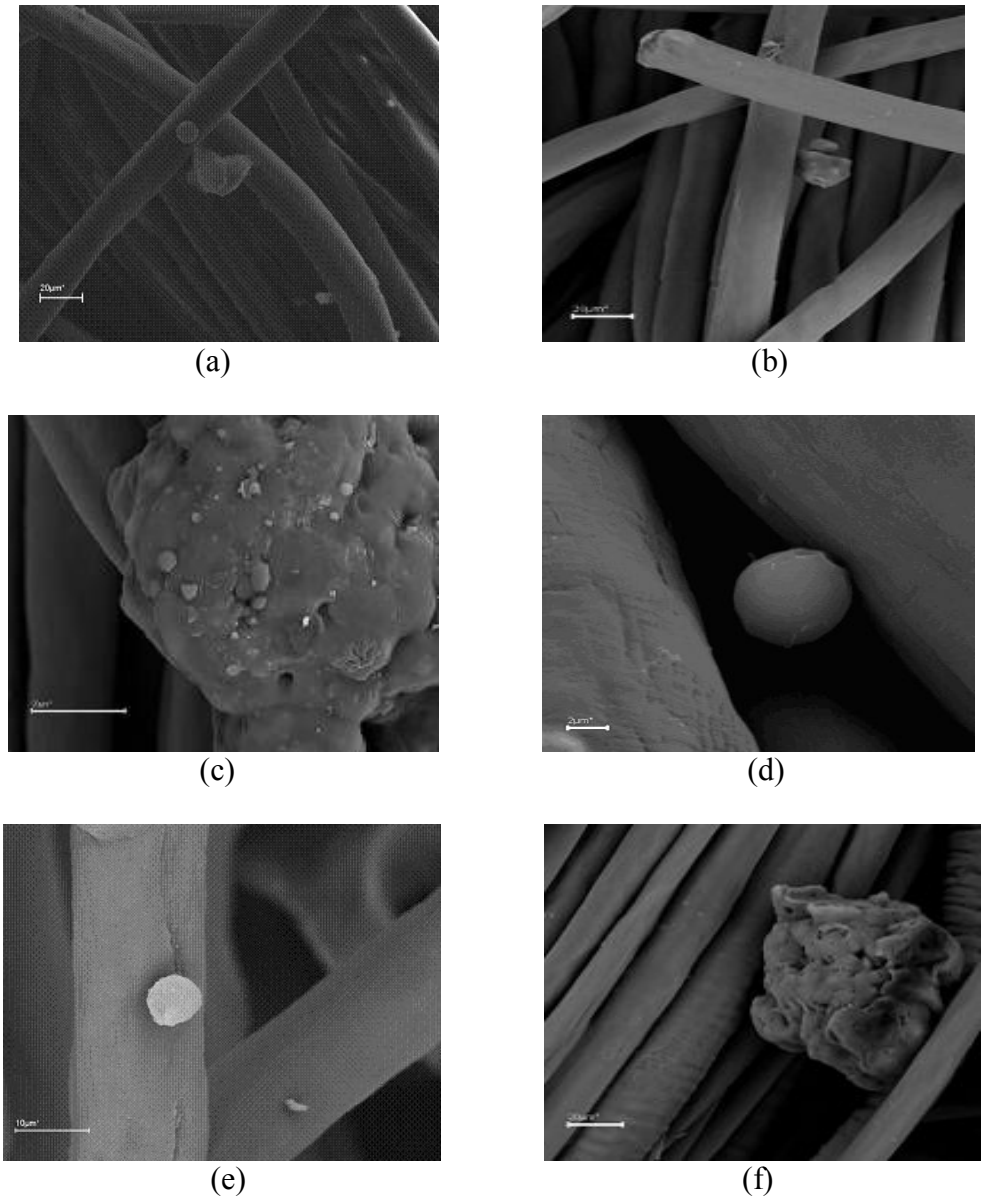


Figure 3.3 SEM images of microcapsules loaded with clove or thyme oils using vacuum drying on the fabric for (a) Test 1 [A(-), B(-), C(-), D(-)], (b) Test 4 [A(+), B(+), C(-), D(-)], (c) Test 5[A (-), B (+), C (-), D (+)], (d) Test 6 [A(+), B(-), C(+), D(-)], (e) Test 7 [A(-), B(-), C(+), D(+)] and (f) Test 8 [A(+), B(-), C(-), D(+)]

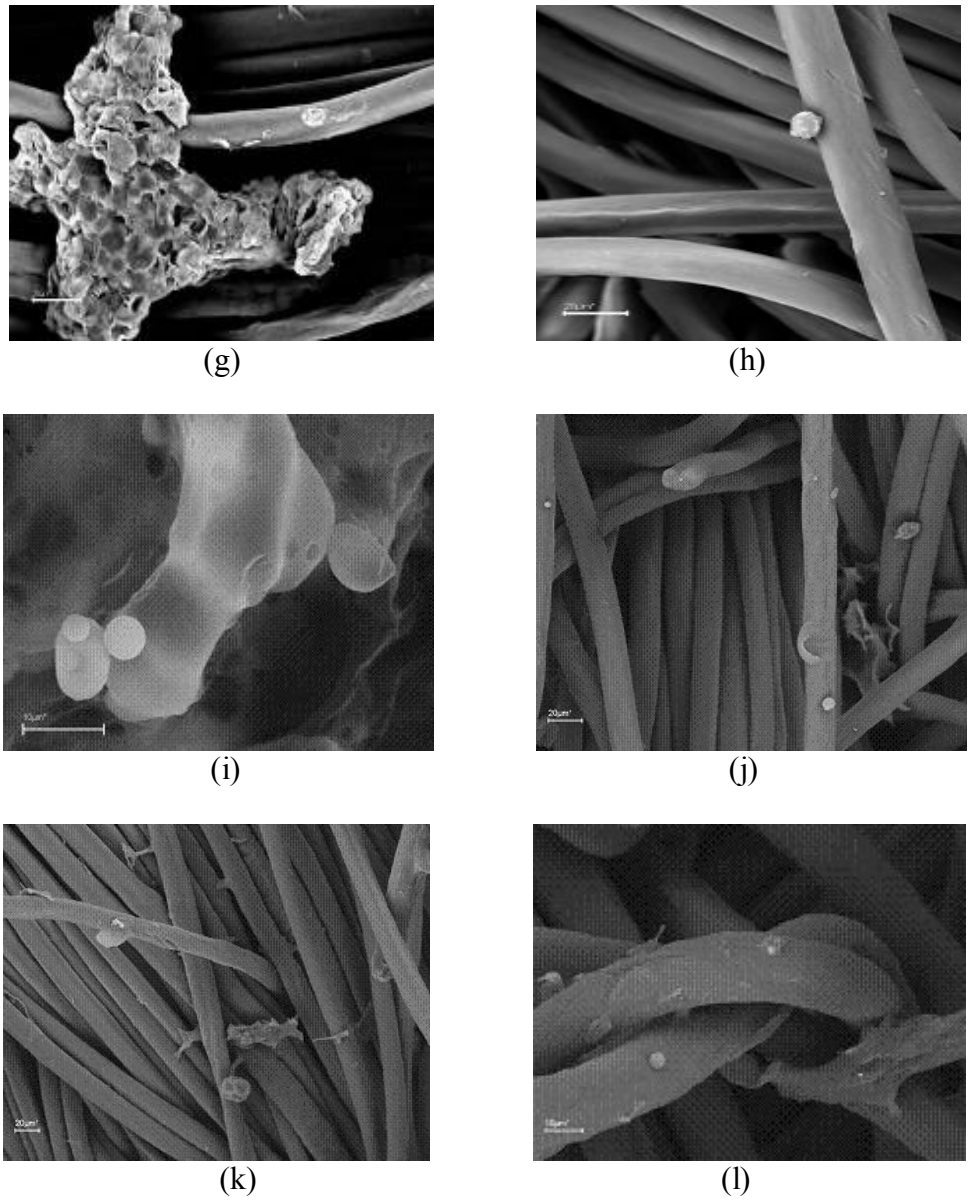


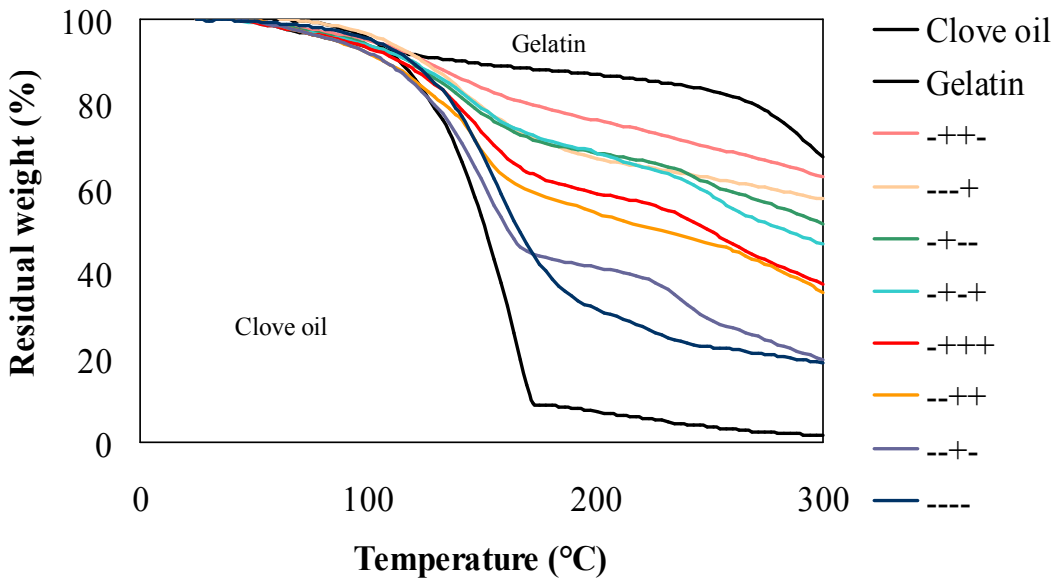
Figure 3.3 SEM images of microcapsules loaded with clove or thyme oils using vacuum drying on the fabric for (g) Test 9 [A(-), B(-), C(-), D(+)], (h) Test 10 [A(-), B(-), C(+), D(-)], (i) Test 11 [A(-), B(+), C(-), D(-)], (j) Test 12 [A(+), B(-), C(-), D(-)], (k) Test 15 [A(+), B(+), C(-), D(+)] and (l) Test 16 [A(+), B(-), C(+), D(+)]

NOTE: A :(+) Thyme, (-) Clove
B: (+) 1:2 (w/w) ratio of protein to oil, (-) 1:5
C: (+) Stirring speed at 1000 rpm, (-) 500 rpm
D: (+) With surfactant span 85, (-) without surfactant

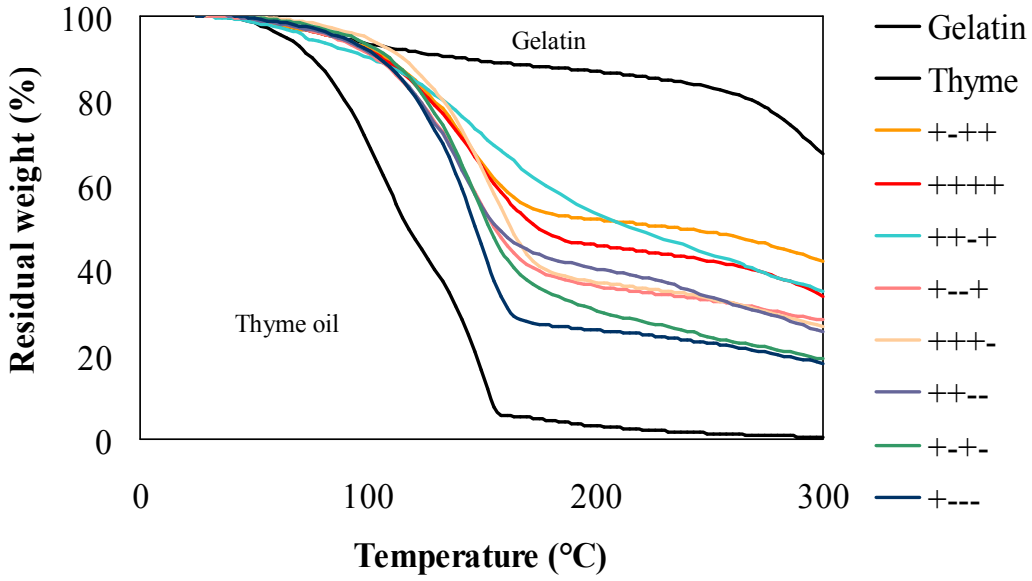
This points out that bigger microcapsules loaded with higher oil content appeared oily and easily agglomerated because of weak shell thickness, whereas smaller microcapsules with relatively lower oil loading can survive through high pressure by the vacuum drying process.

3.3.3 Thermogravimetric Analysis (TGA) to Determine Oil Loading

TGA was used to measure the changes in the weight of microcapsules under thermal heating. Thyme and clove oils exhibited different weight loss patterns with no residuals under 300 °C. The colored lines in Figure 3.4 showed the microcapsules containing thyme or clove oil under 16 different treatments. Pure gelatin lost about 30% of its original weight at 300 °C. Thus, the final weight indicated the residual gelatin weight because oil lost its weight before 300 °C. Microcapsules loaded with thyme oil showed the same pattern of weight loss, whereas pure thyme oil lost its weight mostly at around 160 °C. However, microcapsules containing clove oil lost their weight around 180 °C, as shown in Figure. 3.4. Both microcapsules loaded with clove and thyme oils had ash at the end of TGA testing, indicating residual. This fact demonstrated that thyme and clove oils were the core materials of the gelatin microcapsules. Our study indicated that treatments under conditions of 1:5 RGO, and without surfactant, showed more weight loss than others. In other words, they contained more oils than others. As mentioned above in the size analysis, microcapsules under these conditions demonstrated a larger size than under other conditions. This confirms that bigger microcapsules contain more oil content. However, they can easily burst because of weak shell thickness and capacities of oil weight depend upon fiber types. Of the oils tested, thyme red oil seems to be more consistent in oil loading and more promising to give a high oil yield. The relative oil weights of the microcapsule powders ranged from 50 to 80% w/w for the thyme oil, and 30 to 80% w/w for clove oil.



(a)



(b)

Figure 3.4 Thermogravimetric analysis of microcapsule powder under 16 treatments (a) TGA results of microcapsules loaded with clove oils and (b) TGA results of microcapsules loaded with thyme oil

3.4 Conclusions

The outcome of this study showed that the coacervation microencapsulation method was useful for entrapping thyme and clove oils into the microcapsules enclosed with gelatin within 4 to 85 μm. Through the factorial design method, agitation speed and type of oil (oil viscosity) were the critical variables in controlling the size of the microcapsules. In addition, microencapsulation coupled with vacuum drying can be an effective process to prevent aggregation of microcapsules and to attach microcapsules mechanically on the fabric without any binder. However, microcapsules under 30 μm can be attached successfully as a single unit without aggregation on the fabric by vacuum drying process. TGA testing showed that microcapsules loaded with thyme oil exhibited more consistent or narrow ranged of oil loading (50 to 80% w/w oil content). Conditions with 1:5 RGO, and without surfactant, showed higher oil loading in both TGA tests of microcapsules loaded with clove and thyme oils. Furthermore detailed studies of surfactant and vacuum drying processes are required. Additionally interactions between factors are needed to get more precise results.

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CHAPTER 4

ACARICIDAL ACTIVITY OF ESSENTIAL OILS AGAINST HOUSE DUST MITES USING DIRECT CONTACT BIOASSAYS AND FABRIC TREATED WITH MICROCAPSULES LOADED WITH CLOVE AND THYME OILS

4.1 Introduction

While presently available commercial synthetic acaricides may be highly effective and applied in low concentrations at an affordable price, they have exposed the environment and humans to toxic influences. These synthetic insecticides, like pyrethroids that perform on Na^+ channels of the nerve cell membrane, can have harmful effects that are toxic to economically important insects and aqueous microorganisms (Loucif-Ayad et al., 2008). Besides, these synthetic chemicals are hard to break down, and they build up quickly to a toxic level, which may bring about health risks in the environment.

In the United States, in accordance with the government actions of the Environmental Protection Agency and the Food Quality Protection Act, restrictions have been placed upon the sale of many commercial pyrethrum-based insecticides. Thus, a major market opportunity is growing for low-risk synthetic acaricides (Isman, 1999; 2000). Additionally, natural essential oils from plants would be another source for controlling house dust mites (HDM) because they contain effective phenolic monoterpenoids and they have been found generally safe for use in cosmetic fragrances and food flavoring additives. The use of such essential oils often is not restricted by the Flavor and Extracts Manufacturers Association (FEMA) by generally regarded

as safe (GRAS) (Ashurst, 1995). Additionally, plant essential oils are used in numerous products such as antiseptics, disinfectants, and as antimicrobials. The volatile characteristic of monoterpenoids in the essential oils displays antimicrobial and acaricidal activities (Coats et al., 1991; Kwon and Ahn, 2002; Kim et al., 2003). These compounds have been proven to be relatively nontoxic to humans with little negative impact on non-target environments or humans (Isman, 1999; 2001).

The studied natural acaricidal compounds that work against HDM include: butylidenephthalide from the bulb of *Cnidium officinale Makino* (Kwon and Ahn, 2002); perilla oil (Watanabe et al., 1989); and leaf oil from *Lauraceae* trees (Furuno et al., 1994). Furthermore, the most effective oils against HDM (*Dermatophagoides farinae, Der. f*) among 56 natural plant essential oils were clove, thyme, horseradish, and coriander oils. When administered through direct contact and fumigation, thyme red and clove bud oils caused up to 100% mortality of *Der. f* at 0.07 mg cm^{-2}(Kim, et al, 2004). Saad et al. (2006) reported that the value of LC_{50} clove oil was 29.78 µg, compared to thyme oil of 488.65 µg. In addition, other researchers reported that clove bud oil (Kim et al., 2004; El-zemity et al., 2006); thymol and cinnamaldehyde (El-zemity et al., 2006; Deans et al., 1995); and *Cnidium officinale rhizome* extracts (Kwon and Ahn, 2002) exhibited efficient mortality against HDM. As a consequence of this, many studies have focused on natural plant essential oils as potential and safe agents in lieu of commercial pest control agents. In light of this, the objective of this research was to investigate the acaricidal activity against HDM (*Der. f*) of clove bud and thyme red oil through direct contact bioassays and mortality tests according to AATCC methods 187-2007.

4.2 Materials and methods

4.2.1 Materials

Clove bud oil and thyme red oil were purchased from the Sigma and Aldrich Co. USA. Tangletrap sticky coating was purchased from Tanglefoot® Co. USA. Nylon mesh with a pore size under 50 μm was purchased from Smallparts® CO. USA. House dust mites were donated by the Insect Control Research Inc. MD, USA.

4.2.2 Gas Chromatography-Mass Spectrometry (**GC-MS**) Analysis of Essential Oils

GC-MS was used to identify species and compositions of monoterpenoids in clove bud and thyme red oils. Shimadzu GC-MS with column no. DB-5 (optima-5) was set in order to evaluate the monoterpenoids' toxic activities to HDM. The column was a DB-5 (optima-5) with the following specifications: 30 m length, a 0.25 mm column held at 50 °C, with a planned temperature increase from 50 to 280 °C at 3 °C per minute to 250 °C, and afterward, held for 5 min. The next step was to increase the temperature by 2 °C per minute to 280 °C, and then, held for 3 min. 0.1 μg of clove or thyme oil was injected at 250 °C and the carrier gas was nitrogen at 30 cm per second under a pressure of 99.8 KPa. The detector temperature was 280 °C with a 50 ml min^{-1} of H_2 flow rate, 400 ml min^{-1} of air flow rate. Data were analyzed by GC solution software (Shimadzu) and previous studies (Formacek and Kubeczka, 1982).

4.2.3 Direct Contact Bioassays

Acarcidal activity were conducted according to the method conducted by Park and Shin (2005) using a filter paper contact bioassay to HDM (*Der. f*). About a 3.2 mg amount (0.05 mg cm^{-2}) of clove bud or thyme red oil was applied to cut filter papers of 9 cm in diameter (Whatman no. 1441 125. 125 mm diameter). After drying in the fume hood for 3 hours, each filter paper was set

on the bottom of a 10 cm diameter Petri dish. All treatments and controls received a 50 mg diet of a mixture of albumin and yeast for fifty house dust mites (*Der. f*). Each specimen was covered with a nylon mesh with a less than 50 μm pore size, and the edge of the Petri dishes was brushed with a sticky gel to prevent the escape of HDM. Fifty house dust mites (*Der. f*) were randomly placed on each specimen with an open-lid. These specimens were maintained at 25 °C and over 65% relative humidity and were replicated two times. Mortalities of HDM were counted at 72hrs after treatment under an optical microscope (20 x) using micro-sized forceps. HDM were regarded as dead if they did not move or if they had turned over.

4.2.4 Mortality Study of HDM by Contact with Microcapsules Loaded with Essential Oils

The acaricidal activity on the HDM was evaluated for the textiles treated with microcapsules loaded with clove or thyme oil according to AATCC test method 194-2007

4.2.4.1 Rearing of HDM in the laboratory

HDM colonies were kept at 25 ± 1°C and over 65% relative humidity on 50 mg of albumin powder with dried yeast powder in the bottom of a 10 cm diameter Petri dish covered with a nylon mesh with a pore size smaller than 50 μm.

4.2.4.2 Test Setup

50 mg of nutrient mixture (albumin powder and dried yeast powder) and microcapsules loaded with essential oils were placed on two specimens. Simultaneously, only 50 mg of nutrient mixture was distributed on the acrylic fabric for the controls. Then, the edges of the dishes were coated with the sticky gel and covered with a nylon mesh with a pore size of under 50 μm to prevent mites from escaping, as shown in Figure 4.1. The next step was placing fifty randomly selected house dust mites (*Der. f*) in each specimen using micro-sized forceps. Then, all specimens were placed in a chamber regulated at the optimal conditions for growth of 25 ± 1 °C

and over 65% relative humidity. The following equation was used to determine the mortality of the HDM after 72 hours with two replications as

$$R = \frac{(A - B)}{A} \times 100 \tag{4.1}$$

where R is the percentage of the reduction of HDM (*Der. f*) compared to the control specimen. A and B are the average numbers of HDM (*Der. f*) found on the control specimen and the test specimen respectively. After 3 days of incubation under optimal conditions, the total number of HDM was counted to determine their mortality.

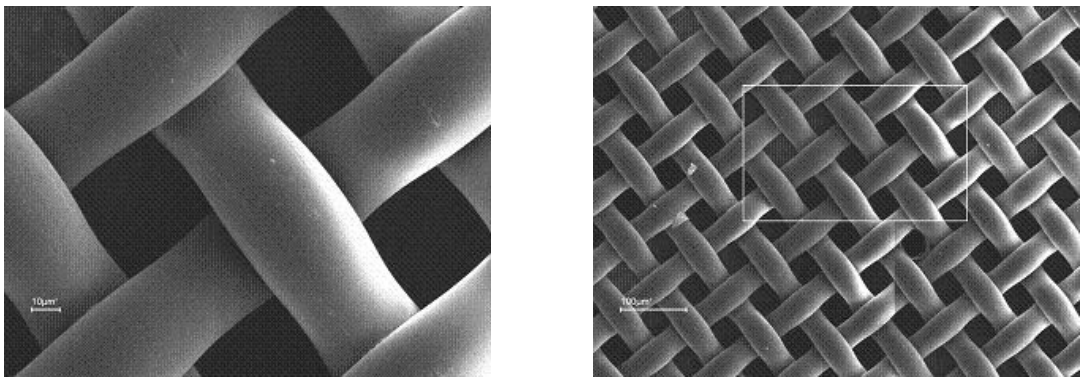


Figure 4.1 Nylon mesh with a pore size smaller than 50 μm to prevent HDM escaping

4.3 Results and discussion

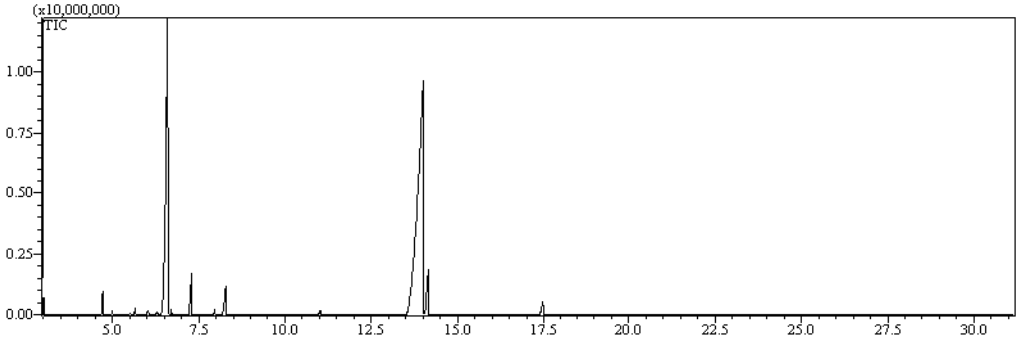
4.3.1 The Chemical Analysis of Clove and Thyme Oils

The objective of GC-MS was to identify the monoterpenoids and the chemical composition of clove bud and thyme red oils. From GC-MS analysis, thymol was the main monoterpenoid, comprising 54% of thyme red oil. It has been indicated to be effective as an antimicrobial and antifungal agent (Pierce, 1999). The second largest constituent, *para-cymene,* also exhibited

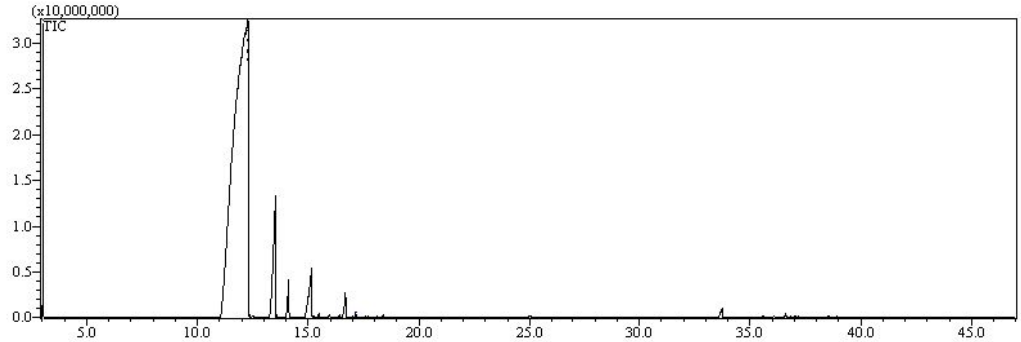
antimicrobial activity (Deans et al., 1995). Eugenol and *B*-caryophyllene were the main phenolic monoterpenoids in the clove bud oil comprising 84% and 12%, respectively (Formacek and Kubeczka, 1982), as shown in Table 4.1 and Figure. 4.2..

Table 4.1 GC –MS analysis of the composition of thyme red and clove bud oils

Thyme red oil Main constituents (%)	Clove bud oil Main constituents (%)
p-Cymene (33.34)	Eugenol (83.66)
r-Terpinene (2.78)	*B*-caryophyllene (12.25)
Linalool (2.14)	Caryophyllene oxide (1.0)
Thymol (54.41)	Eugenol acetate (1.81)
Carvacrol (3.05)	



(a)



(b)

Figure 4.2 Gas chromatograms of (a) thyme red oil (b) clove bed oil

4.3.2 Direct Contact Bioassay

The efficiency of monoterpenoids in the essential oils was observed for the degree of their mortality against HDM (*Der. f*). Studies have reported that there are no significant toxicity differences between *Der. f* and *Der. p* species (Kim et al., 2003; Kwon and Ahn, 2002) and a little difference in their appearance with four hairs on the abdomen in the *Der. f*. Thus, we standardized on the *Der. f* species, which prevails in the U.S. After 72 hours, mortality was determined from the setup shown in Figure 4.3. Both thyme red and clove bud oils were toxic to HDM (*Der. f*) resulting in 100% mortality. Eugenol from clove bud oil has been identified for acaricidal activity by direct contact bioassay (Park and Shin, 2005). In our direct contact test with open lids, clove bud and thyme red were the most effective acaricidal agents on HDM (*Der. f*) with up to 100% mortality at 0.05 mg cm^{-2}, whereas a control maintained the starting number of HDM. Thymol and *para*-cymene (El-zemity et al., 2006; Deans et al., 1995) from thyme red oil and eugenol and *β-caryophyllene* (Kim et al, 2003; El-Zemity et al, 2006) from clove bud oil were the main monoterpenoids to produce acaricidal activity on HDM (*Der. f*).

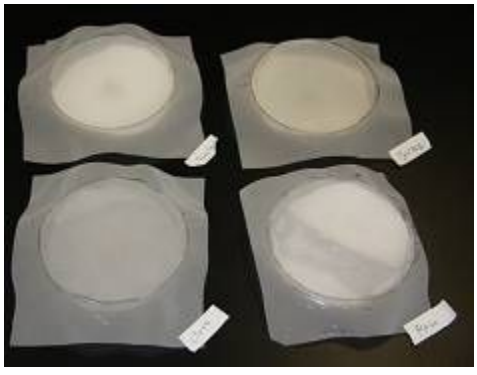


Figure 4.3 Direct contact bioassays of essential oils in HDM

Kim et al. (2004) reported four types of death characteristics of HDM. The HDM's poisoning symptoms can explain the effect of natural terpenoids and synthetic pyrethroids; death

induced by natural essential oils displayed a knockdown type (Furuno et al., 1989) or death related to desiccation (Sanchez-Ramos and Castanera, 2001). However, other synthetic chemicals exhibited death related to uncoordinated behavior (Kim, 2001) or death involving a hollow surface feature on the back with idiosoma which located in the genital (Ignatowiz, 1981). For example, currently applied synthetics pyrethroids, using N,N-diethyl-m-toluamide (DEET) and benzyl benzoate as repellents, induced uncoordinated behavior with desiccation on HDMs (Kim et al, 2003). In our study, the mites exposed to clove and thyme oils, the natural plant extracts, showed a leg movement patterns similar to death induced by natural causes. Dead HDM, as shown in Figure 4.4, demonstrated the knockdown type with forwarding leg movements induced by natural plant sources.

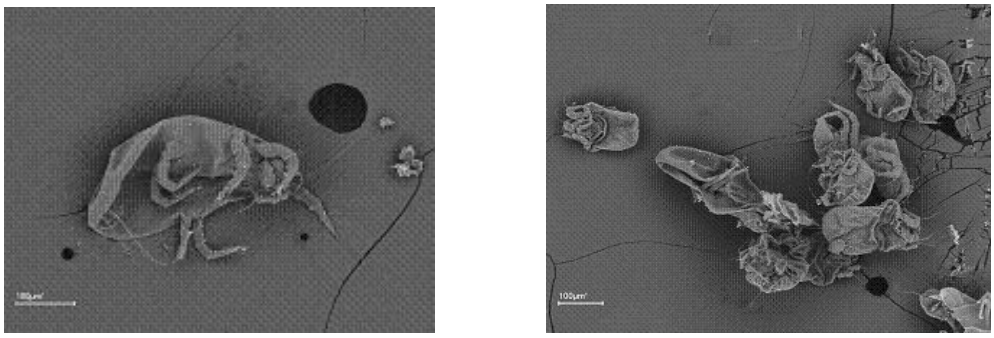
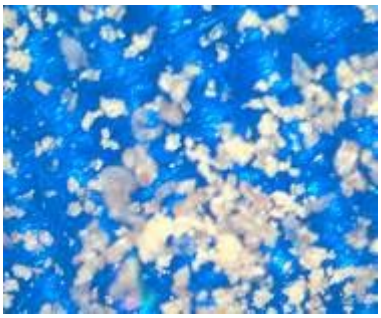


Figure 4.4 The characteristics of dead HDM induced by natural monoterpenoids, clove and thyme oils.

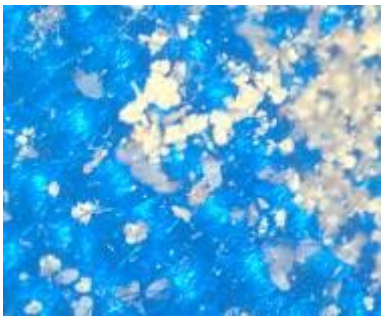
4.3.3 Mortality Tests against HDM on the Fabric treated with Microcapsules that Contained Clove bud or Thyme red oil

This test was used to assess the acaricidal activity against HDM in a long-term examination for textiles with attached microcapsules containing clove bud or thyme red oil. We selected the conditions of microcapsules loaded with thyme oil produced at 1000 rpm, 1:2 RGO, without surfactant; microcapsules loaded with clove oil produced at 1000 rpm, 1:2 RGO, without

surfactant. The average diameter of microcapsules with these conditions were under 8 μm and they showed different oil loading that microcapsules loaded with clove oil showed about 40%, whereas microcapsules loaded with thyme oil showed 60%, as shown in Table 3.2 and Figure 3.4. In each specimen, the mortality of the HDM after 72 hours, with two replications, was determined. Each specimen reduced the number of live house dust mites, shown in Table 4.2. The specimen with clove microcapsules was more effective at reducing the number of HDM than the specimen with thyme microcapsules, showing up to 94% mortality. Similar to the above mentioned direct contact test, dead HDM symptoms were characterized by immobility or an underside shape showing the symptoms of knockdown type death with the forwarding leg movements shown in Figure 4.4.



(a) Treated clove microcapsules



(b) Treated thyme microcapsules

Figure 4.5 Optical microscopy (20 × images) of dead HDM for (a) treated clove microcapsules produced at 1000 rpm of stirring speed, 1:2 (w/w) ratio of gelatin to protein without surfactant and (b) treated thyme microcapsules produced at 1000 rpm, 1:2 (w/w) without surfactant

The chemical structure of an essential oil is important for the activity of certain plant compounds against pests (Rice and Coats, 1994; Holly and Patel, 2005). This study indicated that the acaricidal ability of selected phenolic monoterpenoids such as eugenol from clove oil would be enhanced through the presence of hydroxyl groups because the increased vapor pressure of oils due to this structure enhanced penetration into insects' body (Rice and Coats,

1994). Besides, eugenol in the clove oil has antioxidant activity because it has the most effective and strongest free radical scavenging function from the their chemical composition containing ortho-position electron (Tomaino et al., 2005; Dorman et al., 2000). Thyme oil also exhibits antioxidant activity. However, thyme oil has more reactive than clove oil because of more active methyl group and para position. Therefore, more hydroxyl groups and anti-oxidant property related to phenolic compounds in the eugenol (clove oil) played a critical role in reducing the population of live HDM (Kim et al., 2004; Regnault-Roger and Hamraoui, 1995). Furthermore, clove oil was revealed to be one of the plant derived phenylpropanoids that showed a structural advantage in defense functions against microbial attack and herbivores (Hahlbrock and Scheel, 1989; Varel et al., 2004). In the present study, clove bud oil, containing the phenolic monoterpenoid of eugenol, exhibited more powerful acaricidal activity against *Der. f* over thyme red oil and required a less oil concentration. However, the difference in mortality rate between clove and thyme treatments is not significant ($p = 0.4974$). Therefore, both clove bud oil and thyme oil can control the HDM as natural acaricides.

Table 4.2 Acaricidal activity against HDM (*Der. f*) on the textile treated with clove or thyme microcapsules after 72hr

Specimen	Initial population of HDM	Residual number of HDM (1^{st})	Residual number of HDM (2^{nd})	Mortality (%)
Clove microcapsules	50 ± 1	5	3	94
Thyme microcapsules	50 ± 1	10	8	84
Control	50 ± 1	51	47	2

NOTE: 1^{st} : the first test
2^{nd} : the second test

The fabric treated with microcapsules loaded with essential oils exhibited a lower mortality on HDM than the direct contact tests. This could be explained by the loss of the bioactive principle during coacervation and drying. Clove oil can remain stable and contain bioactive power against HDM due to its antioxidant property from more phenolic compound than thymol and *para cymene*, and enhanced bioactivity through more hydroxyl groups. Thus, clove oil may be preferable for long-term use in terms of environmental exposure.

4.4 Conclusions

Highly complex mixtures of terpenoids, particularly monoterpenoids, and related phenols exist in plant essential oils (Coat et al., 1991; Kong et al., 2007). These can contribute to a variety of biological activities in the propagation and repulsion of HDM. However, little work has been done to study the effect of essential oils and their constituents on the textiles for containing HDM.

Our results showed that it was possible to control HDM using microencapsulated clove and thyme oils containing specific phenolic monoterpenoids such as eugenol and thymol. Among the two encapsulated essential oils, constituents of clove bud oil could be an efficient agent in reducing the population of live house dust mites (*Der. f*) because of the former's propertied anti-oxidation from its allyl-structure and natural defense functions as phenylpropanoids against herbivores. During the production of microcapsules, clove bud oil can maintain stability due to better antioxidant property and lose less phenolic properties affecting the mortality of HDM than thyme red oil. From our experiments, HDM showed the symptoms of knockdown type death with forwarding leg movements induced by natural plant sources in both clove and thyme oils. The results of this study and previous studies (Kim et al, 2003; El-zemity et al., 2006) demonstrated that clove bud oil could be an effective natural alternative to synthetic chemicals in

reducing the population of HDM. This research supports the use of essential oils and their major components as eco-friendly agents for the control of HDM (*Der. f*). For the practical use of essential oils, it is necessary to examine the safety of these materials to humans and to develop formulations to improve their efficacy and stability.

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CHAPTER 5

CONCLUSION AND FUTURE WORKS

This study demonstrated that the clove and thyme essential oils have potential to serve as natural acaricides for controlling house dust mites in the indoor environments. Additionally, the coacervation microencapsulation method was useful for entrapping volatile thyme and clove oils into the microcapsules enclosed with gelatin for long-term uses. The size range of microcapsules was from 4 to 85 μm. Through the factorial design, we recognized the agitation speed and oil viscosity played critical role in governing the size of microcapsules. The higher viscosity and agitation speed produced smaller microcapsules. The oil loading of encapsulated powders was also examined by the thermogravimetric analysis. Microcapsules from thyme red oil showed more consistency in oil loading than microcapsules from clove oil. Microencapsulation coupled with vacuum drying can be an effective process to prevent aggregation of small size microcapsules and to attach microcapsules mechanically on the fabric without any binder. The morphology of larger microcapsules, containing higher oil loading and measuring over 30 μm, showed a ruptured and aggregated appearance on the fiber during the vacuum drying process, whereas smaller microcapsules under 30 μm could survive on the fabric.

Through mortality test on the fabric attached with microcapsules loaded with clove or thyme oils against HDM (*Der. f*), we concluded that clove oil containing phenolic monoterpenoids (approximately 84% eugenol) was more effective in reducing the number of live HDM (up to 94 %) than thyme oil due to the former's propertied antioxidant activity from its

chemical composition, enhanced vapor pressure through more the hydroxyl groups from phenolic monoterpenoids, and natural phenylpropanoid properties against herbivores.

Further detailed studies of surfactant and vacuum drying processes are needed since this method was limited to the low quantity and small size of microcapsules. There needs to be more study using confocal microscope to investigate microcapsules inside fabric. Additionally, structure-activity of the certain monoterpenoids in other essential oils such as horseradish and coriander oils against HDM can be investigated to develop the natural and safer acaricides.

This research presented the use of the essential oils and their major components as eco-friendly agents for controlling HDM that are consumer and environmentally friendly replacements for synthetic acaricides in terms of their impact on the environment and human health.