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Use Of Different Ripening Inhibitors To Enhance Antimicrobial Activity Of Essential Oil Nanoemulsion

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USE OF DIFFERENT RIPENING INHIBITORS TO ENHANCE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL NANOEMULSION

A Thesis Presented by

VICTOR RYU

Submitted to Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Food Science
USE OF DIFFERENT RIPENING INHIBITORS TO ENHANCE
ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL
NANOEMULSION

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By
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ABSTRACT

USE OF DIFFERENT RIPENING INHIBITORS TO ENHANCE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL NANOEMULSION

SEPTEMBER 2017

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Essential oils are complex mixture of both volatile and non-volatile compounds that are extracted from plants through different methods of distillation. Some of the compounds in the essential oil are known to exhibit antimicrobial activity. However, as they are reactive, volatile, and has low water solubility in nature, appropriate delivery system is required. The objective of this study was to formulate antimicrobial nanoemulsion using spontaneous emulsification method with thyme oil with different ripening inhibitors using low energy phase inversion method and test whether they exhibit prolonged stability and sufficient antimicrobial activity.

Oil-in-water antimicrobial nanoemulsions (10 wt%) were formed by titrating a mixture of essential oil and ripening inhibitors into 5mM sodium citrate buffer (pH 3.5). Stable nanoemulsions containing small droplets ($d < 70$ nm) were formed at essential oil-to-ripening inhibitor mass ratios of 4:6. The antimicrobial activity of the nanoemulsions decreased with increasing ripening inhibitor concentration, which was attributed to a
reduction in the amount of hydrophobic antimicrobial constituents transferred to the bacterial cell membranes. The antimicrobial activity of the nanoemulsions also depended on the nature of the ripening inhibitor used: palm ≈ corn > canola > coconut. The origin of this effect was again attributed to differences in the partitioning of antimicrobial agents between the oil droplets and bacterial cell membranes. Information about the partitioning of antimicrobial agents between different hydrophobic domains in a system was obtained using dialysis and chromatography. This study suggested that those oil phases that facilitated the greatest transfer of antimicrobial agents also had the highest antimicrobial efficacy. These results indicated that the type and amount of ripening inhibitor in the oil phase had to be optimized to produce physically stable nanoemulsions with antimicrobial activity.
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CHAPTER 1

LITERATURE REVIEW

The attribute of naturally occurring antimicrobial compositions in essential oils and their activity against foodborne pathogenic microorganism will be discussed. Also, the use of appropriate delivery system utilizing nanoemulsion to encapsulate essential oil will be reviewed.

1.1. Essential Oil

From early as 1960s, the industries have put efforts toward making food more natural and less processed due to higher demand from consumers for natural food and due to released studies about the toxicity of synthetic antimicrobial or antioxidant food additives such as benzoate, sodium sorbate, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) (Carocho et al., 2004, Hyldgaard et al., 2012). Therefore, antimicrobial, antioxidant, and antiseptic, antifungal, and antiviral property of essential oils have been receiving attentions (Burt, 2004). Essential oils are secondary metabolites usually composed of complex volatiles and non-volatiles (Dorman and Deans, 2000, Bakkali et al., 2008). In plant they are used as dispersant to attract insects for pollination (Bakkali et al., 2008). Also, they are used as defense mechanism against other plants, insects, animals, and microorganism as some of them act as allelochemicals, antifeedant, and antimicrobials (Bakkali et al., 2008, Hyldgaard et al., 2012). In vitro and in vivo studies have shown that many essential oils have strong potency against pathogenic bacteria and fungi (Dorman and Deans, 2000, Gutierrez et al., 2009, Sienkiewicz et al., 2012). Relatively strong antimicrobial activity was found in essential
oils extracted from thyme and oregano which are additives categorized to be generally recognized as safe by FDA (Ultee et al., 1999, Lambert et al., 2001, Burt, 2004, Substance Generally Recognized as Safe, 2016). The antimicrobial activity of essential oil is determined by the major components constituted of terpenes and terpenoids with aromatic and aliphatic groups which takes 20 to 70% of the essential oil composition (Bakkali et al., 2008). However, in some cases, minor components synergistically enhance the antimicrobial activity of the major components (Hyldgaard et al., 2012).


Among these components, the ones that have both the hydroxyl group and phenolic ring were the most effective antimicrobial agents (Figure 1.1) (Ultee et al., 2002). The mechanism of antimicrobial activity of each component is listed below.

1.1.1. Thymol

Thymol is one of the major antimicrobial components of thyme and oregano essential oils that exhibit strong antimicrobial activity by disrupting the cell membrane and binding to periplasmic proteins (Pasqua et al., 2010, Lambert et al., 2001). Thymol was reported to disintegrate the outer membrane and cause leakage of ATP in Escherchia coli O157:H7 and Salmonella typhimurium (Helander et al., 1998). Also, thymol was found to upregulate outer membrane protein and impair ATP synthesis through citrate metabolic pathway of Salmonella enterica serovar Thompson MCV1 as it could down regulate enzymes involved in the path way when they were exposed to sub lethal dose of thymol (Pasqua et al., 2010).
1.1.2. Eugenol

Eugenol is one of the major components in clove essential oil (Hyldgaard et al., 2012). It was found to cause cell lysis of *E. coli* O157:H7 and cause deterioration of cell wall of *Pseudomonas fluorescense* when essential oil was added at higher concentration than the minimum inhibitory concentration (MIC) (Pasqua et al., 2007). Eugenol was found to cause less alteration in cell wall composition of saturated and unsaturated fatty acid relative to other essential oils. Therefore, it was suggested to be using different pathways to inhibit cell growth, relative to other antimicrobial components, by binding to proteins (Pasqua et al., 2007)
Figure 1.1. Chemical structure of selected essential oil components (Hyldgaard et al., 2012)
1.1.3. Carvacrol

Carvacrol is a naturally occurring monoterpenoid that has alcohol as functional group. It was reported when both gram negative and gram positive bacteria were exposed to carvacrol, increase in membrane permeability was observed by leakage of $H^+$ and $K^+$, which was suggested to be due to disruption of van der Waals interaction by carvacrol in cell membrane as it occupy more space between lipid acyl chain leading to dissipation of membrane potential (Helander et al., 1998, Ultee et al., 1999, Ultee et al., 2000 (b), Lambert et al., 2001). Therefore, depletion of inner ATP pool occurred as the cell loses the ability to synthesize ATP, which eventually lead to cell death (Helander et al., 1998, Ultee et al., 1999). Helander et al. (1998) concludes that antimicrobial activity of carvacrol is due to disintegration of the outer membrane with observation of release of lipopolysaccharide (LPS) and leakage of ATP in *Escherchia coli* O157:H7 and *Salmonella typhimurium*. However, Ultee et al. (2002) reported that there is more factor involved in antimicrobial activity of cell in addition to expansion and disruption of cell membrane as non-antimicrobial component ρ-cymene, which has similar structure with carvacrol, was also found to expand and disrupt the cell membrane. Therefore, Ultee et al. (2002) asserts that antimicrobial activity of a compound is influenced molecular structure such as hydroxyl group and delocalized electrons at $\alpha$-$\beta$ double bond. They suggest that this structure enables the cycle of release of proton in cytoplasm by diffusion of un-dissociated carvacrol into the membrane, and also the release of potassium ion or other cations outside the membrane as carvacrol act as ion transporter (Figure 1.2) (Ultee et al., 2002). Also, carvacrol was found to inhibit flagellin synthesis in *E. coli* O157:H7 by inducing heat shock proteins (HSP 60) and was found to stop the flagellar motion that
Figure 1.2. Mechanism of how carvacrol act as an ion transporter. The undissociated form of carvacrol diffuses into the cell membrane and releases proton. The dissociated form of carvacrol inside the cytoplasm takes up potassium ion or other cation and diffuses out of the cell membrane and releases those ions. The cycle continues by binding of proton to the dissociated form of carvacrol which again diffuses into the cell membrane (Ultee et al., 2002).
uses proton motive force, which supports the conclusion above (Gill and Holley, 2006, Burt et al., 2007).

1.1.4. trans-Cinnamaldehyde

Cinnamaldehyde is a major component of cinnamon essential oil. It was reported that when *Escherchia coli* and *Staphylococcous aureus* were exposed to cinnamaldehyde, membrane permeability was observed through leakage of protein and alteration of membrane potential (Zhang et al., 2016). However, it was also reported that cinnamaldehyde didn’t exhibit significant damage toward outer membrane of gram negative cells while inhibiting their growth at same concentration with that of carvacrol and thymol, which suggests that it has different antimicrobial mechanism (Helander et al., 1998). Helander et al. (1998), therefore, suggested that trans-cinnamaldehyde enters through porin at outer membrane due to difference in chemical structure compared to carvacrol. Also, Gill and Holley. (2004a) suggested that trans-cinnamaldehyde has different mechanism of antimicrobial activity from ion transporter m-chlorophenylhydrazone (CCP), which has similar mechanism of antimicrobial activity to carvacrol suggested by Ultee et al. (2002). Therefore, it is predicted that the mechanism of antimicrobial activity of cinnamaldehyde is different from thymol and carvacrol, as it has no chemical group that could function similarly.

1.1.5. ρ-Cymene

ρ-cymene is a monoterpane and a biological precursor of carvacrol lacking a hydroxyl group. It is known to have little to no antimicrobial activity when used alone as they have no effect on pH gradient and inner ATP pool of cell membrane (Dorman and
Deans, 2000, Ultee et al. 2002, Burt et al., 2007, Hyldgaard et al., 2012). ρ-cymene was found to have higher partition coefficient (log P_{o/w}) than other active compounds, which enables more of them to partition into cell membrane leading to more expansion (Ultee et al., 2002). However, the expansion of membrane did not have effect on viability of *B. cereus* even though leakage, due to expansion, occurred for ions other than H\(^+\) and K\(^+\) which had no significant change in pH gradient (Ultee et al., 2002). Nevertheless, ρ-cymene was found to have synergistic effect on antimicrobial activity when used with carvacrol, due to their greater ability to partition into cell membrane and cause disorganization and expansion (Ultee et al., 2000a)

1.2. Limitation in Application

Although essential oils show potential as natural antimicrobial agents, there are number of factors that currently limit application of essential oils in foods. First, essential oils are hydrophobic substances with a relatively low water solubility. If essential oil is mixed with a broth, phase separation occurs between aqueous and oil phase which makes the use of stabilizers or emulsifiers necessary for antimicrobial efficiency (Burt and Reinders, 2003). Second, essential oils are vulnerable to oxidation and light. Third, the beneficial attributes of essential oils are often compromised when they are introduced into complex food matrices because of their tendency to interact with other components in the systems. Gutierrez et al. (2009) reported that the antimicrobial efficacy of oregano oil and thyme oil toward *Listeria monocytogenes* was less effective in a model medium with high content of carbohydrate and fat but was more effective in model medium that was acidic and had high content of protein. Also, Ultee et al. (2000a) reported that food ingredients have impact on the antimicrobial compounds as there was
decrease in antimicrobial activity against *B. cereus* in rice when combinations of carvacrol, ρ-cymene, and soya sauce were used, while synergistic effect was observed when combinations of carvacrol and ρ-cymene or carvacrol and soya sauce were used. Fourth, even though essential oils are isolated from the same species, depending on the season, location, extraction method, and the parts of the plant used, the proportion and types of active components differ (McGimpsey et al., 1994, Hedhili et al., 2002). This imposes difficulty in formulating consistent product applied with essential oil. Lastly, volatile nature and strong flavor (aroma and taste) of essential oils restricts the type of food product and amount that can be incorporated into (Hyldgaard et al., 2012). Ultee et al. (2000a) reported when high level of carvacrol was applied to rice to limit *B. cereus*. However, the odor and taste of the product was negatively affected (Ultee et al., 2000a). Also, as a flavoring agent, essential oils are used in low doses (Burdock, 2016). Therefore, development of appropriate delivery system is necessary to successfully incorporate these compounds in food products. The delivery system will be focused primarily on colloidal dispersions in this review.

### 1.3. Nanoemulsion

Emulsion is a system where two or more immiscible liquid become incorporated by dispersing one phase into another usually by mixing oil, water, and surfactants. Emulsions have been used in foods for a long period of time by people and in food industry to form products like milk, butter, sauces, ice creams, and dressings. Nanoemulsion is a thermodynamically unstable emulsion, like conventional emulsion (droplet diameter> 100nm), with small droplet dimensions (droplet diameter of 30 ~ 100nm) at nanometric scale (McClements, 2010, McClements, 2014). Therefore, it needs
external energy to form a colloidal dispersion which can be divided into high-energy and low-energy method (McClements 2012). Small droplet dimensions give favorable characteristics compared to conventional emulsions such as ability to maintain emulsified state for a long period of time, optical transparency, and faster diffusion of encapsulated active components because of the higher surface area (McClements, 2010, McClements, 2014). Compared to microemulsions, nanoemulsions can exist in wider varieties as they are not in equilibrium state, as microemulsion could only exist when the emulsion droplet matches the spontaneous curvature of surfactant at particular condition (Sonneville-Aubrun et al., 2009). Also, nanoemulsions tend to be kinetically stable if the energy barrier between emulsified state is > 20kT (McClements, 2012).

Unlike conventional emulsion and nanoemulsion, microemulsion is an emulsion that is thermodynamically stable and forms spontaneously (McClements, 2010, McClements, 2014). However, in practice, to reach of thermodynamically stable state to form microemulsion, it requires instruments that could exert force to overcome kinetic energy barrier and slow mass transport of material being encapsulated (McClement, 2012a). The surfactants that is used in microemulsion is limited to small molecule surface active agents as it requires the ability to induce “ultralow interfacial tension at particular monolayer curvature.” (McClement, 2012a). Microemulsion (droplet diameter of 10nm ~ 100nm), also, differ from conventional emulsions (droplet diameter >100nm) in having smaller droplet diameters. (McClements, 2010, McClements, 2014). Therefore, it has favorable characteristic compared to conventional emulsion, such as being stable for long period of time, optical transparency, and large surface area. However, compared to nanoemulsion it tends to be kinetically unstable as microemulsion is spontaneously
formed and kept stable if there is little to no change from the optimal conditions (McClement, 2012a).

1.4. High-Energy and Low-Energy Emulsification

There are two ways of exerting energy to form a nanoemulsion. One is high-energy method that requires a specific instrument to exert intense disruptive force to overcome the kinetic barriers. The other is low-energy method which relies on the method that uses low energy such as low mechanical force or temperature.

1.4.1. High-Energy Method

1.4.1.1. Microfluidizer

To the present day, the most common way to produce nanoemulsions is using high-energy methods involving intense mechanical disruptive forces e.g., high pressure homogenizers, microfluidizers, and sonicators (Anton et al., 2008, McClements and Rao 2011). These intense forces deform and disrupt macromolecule to create small droplets, and they also facilitate surfactant adsorption to the interface to stabilize them (Anton et al., 2008). Microfluidizer generates disruptive forces, such as high shear force, by colliding the preformed macroemulsion in two different channels toward each other in a conjoining compartment (Figure 1.3). According to Horiba Scientific (2010), M-110P microfluidizer could generate shear rate up to $10^7$ s$^{-1}$ by colliding two fluids using high pressure pump (up to 40,000 psi). Studies about encapsulation of essential oil or flavor oil by microfluidizers have been done to create nanoemulsions (Rao and McClements, 2011, Chang et al., 2012, Salvia-Trujillo et al., 2013)
Figure 1.3. The depiction of the chamber where high shear occurs in the microfluidizer (Horiba Scientific, 2010)
1.4.2. Low-Energy Method

1.4.2.1. Phase Inversion Temperature (PIT) Method

Formation of emulsion by altering volume ratio between dispersed and continuous phase, and by altering temperature was first introduced by Shinoda and Saito (1968) using tertiary system of water, cyclohexane, and nonionic surfactant. At constant ratio between water and oil, different dispersed phases were observed when there was an alteration in temperature which was related to the changes in solubility of non-ionic surfactants (Shinoda and Saito, 1968). This is known to be caused by the hydration and dehydration of amphiphilic surfactant head group at low and high temperature, which alters the polarity of the surfactant and also its’ molecular geometry (Rao and McClements, 2011). For instance, if temperature was increased above a certain point called PIT in oil-in-water (O/W) emulsion, amphiphilic surfactant head group will be dehydrated and the surfactants will be more soluble in oil phase as they will be less polar. This is due to the molecular geometry of having smaller head group, and this forms favorable structure for formation of water-in-oil (W/O) emulsion. The intermediate temperature when phase inversion occurs is called either PIT or HLB temperature (Sloans and Sole, 2012). This is the region where the curvature of the surfactant is almost at zero favoring neither the oil phase nor the aqueous phase, and therefore where formation of bicontinuous microemulsion occur (Sloans and Sole, 2012). When bicontinuous microemulsion form, formation of emulsion droplet is favorable due to very low surface tension. However, as surfactant curvature is almost at zero, the formed droplets are susceptible to coalescence. At this point, rapid heating (+20°C from PIT) or cooling (-30°C from PIT) should occur to form stable nanoemulsion and if the process occurs fast
enough, the surfactants in the interface of oil and water will be maintained (Anton et al., 2007; Rao and McClements, 2011). Once this irreversible process occurred, the nanoemulsion stays stable by steric stabilization (Anton et al., 2007). Therefore, the small and monodispersed nanoemulsion formulation is dependent on surfactant structure of bicontinuous microemulsion formed at PIT (Anton et al., 2007; Sloans and Sole, 2012). Also, surfactants that can change its’ configuration depending on temperature can only be used (Sloans and Sole, 2012).

1.4.2.2. Emulsion Inversion Point (EIP) Method

Shinoda and Saito (1968), also introduced that phase changes occur depending on the volume ratio between water and oil. The EIP method formulate nanoemulsion by adding continuous phase of the final nanoemulsion into the mixture of surfactant and the dispersed phase of the final nanoemulsion. Phase inversion is caused by hydration or dehydration of surfactant head group which alters the surfactant curvature to form bicontinuous phase (Roger et al., 2010; Slonas and Sole, 2012). For instance, formulation of O/W nanoemulsion by EIP method is done by addition of aqueous phase to the mixture of oil and surfactant. Addition of the aqueous phase creates swollen reverse micelles inside the oil droplets which swells as more aqueous phase is added (Roger et al., 2010). Once the adequate aqueous phase is added, hydration of surfactant will cause the reverse surfactant micelles to form a lamellar or bicontinuous phase. Faster coalescence of aqueous phase inside the reverse surfactant micelles will facilitate the disintegration of oil droplet into smaller oil droplets and into micelles (Roger et al., 2010; Rao and McClements, 2011). Therefore, compared to PIT method, EIP method tend to create particles of wider distribution in size, and more micelles that will coexist with the
emulsion droplets containing oil (Roger et al., 2010). The advantage of using EIP method over PIT is that it is an isothermal process, which allows incorporation of ingredients that are susceptible to high temperature.

1.4.2.3. Spontaneous Emulsification Method

Spontaneous emulsification occurs in a system that is not initially at equilibrium by both condensation and dispersion upon contact (Miller, 1988). The occurrence of dispersion was proposed to happen by breaking off of droplets by interfacial turbulence which causes significant capillary waves or by temporary negative value in interfacial tension (Miller, 1988). Condensation was proposed to occur from local supersaturation by diffusion which cause formation of droplets (Miller, 1988). In a system where surfactant is incorporated, breaking off of emulsion droplets was reported from swelling intermediate liquid crystalline phase (Miller, 1988).

More optimized method was suggested by Anton and Vandamme (2009) to cause spontaneous emulsification or spontaneous self-emulsification, which triggers formation of smaller O/W nanoemulsion by dilution or titration. O/W nanoemulsion, for instance, is created by diluting or titrating mixture of oil and water-soluble surfactant into the aqueous phase (Anton and Vandamme, 2009, Chang et al., 2013, Komaiko and McClements, 2014). When the titrated or diluted phase makes contact with the continuous phase, components that could be solubilized in the continuous phase such as water soluble surfactants rapidly diffuse creating interfacial turbulence (Komaiko and McClements, 2014, Solans and Sole, 2012). The rapid diffusion of surfactants and interfacial turbulence creates liquid crystalline phase in the interface between the oil and water, which eventually breaks off and forms nanoemulsion (Figure 1.4) (Komaiko and
McClements, 2014, Solans and Sole, 2012). This method is relatively simple compared to other emulsification method and industrial scale up is favorable due to low energy requirement and no need for sophisticated instrument is needed for emulsification (Vandamme and Anton, 2010). For instance, Vandamme and Anton (2010) proposed an emulsification device for delivery of drug for domesticated animals through their water source by low energy emulsification of two immiscible phases using liquid turbulence in chambers. However, compared to high energy emulsification, they don’t seem to show clear trends by direct adjustments of parameters, but were reported to be more influenced by the physicochemical characteristics of each of the components that forms the nanoemulsion (Bouchemal et al., 2004, Anton, 2008).

1.5. Spontaneous Emulsification

The spontaneous emulsification method that will be discussed in this section is focused on the method devised by Chang et al. (2013) of creating O/W nanoemulsion modified from that of Anton and Vandamme (2009) (Figure 1.4). A number of studies have shown that essential oil nanoemulsions can be formed by spontaneous emulsification, and that they have good antimicrobial activity (Chang et al., 2013, Tian et al., 2016, Landry et al., 2014, 2015). Recently, in vivo study of carvacrol nanoemulsions have shown that they are highly effective at inhibiting the growth of Salmonella Enteritidis or E. coli O157:H7 then conventional chlorination treatment in contaminated mung bean, alfalfa seed, broccoli, radish seeds (Landry et al., 2014, 2015).
Figure 1.4. Schematic procedure of formulation of nanoemulsion by titrating organic phase (oil + surfactant) into aqueous phase stirred with magnetic stirrer. A proposed molecular view is included (Komaiko and McClements, 2015).
1.5.1. Potential Problems

1.5.1.1. Flocculation and Coalescence

Flocculation and coalescence occur when repulsive force between colloids are weaker than the attractive forces. Flocculation is a phenomenon which colloids adhere or flock together while maintaining separate inner phases. Coalescence, which usually occurs after flocculation, is a phenomenon which separate inner phases of two or more emulsion droplets merge together to form a bigger sized droplet. The attractive forces could be due to difference in charges on surfactants, hydrophobic effect, and electrolyte concentration.

Flocculation and coalescence can be prevented by steric repulsion, Gibbs Marangoni effect, and electrostatic repulsion, which relies on surfactant characteristic. Nanoemulsion tend to have high steric repulsion due to their small dimensions which enables the droplet to have high ratio of steric layer compared to droplet diameter. Therefore, it acquires high steric stabilization. Gibbs Marangoni effect occurs when there is uneven adsorption of surfactant molecules on the interface of emulsion droplets which alters Gibb’s elasticity (Wooster et al., 2008). Once the emulsion droplets approach each other, due to the lower concentration of surfactant at that interface where the droplets are approaching, influx of aqueous phase occurs. The influx occurs due to interfacial gradient as there is uneven adsorption of surfactant on the interfacial layer which prevents the droplets to coalesce or to flocculate. Electrostatic repulsion opposes the attraction of Van der Waals force by using ionic surfactants to create charged surfaces on the emulsion droplets. However, one should take careful consideration of the concentration of nanoemulsion and salt concentration of the surrounding medium as it could induce
electrostatic screening which could cause destabilization of the emulsion that is stable because of surface charge.

1.5.1.2. Ostwald ripening

Nanoemulsion is reasonably resistant to flocculation and coagulation due to their nanometric dimensions (Anton et al., 2008). Ostwald ripening (OR) is a phenomenon which mass transfer of encapsulated substances from small droplets to large droplets through the surrounding medium occur. OR occurs in emulsion that has droplets with sub-micron dimensions and in conventional emulsions that has dispersed phase with high solubility (Taylor, 1998). For emulsions that have droplets with sub-micron dimensions, the chemical potential of the encapsulated compound changes as it becomes more soluble as the droplet diameter decrease due to difference in Laplace pressure. Therefore, although essential oils are constituted with hydrophobic compounds, many of the compounds are relatively soluble in aqueous phase when they are encapsulated in nanoemulsion which makes them susceptible to OR.

To formulate a stable nanoemulsion, slowing the rate of OR is necessary. One way is decreasing the solubility of encapsulated components by changing its composition. Ripening inhibitors are compounds, that have low water solubility, incorporated into the oil phase prior to nanoemulsion formation to retard OR (Wooster et al. 2008). Ripening inhibitors don’t diffuse out of the droplet as fast as the compounds with appreciable water solubility, therefore it can be assumed that the water-soluble compounds will be more concentrated in bigger droplets in nanoemulsion incorporating ripening inhibitor (Taylor, 1998). At some point, the chemical potential of the droplets will be similar to each other which causes OR to occur at a slower rate (Taylor, 1998). Nevertheless, essential oil
nanoemulsion must still be carefully formulated as there are very few reports of successful incorporation of ripening inhibitors such as long chain triglyceride with large molar mass into nanomeulsion that shows long term stability. Also, the antimicrobial efficacy of nanoemulsions depend on the ripening inhibitors. For example, previous study has shown that the minimum inhibitory concentration (MIC) of thyme oil nanoemulsions depends on the type and amount of ripening inhibitor they contained (Chang et al., 2012). Other formulation factors should be considered as well, as OR was found to be affected by volume fraction between nanoemulsions and the continuous phase, concentration of electrolytes, steric barrier, and existence of micelles (Taylor, 1998)

1.5.2. Formulation Factors

1.5.2.1. Surfactant-Oil-Water Ratio and Type

Formation of bicontinuous microemulsion, when contact between mixture of surfactant and oil with aqueous phase, occurs at certain surfactant-oil-water (SOW) ratio and it also depends on the types of surfactant, oil, and water. Many studies indicate that the different kinds of liquid crystalline phase form when mixing SOW, but the formation of bicontinuous microemulsion is critical in formulating small droplet emulsion (Alexandridis et al., 1998, Sloans and Sole, 2012, Prasert and Gohtani, 2016). SOW ratio is important as some nanoemulsion such as the one encapsulating carvacrol using 10% oil phase and 10% Tween 80 requires its oil phase to be constituted of 15~40% carvacrol to create small emulsion droplets (d < 100 nm) with the rest of the oil phase constituting of MCT (Chang et al., 2013). The formation of small droplets seems to depend on the individual physicochemical properties of constituting components as the nanoemulsion encapsulating different compounds have different diameters and stability. For instance,
vitamin E acetate nanoemulsion with the same SOW ratio as the carvacrol nanoemulsion, created emulsion droplets \((d < 100\text{nm})\) when 40–80\% of the oil phase was constituted of vitamin E acetate (Saberi et al., 2013). Also, depending on the types of oils incorporated, droplet size was influenced in order from largest: long chain triglyceride > flavor oil > medium chain triglyceride (MCT) (Komaiko and McClements, 2015). It was reported that there is no clear correlation between the size of the emulsion droplet and different physicochemical parameters of encapsulated oils such as viscosity, refractive index, density, and interfacial tension suggesting that there is complex interaction between different constituents of nanoemulsion (Bouchemal et al., 2004, Komaiko and McClements, 2015).

1.5.2.2. Surfactant

Surfactant types and surfactant to oil ratio are also known to influence size of the emulsion droplets and their stability. Currently, synthetic surfactant Brij, Span and Tween are known to be utilized to form nanoemulsion through spontaneous emulsification described above (Komaiko and McClements, 2014, Komaiko and McClements, 2015). Therefore, only surfactants with small molecular mass was found to be able to formulate nanoemulsion using spontaneous method. This is due to the fact that the diffusion of surfactants from the oil phase to the aqueous phase is important in formation of spontaneous nanoemulsion as smaller molecules tend to diffuse faster. Surfactant is largely characterized by its structure usually shown as hydrophilic lipophilic balance (HLB) or hydrophilic lipophilic difference (HLD). However, the optimum HLB and HLD to form small emulsion droplet was found to be conflicting among the studies (Komaiko and McClements, 2015). However, according to Komaiko and McClements (2015), MCT
nanoemulsion created with Tween 80 had the smallest droplet size relative to the spontaneous emulsions created with other surfactants. This was suggested to be due to the optimal surfactant curvature that Tween 80 has when it attaches to the interface between oil and aqueous phase as it has one unsaturated tail while other Tweens have saturated linear tail or more unsaturated tails (Komaiko and McClments, 2015).

Surfactant to oil ratio is important as enough surfactant should be present to adhere to interface of oil and continuous phase. Generally, more surfactant is required when forming smaller emulsion droplets as the surface area increases and because of the need to create steric repulsion to make the droplets stable. However, there are reports that excess usage of surfactant could lead to destabilization of the emulsion. Komaiko and McClements (2014) reports that at surfactant to oil ratio at 0.375 formulates the nanoemulsion with smallest droplet while destabilization occurs if more or less surfactant is added. This is also dependent on the encapsulated component as the smallest emulsion droplet forms when surfactant to oil ratio of vitamin E acetate nanoemulsion is at 1 (Saberi et al., 2013). Therefore, again it emphasizes that size of emulsion droplet of the nanoemulsion is largely dependent on the physicochemical characteristics of each of constituting components.

1.6. Mechanism of Antimicrobial Activity of Nanoemulsions

There are very few studies about the mechanism how encapsulated antimicrobial components, such as essential oils, act on cells. There are two proposed ways how nanoemulsion acts as a delivery system. First, nanoemulsion could make physical contact with cell membrane and release the active components. Encapsulated essential oils are predominantly hydrophobic. Therefore, the active components of essential oil could
partition into the hydrophobic part of the cell membrane. Also, if the nanoemulsion has
small enough diameter, there is a possibility that they would travel inside the membrane
through porins. Once destabilization occurs inside the cell membrane, significant pressure
is released as emulsion droplets with smaller diameter has larger Laplace pressure. This
suggests that antimicrobial activity could be increased if essential oils are encapsulated in
nanoemulsion. Second, essential oils have appreciable solubility in water and therefore, it
enables active components to travel through the aqueous medium and diffuse into the cell
membrane. Direct application of essential oil into aqueous media could limit their
efficiency as they are predominantly hydrophobic. However, nanoemulsion suspends the
essential oil inside the aqueous media, which enables it to diffuse out and diffuse in to
one hydrophobic phase to another for a prolonged time. Therefore, prolonged diffusion
and accumulation of essential oil into bacteria cell membrane could enhance the
antimicrobial activity. In one of the studies it was reported that the antimicrobial activity
of nanoemulsion, encapsulating different kinds of antimicrobial agents in essential oil,
correlated with the amounts of active components that were detected in the aqueous
phase (Donsi et al., 2012).

1.7. Conclusion

Delivery of antimicrobial essential oil using nanoemulsion formulated with low
energy spontaneous emulsification method is attracting attention. Essential oil is a mix of
natural chemical compounds with favorable characteristic. However, it requires an
appropriate delivery system to prolong its efficacy and to retard its degradation in an
aqueous environment. Nanoemulsion is an appropriate delivery system as it could both
retard degradation of essential oil and prolong its efficacy as they tend to be kinetically
stable for a long period of time. Nanoemulsions formulated with spontaneous emulsification method has few advantage over ones produced by other low energy emulsification method. Compared to EPI method, it tends to create nanoemulsion with more monodispersed and smaller emulsion droplet size. Compared to PIT method, it can encapsulate compounds that are sensitive to high temperature as it is an isothermal process. However, common to all low energy emulsification method, the incorporation of high amount of surfactant is a problem. Also, there are limited variety of surfactants that could be applied in spontaneous emulsification as diffusion toward continuous phase from the dispersed phase should occur quickly when there is contact between oil and aqueous phase. Nevertheless, it is a method that can formulate nanoemulsion economically as it adapts simple procedures and low mechanical force.
CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The materials used to form the oil phase of the antimicrobial nanoemulsion were thyme oil (Sigma-Aldrich W306509 – 1KG – K) and five different ripening inhibitors. The main ripening inhibitor used was medium chain triglyceride (MCT) oil (Miglyol 812), which was purchased from Sassol Germany GmbH, Witten, Germany. The manufacturer reported that the fatty acid composition of this oil was 58.1 % caprylic acid (C8:0) and 41% capric acid (C10:0). The other ripening inhibitors used were corn oil (Stop and Shop, Foodhold U.S.A, LLC Landover, MD), canola oil (Stop and Shop, Foodhold U.S.A, LLC Landover, MD), palm oil (Deganim, Equador), and coconut oil (Carrington Farms, Closter, NJ). The coconut oil was reported to contain 93% w/w saturated fatty acids comprising mainly of lauric acid (C12:0, 30.7%w/w), caprylic acid (C8:0, 30% w/w), and capric acid (C10:0, 24.3% w/w) (Carrington Farms, Closter, NJ). Consequently, the MCT and coconut oil can be considered to be mainly medium chain triglycerides, whereas the corn, canola, and palm oils can be considered to be long chain triglycerides (LCT). Tween 80 (T80) (Sigma-Aldrich P1754-500G) was used as a non-ionic surfactant to facilitate nanoemulsion formation and stability. For analysis of partitioning behavior of components in nanoemulsion by HPLC, three components of thyme oil in analytical standards, thymol (Sigma-Aldrich 72477), carvacrol (Sigma-Aldrich 42632), ρ-cymene (Sigma-Aldrich 30039) were purchased.
2.2. Methods

2.2.1 Formation of antimicrobial nanoemulsion

The antimicrobial nanoemulsions were prepared according to the optimized formulation and fabrication method described by Chang et al, (2013). The oil phase was prepared by mixing essential oil and ripening inhibitor together. Control emulsions without essential oil were prepared with MCT alone. This oil phase was then mixed with an equal mass of Tween 80 to obtain a surfactant-to-oil ratio (SOR) of 1:1. Then, the surfactant-oil mixture was titrated at a rate of 2 mL/min into the water phase (5 mM sodium citrate buffer), which was being continually stirred at 600 rpm using a magnetic stir bar throughout the titration and for an additional 15 min. The resulting nanoemulsion was sterilized by filtering it through a 0.45 µm syringe filter (Cole-Palmer Cat# 02915-22) and was then stored in separate sterile containers within 4 and 20 °C incubators. For each oil phase composition, three replicates were prepared.

2.2.2. Bacterial culture conditions

Three strains of *Salmonella enterica* subspecies *enterica* (*Salmonella* sp.), representing serovars Enteritidis (BAA-1045) Gaminara (BAA-711) and Michigan (BAA-709) were obtained from the American Type Culture Collection (Manassas, VA). Long term storage of each culture was stored at -80 °C in a mixture of tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% v/v glycerol. Monthly, working stock of each culture were prepared by streaking from frozen stocks onto tryptic soy agar (TSA; BD Diagnostic Systems,). After incubation at 37°C, working stocks were stored at 4°C for a month. For experiments, colonies were selected from
working stock plates, and inoculated individually into TSB and incubated at 37 °C for 18 hours on a 125 rpm shaker.

2.2.3. Determination of minimal inhibitory concentration

The antimicrobial efficacy of the essential oil nanoemulsions was obtained by measuring their minimal inhibitory concentration (MIC) against a cocktail of three strains of *Salmonella sp.* prepared from 18 hours growth of each culture. To prepare the cocktail, each culture was diluted 1:10, and the optical density at 600 nm (OD600) adjusted to OD600=0.2. A cocktail was prepared by mixing 6ml of each culture to produce a cocktail with approximately 9 log CFU/ml. The levels in the cocktail confirmed by dilution and plating on TSA utilizing the drop plate method (Herigstad et al., 2001). The cocktail was diluted in TSB (10^{-3}) and mixed with various concentrations of nanoemulsions to produce an initial inoculum of approximately 6 log CFU/ml. After incubation (37 °C for 24 hours), the level of bacteria in each nanoemulsion was determined using the drop plate technique. MIC was defined as the lowest tested concentration of nanoemulsion that prevented the growth of the *Salmonella sp.* cocktail after a 24 hours incubation period.

2.2.4. Storage stability of antimicrobial nanoemulsion

The antimicrobial nanoemulsions were stored at 4 and 20°C in an incubator for 30 days. Visual observation of the nanoemulsions was carried out by tilting the tube to a 45° angle for observation of creaming every two days. Pictures were taken before each observation. Mean particle diameter (Z-averages) and PDI (Polydispersity Index) were measured using dynamic light scattering (Zetasizer Nano ZS, model ZEN 3600, Malvern
Instrument, UK). This instrument directs a laser beam (633 nm) at the sample and measures the fluctuation in the intensity of the scattered light using a detector at 173°. The particle size distribution is then obtained by mathematical analysis of the intensity-time pattern using the instrument software.

2.2.5. Partitioning of active components

The partitioning of the active components in the essential oil between the nanoemulsion droplets and the surrounding aqueous phase was measured using equilibrium dialysis and HPLC. A buffer solution or MCT emulsion was placed inside a dialysis bag, which was then placed into a beaker containing an essential oil nanoemulsion. The amount of active components from the essential oil that diffused into the dialysis bag was then measured by HPLC.

Dialysis was carried out using a cellulose ester (CE) dialysis membrane (spectra/Por® Biotech, US) with a molecular weight cut off (MWCO) of 100 ~ 500 daltons. Two dialysis membranes were cut 12 ~ 15 cm and then soaked in deionized water for 30 min. The membranes were then rinsed with distilled water and filled with either 1 ml MCT emulsion (10% oil phase, based upon the 4000 ppm nanoemulsion) or 1 ml of buffer solution (5 mM sodium citrate buffer, pH 3.5). The membrane openings were sealed shut (spectra/Por® Biotech, US), and then the sealed membranes were submerged in a beaker containing 594 ml of essential oil nanoemulsions (4000 ppm oil phase) with different ripening inhibitor compositions. The oil phase compositions studied were: 4:6 thyme oil: MCT; 4:3:3 thyme oil: MCT: corn oil; 4:3:3. Samples collected from inside the dialysis tubing were filtered through 0.20 µm PTFE filters (Fisher Scientific Cat# 970002). Dialysis tubings containing either MCT emulsion or buffer was suspended
in nanoemulsion and were stirred at 300 rpm for 24 hours, after which the contents were used for HPLC analysis for the presence of antimicrobial components. In addition, the particle sizes were measured using dynamic light scattering. No emulsion droplets were detected in the buffer, and based upon particle size, no antimicrobial nanoemulsion was detected in the MCT control emulsion, indicating that the dialysis tubing had no leakage during the experiment.

2.2.6. Rate of partitioning of active components

The rate of partitioning of each component was measured by using equilibrium dialysis and HPLC. Eight 1 ml MCT emulsion (10% oil phase, based upon the 4000 ppm nanoemulsion) were enclosed (spectra/Por® Biotech, US) in a cellulose ester (CE) dialysis membrane (spectra/Por® Biotech, US). Then they were submerged in a beaker containing 592 ml of 4:6 thyme oil:MCT nanoemulsions (4000 ppm oil phase) stirred at 300 rpm by a stir bar (Figure 3.1). The samples from inside the dialysis tubing were collected at 1, 10, 30, 60, 180, 360, 720, 1080, 1440 (=24 hours) minutes and were filtered through 0.20 µm PTFE filters (Fisher Scientific Cat# 970002) for HPLC analysis.

2.2.7. High Pressure Liquid Chromatography

The concentration of essential oil components collected from inside of the dialysis tubing was analyzed using High Performance Liquid Chromatography (HPLC, Model LC-2030C 3D, Shimadzu Corporation, Japan) with a based upon a method previously described (Hajimehdipoor et al., 2010). The HPLC was equipped with an auto-sampler, a Hypersil ODS-2 (C18) column (200 mm length and 4.6 mm internal diameter), and was
Figure 3.1. Depiction of establishing two separate hydrophobic system using dialysis tubing for partitioning experiments.
operated with a mobile phase of acetonitrile and water (50/50 vol/vol) at flow rate of 1 ml/min. A photo diode array detector was used to measure UV spectra of the samples were measured at wavelength of 254 nm was used in measuring the peak areas of all components. Standards and samples were injected at a volume of 10 µl.

2.2.8. Data analysis of partitioning rate

The data acquired from HPLC were analyzed using Mathematica 11.01 (Wolfram Research, Inc. Champaign, Illinois). The concentration ratio was calculated by dividing the momentary concentration, C(t), by the concentration attained after 24 hour diffusion, $C_\infty$. The changes in the concentration ratio as a function of time were characterized with the two parameter Peleg’s model Eq. 1 (Peleg, 1988), which has been used in mass transfer applications, for example, Pavelkic et al., 2015.

$$\frac{C(t)}{C_\infty} = \frac{t}{k_1 + k_2 t} \quad (1)$$

where $k_1$ is a rate constant, inversely proportional to the partition rate, and $k_2$ is a capacity constant, which is inversely proportional to the asymptotic diffused concentration ratio. The experimental data were fitted with Eq. 1 using a nonlinear fitting routine available in Mathematica and the goodness of fit was evaluated based on the mean squared error (MSE) and the adjusted $R^2$.

2.2.9. Statistical analysis

Statistically analysis software (SPSS, Version 24, SPSS Inc., USA) was used to analyze the data. Means were subjected to independent T-test and Duncan’s least significant difference (LSD) and a P-value < 0.05.
CHAPTER 3

EFFECT OF RIPENING INHIBITOR TYPE ON FORMATION, STABILITY, AND ANTIMICROBIAL ACTIVITY OF THYME OIL NANOEMULSION

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3.1. Abstract

Incorporation of essential oil into novel colloidal delivery system is gaining interest due to their relatively simple steps of formulation. Essential oils are complex mix of volatile and non-volatile compounds that has antimicrobial, antifungal, antioxidant, and antiviral properties. However, due to their hydrophobic nature, reactivity, and susceptibility to oxidation, it needs a proper delivery system. The objective of this research was to study the influence of the amount and types of ripening inhibitor on the formation, stability, and activity of antimicrobial thyme oil nanoemulsions formed with spontaneous emulsification. Oil-in-water antimicrobial nanoemulsions with oil phase (10 wt%), surfactant (10 wt%), and 5mM sodium citrate buffer (80 wt%, pH 3.5) was created by titrating mixture of oil and surfactant into aqueous buffer. Nanoemulsion with small emulsion droplets ($d < 70$ nm) were formed at essential oil-to-ripening inhibitor mass ratios of 4:6, when medium chain triglyceride (MCT) was used as ripening inhibitor. However, as more ripening inhibitor was incorporated into the oil phase, antimicrobial activity of the nanoemulsions decreased, which was attributed to a reduction of transfer of hydrophobic antimicrobial constituents into the bacterial cell membranes. The antimicrobial activity of the nanoemulsions also depended on the type of the ripening inhibitor incorporated: palm $\approx$ corn $>$ canola $>$ coconut. The difference in antimicrobial
efficacy between nanoemulsions was attributed to be the difference and type of ripening inhibitor. In combination of using dialysis and chromatography, the behaviors of partitioning antimicrobial agents were studied. This experiment demonstrates that high antimicrobial activity of nanoemulsions incorporating ripening inhibitors was due to their physicochemical property to transfer more antimicrobial agents into the hydrophobic domain. These results indicated that the type and amount of ripening inhibitor in the oil phase had to be optimized to produce physically stable nanoemulsions with antimicrobial activity.

3.2. Introduction

Essential oils consist of a complex mixture of different volatile and non-volatile compounds that are normally extracted from different parts of plants (Dorman and Deans, 2000, Bakkali et al., 2008). Many of the compounds in essential oils have bioactive properties that are useful for certain industrial and medical applications, such as antimicrobial, antiviral, antifungal, and antiseptic properties (Burt, 2004). The fact that essential oils are natural products has led to particular interest within the food industry, since many consumers are interested in purchasing foods that do not contain synthetic ingredients. *In vitro* and *in vivo* studies have shown that many essential oils have strong potency against pathogenic bacteria and fungi (Dorman and Deans, 2000, Gutierrez et al., 2009, Sienkiewicz et al., 2012). Essential oils isolated from the herb thyme have relatively strong antimicrobial activity, and are suitable for application in food products (Burt, 2004). Nevertheless, it has been reported that thyme contains different proportions of active components depending on the season, location, extraction method, and the parts of the plant from which it was extracted (McGimpsey et al., 1994, Hedhili et al., 2002).
The current study focuses on using thyme oil extracted from *Thymus vulgaris* as an antimicrobial agent in nanoemulsion-based delivery systems. The major components in this source of thyme oil have been reported to be thymol, carvacrol, β-caryophyllens, γ-terpinene, and ρ-cymene (Hedhili et al., 2002, Burt, 2004, Bakkali et al., 2008, Sienkiewicz 2012 et al., Borugă et al. 2014). The mechanism of antimicrobial activity of essential oils (such as oregano oil) against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was attributed to the presence of carvacrol and thymol constituents, which increased the permeability of the bacterial membranes thereby leading to the leakage of inorganic ions, ATP, and amino acids (Lambert et al., 2001, Ultee et al., 2002). This mechanism was supported by another study using flow cytometry and specific fluorescent dyes to detect the leakage of specific components from cells exposed to carvacrol and thymol (Xu, 2008).

Despite their potential as natural antimicrobial agents, there are a number of factors that currently limit the widespread utilization of essential oils in foods. Firstly, essential oils are typically hydrophobic substances with a relatively low water solubility, which means that they have to be introduced into foods using appropriate delivery systems (such as in organic solvents, microemulsions, or emulsions). Second, the antimicrobial activity of essential oils is often compromised when they are introduced into complex food matrices because of their tendency to interact with other components in the systems. Third, many essential oils have a strong flavor (aroma and taste), which restricts the type of food products that they can be successfully incorporated into. Many of these challenges can be overcome using well-designed colloidal delivery systems.
Numerous studies have shown that essential oils can be successfully encapsulated within nanoemulsion-based delivery systems (Chang et al., 2012, 2013, Donsì et al., 2012, Liang et al., 2012). These delivery systems consist of small \((d < 200 \text{ nm})\) oil droplets dispersed within an aqueous medium (McClements, 2010, 2014). This type of delivery system is particularly useful for incorporating essential oils into a wide range of aqueous-based food products. In particular, antimicrobial nanoemulsions can be designed to be optically transparent, physically stable, and suitable for rapidly delivering essential oils to bacteria surfaces. Conventionally, nanoemulsions are produced using high-energy methods that involve mechanically applying intense disruptive forces to a mixture of oil and water to break up the oil phase into tiny droplets, e.g., high pressure homogenizers, microfluidizers, and sonicators (McClements and Rao, 2011). However, nanoemulsions can also be formed using low-energy methods, such as “spontaneous emulsification”, which simply involves titrating a mixture of oil and surfactant into water (Komaiko and McClements, 2014). This method is highly advantageous for certain commercial applications because it is simple to implement and does not require the use of expensive or sophisticated manufacturing equipment, such as that needed for high-energy homogenization. A number of studies have shown that essential oil nanoemulsions can be formed by spontaneous emulsification, and that they have good antimicrobial activity (Chang et al., 2013, Tian et al., 2016, Landry et al., 2014, 2015). For instance, carvacrol nanoemulsions were shown to be highly effective at inhibiting the growth of *Salmonella Enteritidis* or *E. coli* O157:H7 in contaminated mung bean, alfalfa seed, broccoli, radish seeds (Landry et al., 2014, 2015). Indeed, the antimicrobial nanoemulsions were reported to be more effective than conventional chlorination treatments for this application.
One of the major challenges that must be overcome during the development of essential oil nanoemulsions is the tendency for the oil droplets to grow during storage due to Ostwald ripening (OR). Droplet growth occurs through this mechanism as a result of the diffusion of oil molecules from the smaller droplets to the larger droplets through the intervening aqueous phase due to differences in the chemical potential of the oil inside droplets with different dimensions (Taylor, 1998, McClements, 2014). OR leads to an increase in the mean particle size over time, which eventually causes emulsion instability due to creaming and phase separation. Essential oil nanoemulsions are particularly prone to OR because even though the oil phase is predominantly hydrophobic it still has a significant solubility in the aqueous phase. Droplet growth due to OR can be retarded by incorporating water-insoluble oils, known as ripening inhibitors, into the oil phase prior to nanoemulsion formation (Wooster et al. 2008). Nevertheless, essential oil nanoemulsions must still be carefully formulated since the presence of the ripening inhibitor can reduce the efficiency of nanoemulsion formation, as well as reducing the antimicrobial efficacy of the essential oils. For example, a previous study has shown that the minimum inhibitory concentration (MIC) of thyme oil nanoemulsions depends on the type and amount of ripening inhibitor they contained (Chang et al., 2012). Even so, the effect of different ripening inhibitor types on nanoemulsion performance has not previously been established, and the impact of ripening inhibitor type on the mechanism of action of essential oils has not be investigated. The purpose of the current research is therefore to examine the impact of ripening inhibitor type on the formation, stability and antimicrobial activity of antimicrobial thyme oil nanoemulsions, and to provide some insight into the potential physicochemical mechanisms involved.
3.3. Results and Discussion

3.3.1. Influence of ripening inhibitor level on thyme oil nanoemulsion properties

Previous studies suggest that level of ripening inhibitor in the oil phase is critical in forming stable antimicrobial nanoemulsions (Komaiko and McClements, 2015, Chang et al., 2012). This was further examined by initially testing different ratios of thyme oil (antimicrobial) and MCT (ripening inhibitor) on the formation and stability of antimicrobial nanoemulsions. MCT was selected because it has previously been shown to be successful at stabilizing carvacrol nanoemulsions against Ostwald ripening (Chang et al., 2013), and carvacrol is a major antimicrobial component within thyme oil. The overall oil content of the nanoemulsions was fixed at 10 wt%, but the amount of thyme oil in the oil phase was varied from 0 to 100%. Nanoemulsions formed from pure thyme oil were highly unstable, with visible creaming and phase separation occurring within a few hours of preparation, which can be attributed to rapid droplet growth caused by Ostwald ripening (Figure 3.2). When the thyme oil level was reduced, and MCT added as a ripening inhibitor the mean droplet diameter of the nanoemulsions initially decreased with a minimum droplet size of 50±1.8 nm (being observed at about 40% thyme oil / 60% MCT (Figure 3.2). At thyme oil levels greater than 50 % of the oil phase, there was an increase in mean droplet diameter during storage and visible creaming was observed after a few hours, which suggested that there was insufficient MCT present to completely inhibit Ostwald ripening. In contrast, when the thyme oil level was ≤50 wt% of the oil phase, the mean droplet diameter of the nanoemulsions did not change appreciably during storage and there was no visible evidence of creaming (Figure 3.2). This suggested that once the droplets had been formed by spontaneous emulsification they remained stable to
Figure 3.2. The effect MCT ripening inhibitor and thyme oil upon the average droplet diameter (nm) at day zero and minimum inhibitory concentration (MIC). The MIC was calculated based on the concentration of thyme oil in the nanoemulsions. The number of days before visible phase separation is labeled next to the average droplet diameter (as measured at day 0), The “*” symbol means that the MIC was above the upper threshold value (10,000 ppm).
Ostwald ripening. Similar effects have been reported in other studies of nanoemulsions prepared using spontaneous emulsification (Chang 2013, Saberi 2013).

The initial decrease in mean particle diameter with increasing thyme oil level (Figure 3.2) can be attributed to the fact that the presence of the essential oil facilitated the formation of small droplets during spontaneous emulsification. On the other hand, the increase in mean particle diameter observed at high thyme oil levels can be attributed to rapid droplet growth due to Ostwald ripening, i.e., the movement of oil molecules from small to large droplets through the intervening aqueous phase (Taylor, 1998). The most important factor determining the susceptibility of a nanoemulsion to this instability mechanism is the solubility of the oil phase in the aqueous phase. The major constituents of thyme oil have appreciable water-solubility (thymol = 1g/L (Sigma Aldrich, 2016), carvacrol = 301.1 mg/L (Sigma Aldrich, 2016) and p-cymene = 23.4 mg/L at 25°C (Sigma Aldrich, 2015)), and so they are particularly prone to Ostwald ripening. Previous studies have also reported that nanoemulsions formed using relatively polar oils (such as flavor or essential oils) are highly unstable to Ostwald ripening (McClements et al., 2012, Chang et al., 2012, 2013). The presence of sufficient levels of MCT in the oil phase of the nanoemulsions inhibits Ostwald ripening due to a compositional ripening effect, i.e., as the essential oil moves from small to large droplets it causes the droplets to have different internal compositions, which generates an osmotic pressure that opposes further changes in droplet size (McClements et al., 2012). Nevertheless, if too much MCT is added to the oil phase then the efficiency of spontaneous emulsification is decreased (leading to larger droplets). In addition, the total amount of the antimicrobial agent present within the nanoemulsions would be reduced. Consequently, an optimum MCT
level is required (around 40%) to form stable nanoemulsions containing small droplets with high antimicrobial loadings.

The composition of the oil phase also had a major impact on the antimicrobial activity of the nanoemulsions, as determined by measuring their MIC against a cocktail of *Salmonella sp.* strains (**Figure 3.2**). The MIC was calculated in parts per million (ppm) based on the thyme oil level present in the nanoemulsions. As the thyme oil level in the nanoemulsions increased (corresponding to a decrease in MCT level), there was a decrease in the MIC, indicating that their antimicrobial efficacy was increased (**Figure 3.2**). If the ripening inhibitor did not have an impact on the antimicrobial efficacy, then all of the nanoemulsions should have had the same MIC. The observed effect can therefore be attributed to the impact of the ripening inhibitor on the partitioning of the antimicrobial constituents between the nanoemulsion droplets and the bacterial cell membranes. At high MCT levels (low thyme oil levels), more of the antimicrobial constituents may be located in the nanoemulsion droplets, and are therefore unavailable to interact with the microbial cells. Conversely, at low MCT levels (high thyme oil levels), more of the antimicrobial constituents will be solubilized in the microbial cell membranes (rather than in oil droplets), thereby disrupting their normal function. Other studies have also reported that the presence of an oil phase can reduce the antimicrobial activity of essential oils, which was attributed to the partitioning of the hydrophobic antimicrobial molecules into the oil phase and therefore away from the bacteria (Gutierrez et al., 2008, Mejlholm and Dalgaard, 2002). In summary, the optimum level of thyme oil required to form nanoemulsions with small initial droplet sizes, good long-term
stability, and high antimicrobial efficacy, which was around 50% thyme oil and 50% MCT.

3.3.2. Influence of ripening inhibitor fatty acid chain length on nanoemulsion properties

In this series of experiments, the impact of using ripening inhibitors with different molecular characteristics (average fatty acid chain lengths) on nanoemulsion formation, stability, and antimicrobial activity was examined. In particular, varying the ratio of medium chain triglycerides (MCTs) to long chain triglycerides (LCTs) in the ripening inhibitor phase was examined. LCTs are known to be particularly effective at inhibiting OR because of their extremely low water-solubility (Wooster et al., 2008, Chang, 2012, McClements et al., 2012). On the other hand, LCTs are known to be ineffective at forming small droplets using the spontaneous emulsification method (Komaiko and McClements, 2015). Currently, little is known about the LCT-to-MCT ratio on the antimicrobial efficacy of essential oil nanoemulsions. Corn oil was utilized as a commonly used food-grade representative of LCTs. Based on the results of the previous section, the influence of LCT-to-MCT ratio was examined for nanoemulsions containing 40% thyme oil and 60% ripening inhibitor (Figure 3.3).

Using Corn oil as a LCT source, the mean droplet diameter of the nanoemulsions increased as the concentration of corn oil increased. As the proportion of LCT-to-MCT used as the ripening inhibitor was increased, (Figure 3.3). The mean droplet diameter was relatively small (< 100 nm) from 0 to 50% LCT, but relatively high when 100% LCT was used (> 250 nm). The nanoemulsions that contained ≤ 50% LCT remained stable to
Figure 3.3. MIC and droplet size of emulsions made with thyme oil (40%) mixed with 60% ripening inhibitors (corn oil and MCT). Emulsion were prepared with 40% thyme oil and 60% ripening inhibitor with varying amounts of long chain triglyceride (LCT, Corn oil) and mid-chain triglyceride (MCT oil). The MIC was based on the concentration of thyme oil in the system. The number of days before visible phase separation due to creaming was observed is given below or above the average droplet diameter (as measured at day 0). The “*” symbol means that the MIC was above the upper threshold value (10,000 ppm).
creaming for over 30 days, while those containing higher LCT levels creamed after only a few days storage. Previous studies suggest that it is difficult to form nanoemulsions by spontaneous emulsification when LCT is used as the oil phase because it suppresses the generation of the bicontinuous microemulsions believed to be an important intermediate phase involved in the generation of fine oil droplets (Saberi et al., 2013, Komaiko and McClements, 2015). The fact that appreciable creaming was observed at higher LCT levels can therefore be attributed to the relatively large initial size of the oil droplets formed in these systems.

The composition of the ripening inhibitor (LCT-to-MCT ratio) also had a pronounced influence on the antimicrobial activity of the thyme oil nanoemulsions. As the proportion of corn oil increased, the MIC decreased (Figure 3.3), which suggested that nanoemulsions with higher LCT-to-MCT ratios were more effective at killing bacteria. There are a number of possible mechanisms that may account for this observation: (i) the nature of the oil type might influence the partitioning of the antimicrobial agents between the oil droplets and bacterial cell membranes; (ii) different oils may contain minor constituents that are themselves antimicrobial agents; (iii) differences in the size of the oil droplets might have influenced their ability to interact with the bacterial surfaces.

3.3.3. Influence of ripening inhibitor type and storage temperature on nanoemulsion properties

Four different commonly used commercial food oils were used as part of the ripening inhibitor phase: canola oil, corn oil, palm oil (all LCTs) and coconut oil (a MCT). These oils were mixed at a 1:1 mass ratio with MCT to form nanoemulsions that
contained 40% thyme oil and 60% ripening inhibitor phase as part of the oil phase. The change in droplet size of the nanoemulsions was then measured when they were stored at either 4 or 20 °C for 30 days, as well as their antimicrobial efficacy (Table 3.1). The nanoemulsions prepared from coconut oil and MCT had a similar mean droplet diameter, polydispersity index, and MIC as the ones prepared using only MCT, which might be expected since coconut oil mainly consists of medium chain triglycerides and would therefore be expected to have similar physicochemical properties as MCT. There were no major differences between the nanoemulsions formulated with different kinds of commercial LCTs (canola, corn, or palm oil). However, the nanoemulsions formed using corn oil and palm oil had relatively small initial particle sizes and had the lowest MIC values, which means that they should be the most effective antimicrobial systems. There was little change in the mean particle diameter or polydispersity index when the nanoemulsions were stored at 4 °C for 30 days, but there was an appreciable increase in particle size when they were stored at 20 °C (Figure 3.4, Figure 3.5). This effect may be attributed to an increase in either Ostwald ripening and/or coalescence with increasing temperature. The polyoxyethylene head groups of non-ionic surfactants (like Tweens) are known to become progressively dehydrated as the temperature is raised, which alters the optimum curvature of the surfactant monolayer and reduces the range of the steric repulsion. Both of these effects can promote droplet coalescence at elevated temperatures (Saberi et al., 2015). Despite the increase in mean particle diameter during storage, all of the nanoemulsions appeared visibly stable to creaming throughout the 30-day storage period, which can be attributed to their small droplet sizes.
Figure 3.4. Influence of oil phase composition on the particle size distribution at day 1 and day 30. 10 wt% oil-in-water nanoemulsions were used containing the following oil phase compositions: 40% thyme oil and 60% MCT (4:6 Thyme:MCT), 40% thyme oil, 30% MCT, and either 30% corn oil or canola oil (4:3:3 thyme:MCT:corn, or 4:3:3 thyme:MCT:canola, respectively) at 20°C. For the sake of clarity, the successive relative intensity values were shifted up the y-axis using fixed increments of 15%.
Figure 3.5. Influence of oil phase composition on mean droplet diameter during storage at 20°C. 10 wt% oil-in-water nanoemulsions were used containing the following oil phase compositions: 40% thyme oil and 60% MCT (4:6 Thyme:MCT), 40% thyme oil, 30% MCT, and either 30% corn oil or canola oil (4:3:3 thyme:MCT:corn, or 4:3:3 thyme:MCT:canola, respectively).
Table 3.1. Minimum inhibitory concentrations (MIC), mean droplet diameters, polydispersity indexes, and of nanoemulsions containing different types and amounts of ripening inhibitors.

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Ripening Inhibitor(s)</th>
<th>MIC (ppm)</th>
<th>Mean Droplet Diameter Z-average (nm)</th>
<th>Polydisperity Index (PDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Thyme</td>
<td>None</td>
<td>425</td>
<td>Day 0: 25 ± 3.0</td>
<td>Day 0: 0.23 ± 0.01</td>
</tr>
<tr>
<td>40% Thyme</td>
<td>60% MCT</td>
<td>&gt;10,000</td>
<td>Day 0: 50 ± 1.8</td>
<td>Day 0: 0.27 ± 0.04</td>
</tr>
<tr>
<td>40% Thyme</td>
<td>30% MCT 30% Coconut</td>
<td>&gt;10,000</td>
<td>Day 0: 47 ± 1.7</td>
<td>Day 0: 0.19 ± 0.08</td>
</tr>
<tr>
<td>40% Thyme</td>
<td>30% MCT 30% Corn</td>
<td>7000</td>
<td>Day 0: 61 ± 9.1</td>
<td>Day 0: 0.26 ± 0.02</td>
</tr>
<tr>
<td>40% Thyme</td>
<td>30% MCT 30% Palm</td>
<td>6000</td>
<td>Day 0: 52 ± 0.72</td>
<td>Day 0: 0.29 ± 0.02</td>
</tr>
<tr>
<td>40% Thyme</td>
<td>30% MCT 30% Canola</td>
<td>10,000</td>
<td>Day 0: 51 ± 2.7</td>
<td>Day 0: 0.27 ± 0.07</td>
</tr>
</tbody>
</table>

1 Tween 80 was used in fixed ratio of 1:1 with the oil phase and all of the nanoemulsions with one or more ripening inhibitors were stable over 30 days.

2 Without the addition of ripening inhibitors, emulsions were unstable and formed a creaming layer at day 0.

3 The MIC >10,000 ppm showed no microbial inhibition.

4 Capital letters were used to indicate differences between measurements at day 0 and 20, means with the same letter letters indicate data was not significantly different (P≥0.05). Means with same lower case letter in the same column are not significantly different (P≥0.05).
3.3.4. Influence of ripening inhibitors on partitioning

It was hypothesized that the observed differences in antimicrobial activity of thyme oil nanoemulsions with different oil phase compositions was due to differences in the partitioning of hydrophobic antimicrobial agents between the oil droplets and the bacterial cell membranes. For this reason, the partitioning of the antimicrobial agents arising from the thyme oil was analyzed using a combination of dialysis and chromatography. The pore size of the dialysis bag was chosen to be small enough to prevent the free movement of nanoemulsion droplets through it. Consequently, it was possible to establish two hydrophobic domains in the same system: (i) the droplets in the thyme oil nanoemulsion; (ii) the droplets in the MCT emulsion. This system was used as a model to study the transfer of hydrophobic antimicrobial components from the thyme oil droplets to another hydrophobic domain (representative of bacteria cell membranes). In the case of the MCT emulsions, the oil droplet concentration was the same inside and outside of the dialysis bag (based on the oil phase of the emulsion). HPLC analysis of the pure thyme oil indicated that it contained, 1.27, 1.06, and 0.098 mmol/ml of thymol, ρ-cymene, and carvacrol respectively. To observe whether there will be a transfer of active ingredients through the continuous phase, eight dialysis bags of MCT emulsion were placed in 4:6 thyme oil:MCT nanoemulsion. The concentration of components inside the dialysis bags was then measured by HPLC at different time points and the changes in concentration ratios within the MCT emulsion are shown in Fig. 5. The concentration curves were fitted with Eq. 1 and the parameters of the model for each antimicrobial agent are reported in Table 3. The kinetic parameters ($k_1$) indicated that the overall partition rate from the external hydrophobic domain could be ranked in order of carvacrol
Figure 3.6. Normalized concentrations of antimicrobial thyme oil constituents present in MCT emulsions collected from inside a dialysis tube that was submerged in nanoemulsion with oil composition of 4:6 Thyme:MCT at different time points for 24 hours. A) thymol, B) p-cymene and C) carvacrol.
Table 3.2. Fit parameter and goodness of fit for model Eq. (1)

<table>
<thead>
<tr>
<th>Eq. 1 Parameters and Goodness of Fit</th>
<th>Thymol</th>
<th>p-Cymene</th>
<th>Carvacrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ (min)</td>
<td>359.7</td>
<td>524.2</td>
<td>113.0</td>
</tr>
<tr>
<td>$k_2$ (-)</td>
<td>0.71</td>
<td>0.55</td>
<td>0.92</td>
</tr>
<tr>
<td>Mean Square Error (MSE)</td>
<td>0.0020</td>
<td>0.0063</td>
<td>0.021</td>
</tr>
<tr>
<td>Adjusted $R^2$</td>
<td>0.995</td>
<td>0.982</td>
<td>0.995</td>
</tr>
<tr>
<td>$1/k_1$ (min$^{-1}$)</td>
<td>0.0028</td>
<td>0.0019</td>
<td>0.0089</td>
</tr>
</tbody>
</table>
The value of $k_2$ suggest that the carvacrol partition is the most effective ($k_2$ closer to 1) and that the full capacity of the MCT emulsion has not been completely reached in the case of other two antimicrobial agents. The observed differences in the rate of transfer of the active components from the external to internal droplets may be attributed to thermodynamic and kinetic effects. Entropy of mixing effect will determine the final composition of the system, and therefore the driving force for the process. Mass transfer through the aqueous phase will determine the kinetics of the process, with the rate increasing as the water-solubility and diffusion of the active components increases. The logarithm of the octanol/water equilibrium partition coefficient of the active components has been found to be: $\log P_{\text{O/W}} = 3.64, 3.30, 4.10$ for carvacrol, thymol, and $\rho$-cymene, respectively (Banerjee et al., 1980, Griffin et al. 1999, Ultee et al., 2002). The water solubility of the active components has been reported to be 301, 1000, and 23 mg/L for carvacrol, thymol, and $\rho$-cymene, respectively (Sigma Aldrich). As expected, there is a correlation between the oil-water partition coefficient and the water-solubility: the higher the logP value, the lower the solubility. However, there was not a correlation between the rate of transfer and water solubility of active components. All of the active component molecules have fairly similar translational diffusion coefficient(Chemspider.com). It is possible that there may have been differences in the ability of the different types of active components to cross the oil-water interfaces of the oil droplets, but further work is needed in this area to establish the molecular origin of this effect. The difference in the amount of partitioned antimicrobial components in Table 3.3 and Figure 3.6 at 24 hours was predicted to be $>\text{thymol} > \rho$-cymene (Table 3.2, Figure 3.6).
Table 3.3. Concentrations of antimicrobial thyme oil constituents within the dialysis tubing collected either with MCT emulsions or buffer after 24 hours incubation.

<table>
<thead>
<tr>
<th>External Nanoemulsion Oil phase</th>
<th>Internal Aqueous phase</th>
<th>Internal MCT emulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential oil</td>
<td>Carrier oil</td>
<td>MIC (ppm)</td>
</tr>
<tr>
<td>40% Thyme 60% MCT</td>
<td></td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>40% Thyme 30% MCT 30% Corn</td>
<td></td>
<td>7000</td>
</tr>
<tr>
<td>40% Thyme 30% MCT 30% Canola</td>
<td></td>
<td>10,000</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Lower case letters were used to indicate statistical analysis of data within columns. Uppercase letters indicate statistical analysis between concentrations in the aqueous phase and MCT emulsions for each chemical component. Means with same letter are not significantly different (p<0.05). \textsuperscript{2}nd Not detected
due to difference in parameters of thickness and pores between the batches of dialysis tubing.

Next, the effect of ripening inhibitors on the partitioning of active components at 24 hours were then measured. A thyme oil nanoemulsion (formed using different ripening inhibitors) was placed within a beaker, and then a dialysis bag containing either buffer solution or MCT emulsion was submerged. After 24 hours, the concentration of antimicrobial thyme oil constituents (thymol, carvacrol, and ρ-cymene) that had diffused into buffer solutions or MCT emulsions collected from inside the dialysis tubes was measured using HPLC (Table 3.3). As expected, higher levels of all three major thyme oil constituents were transferred into the dialysis tube containing the MCT emulsions than the one containing the buffer solution (Table 3.3), which can be attributed to the fact that these constituents are predominantly hydrophobic, determined by partitioning coefficient. Nevertheless, there were detectable amounts of thymol and carvacrol within the buffer solutions, shows that they are relatively water-soluble, and suggests these compounds may diffuse from the oil droplets to the bacterial membranes through the intervening aqueous phase. Moreover, an appreciable amount of all measured antimicrobial constituents were transferred from the thyme oil nanoemulsion droplets to the MCT emulsion droplets, suggesting that transfer through the aqueous phase occurred for all three antimicrobial constituents.

The amount of the antimicrobial constituents transferred into the MCT emulsions trapped in the dialysis bag depended on the ripening inhibitor composition in the thyme oil nanoemulsions outside the dialysis bag (Table 3.3). In particular, there appeared to be a higher level of all three antimicrobial components transferred from the thyme/corn oil/MCT nanoemulsions than in those containing only thyme/MCT or thyme/canola oil/MCT. This was especially true for ρ-cymene, which is the most hydrophobic of the three antimicrobial
constituents, and is known to have synergistic effect with other antimicrobial components in disrupting microbial cell membranes (Ultee et al., 2002). Interestingly, the antimicrobial efficacy of the nanoemulsions containing corn oil/MCT was also the highest, i.e., they had the lowest MIC (7000 ppm) of the three samples examined (Table 3.1). A possible explanation of this phenomenon is that a larger fraction of antimicrobial constituents was transferred into the bacterial membranes for the nanoemulsions containing corn oil.

3.4. Conclusion

In this study, the formation, stability, and antimicrobial efficacy of thyme oil nanoemulsions was found to depend on the type and level of ripening inhibitor used to prevent Ostwald ripening. An optimum amount of ripening inhibitor (around 40% of the oil phase) was required to form nanoemulsions containing small oil droplets that were stable during storage, but still maintained antimicrobial activity. Nanoemulsions formulated using MCT mixed with either corn or MCT oil were the most effective at inhibiting microbial growth (lowest MIC). This effect was attributed to their ability to deliver higher levels of hydrophobic antimicrobial constituents (such as thymol, carvacrol, and ρ-cymene) from the thyme oil droplets into the microbial cell membranes. The transfer of these hydrophobic compounds from the delivery systems may have occurred through two different mechanisms. First, the antimicrobial constituents may be directly transferred from the oil droplets to the bacterial cells through the intervening aqueous phase. Second, the antimicrobial constituents may be transferred when the oil droplets collide with the surfaces of the microbes. The fact that an appreciable amount of the antimicrobial constituents was transferred from the thyme oil nanoemulsions to MCT emulsions suggests that the collision of oil droplets with microbial surfaces is not necessary for transfer.
Our results have important implications for the formulation of effective antimicrobial delivery systems for essential oils.
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Substance Generally Recognized as Safe, 21 C.F.R. pt. 182 (2016)


