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Food Colloids As Carrier Systems For Antimicrobials

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FOOD COLLOIDS AS CARRIER SYSTEMS FOR ANTIMICROBIALS

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by

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DEDICATION

My family and my teachers.

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ABSTRACT

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Colloidal dispersions such as oil-in-water or water-in-oil emulsions have found widespread use in the food industry. Oil-in-water emulsions consist of three principal components i.e. oil dispersed in the form of droplets, water surrounding the droplets as the continuous phase, and emulsifiers comprising the interface. Because of the complicated interaction among components, it is often difficult to predict the physicochemical properties and final functionalities of emulsions. Nevertheless, the structural and functional features of emulsions allow scientists to create many unique emulsions that may serve as suitable carriers for lipophilic functional compounds. These functional compounds may include antioxidants, flavors, colors and antimicrobials, the latter which is the principal topic of this thesis. Incorporation of food antimicrobials in emulsions could create value-added emulsions that may improve the safety and quality of a variety of foods, but to date, few systematic studies on their formulation have been reported.

The objective of this thesis was therefore to formulate food emulsions that are physicochemically stable and able to deliver antimicrobial compounds to microbial target

sites. Two antimicrobial agents, N-α-lauroyl-L-arginine ethyl ester monohydrochloride, (LAE) and eugenol were used as model compound to be incorporated into the colloidal food dispersion. The two antimicrobials were selected because they are either amphiphilic (LAE) or predominantly lipophilic (eugenol). When emulsions were formulated with eugenol, an essential oil component, it was found that O/W emulsions were only stable when emulsions were formulated with other lipids (hexadecane, dodecane, tetradecane, and corn oil). Above a critical loading of the carrier lipid with eugenol, Ostwald's ripening led to rapid destabilization while above this critical loading concentration, the ripening rate was greatly reduced and depended on type of carrier lipid and concentration of eugenol. Alternatively, when emulsions were formulated with LAE as emulsifier, results indicated that emulsions were not stable to aggregation and coalescence. Consequently, LAE had to be combined with a nonionic surfactant (Tween 20) to improve the emulsion stability. Higher Tween20 composition led to more stable emulsions droplets. Both systems (emulsions with either eugenol or LAE) had high antimicrobial efficacies and were able to completely inhibit microbial growth at concentrations that depended on the type of microorganisms and formulation of the emulsions. Generally, eugenol were able to more effectively inhibit the growth of *E.coli* O157:H7 while LAE containing emulsions were more effective against *L.monocytogenes.* Finally, a food emulsion was formulated that contained both antimicrobial agents; eugenol in the lipid phase and LAE in the droplet interface. Interestingly, stability of these emulsions depended both on the LAE and eugenol loading. The antimicrobial activity in this double antimicrobial emulsion was high but was principally influenced by the interfacial formulation that is the ratio of LAE to Tween 20. The combined emulsion

similar to the LAE stabilized emulsion more effectively inhibited growth of *L.monocytogenes*.

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CHAPTER 1

INTRODUCTION

Foodborne illnesses have been a vital concern of public health officials due to the high costs and losses of productivity associated with their outbreak. Foodborne illnesses are caused by ingesting bacteria, fungi, parasites, viruses, toxins, or other harmful substances present in contaminated foods. Foodborne diseases associated with shiga toxin-producing *Escherichia coli* and the *Salmonella* bacterium have been estimated to cost the national economy annually $445,857,703 and $2,467,322,866 in 2006, respectively [3]. Therefore, there is a high interest to develop new methodologies to reduce or eliminate foodborne pathogens from food matrices. One strategy that is actively pursuit is to develop novel delivery systems for food antimicrobials that would enable the more widespread use of available antimicrobials compounds in a wide variety of food systems while maintaining or improving their efficacies [4].

Food antimicrobial agents are compounds that have been defined as “chemical compounds present in or added to foods, food packaging, food contact surface, or food processing environments that inhibit the growth of, or inactive pathogenic or spoilage microorganism” [5]. They can be used to preserve quality and enhance safety of a variety of food [6]. Naturally-occurring antimicrobials can be obtained from animal, plant, microbial and mineral source. Of these, antimicrobial essential oils obtained from plant sources are of particular interest because they also exhibit strong antimicrobial activities and they are used as flavors. Essential oils and their extracts are aromatic liquids composed of a great variety of more or less volatile low-molecular weight compounds

that typically have a preference to associate with lipid phases. Many of these compounds are phenolics which have been linked to their remarkable antibacterial properties. Most importantly, essential oils and their extracts have been are classified by the US FDA as "Generally Recognized as Safe" (GRAS) and thus can be readily used in a food product. For example, eugenol is an essential oil component extracted from clove [7, 8]. It has been shown to inhibit growth of *Listeria monocytogenese* [9, 10], *E.coli* O157:H7 and *Vibrio paraheamolyticus* amongst others. The compound has thus attracted increased attention as a potential inhibitory agent to improve the microbial safety of product. Eugenol is soluble in ethanol or ether but only sparingly soluble in water with typical water solubility of less than 0.01%.

Another interesting and novel antimicrobial that has recently emerged is the ethyl ester of lauramide of arginine monohydrochloride, also referred to as lauric arginate (LAE), available commercially under the trade name Mirenat ($C_{20}H_{41}N_4O_3Cl$) [1, 11]. LAE is a synthetically-derived antimicrobial that can be added to all perishable food including meat product [12, 13]. Most interestingly, LAE is a cationic surfactant in addition to being a strong antimicrobial that is it adsorbs readily at surfaces due to its amphiphilic characteristics and it is positively charged. The L-arginine part of the molecules constitutes the hydrophilic head group of the surfactant while the fatty acid chain of lauric group provided the hydrophobic tail of the surfactant. Synthetic chemical preservatives have typically been rejected because of concerns with their toxicological safety, however, in the case of LAE, the link between the arginine group and the lauric group is a simple ester bond that is readily hydrolyzed by the human digestion system generating arginine and a fatty acid, which is then further digested [14]. The advantage

of using LAE is thus its low toxicity and its remarkable antimicrobial efficacies. LAE was approved as GRAS on September 1st, 2005. In addition, USDA approved its use in meat and poultry products at level up to 200 parts per million (ppm) of ethyl-N-Alpha-lauroyl-L-arginate hydrochloride [11]. The antimicrobial properties of LAE have been attributed to its action on the cytoplasmic membrane of microorganism, that is the positively charged compound binds to the negatively charged bacterial membrane and induces leaks that alter the membrane potential required to generate ATP [15].

Oil-in-water emulsions are liquid-liquid dispersions of two completely or partially immiscible liquids (in this case oil and water) with one phase being dispersed in the second in the form of droplets. Emulsions are a widely used class of food products and examples include products such as milk, cream, salad dressing, mayonnaise, beverages, and sauces. In addition to the food industry, they are heavily used in other industries in products such as pharmaceuticals, petrochemicals, and cosmetics [16, 17]. Emulsions are not only used to change texture, flavor and appearance of foods, but they are also used to delivery biologically functional components that are lipophilic in nature such as antioxidants, flavors and nutraceuticals. Interestingly, only few studies have been conducted though to investigate their suitability to act as carrier systems for antimicrobials. In principle, emulsions could serve as antimicrobial delivery systems in two ways: (1) by containing an amphiphilic antimicrobial (such as the above mentioned lauric arginate) adsorbed at the emulsion droplet interface or (2) by containing a lipid antimicrobial (such as the above mentioned eugenol) that is part of the lipid phase. Moreover, these two approaches could possibly be combined two produce a “double” antimicrobial emulsion based preservation system with improved efficacies.

In this study, we hypothesize that the effectiveness of an emulsion based antimicrobial delivery systems depends on the emulsion characteristics, e.g. droplet size and concentration and their interaction with the surrounding environment, e.g. their interaction with bacterial surfaces (e.g. electrostatic, Van der Waals, etc) to facilitate direct contact with microorganisms or through molecular transport of encapsulated compounds through the intervening continuous phase to the surface of the microorganisms (e.g. via partitioning of active compounds from the lipid phase into the water phase) [18-22]. Surface charges of bacterial cells and emulsion droplets thus may play a key role in emulsion stability [19]. Most importantly, for the emulsions to function as carriers, they have to maintain their physical stability i.e. the ability of the emulsion to maintain its dispersed state under a variety of environmental stresses such as pH, salt, temperatures [19, 23-25]. Surface charges of bacterial cells and emulsion droplets played key role in emulsion stability [19].

The objective of this study was to test the above presented hypothesis by formulating emulsions composed of an amphiphilic antimicrobial (lauric arginate), a lipid antimicrobial (eugenol) or both. To this purpose, the influence of formulation and environmental conditions on emulsion stability and antimicrobial efficacy was determined. The goal of the project was to design a colloidal system that can achieve adequate stability while exhibiting antimicrobial activity over a desirable period of time.

CHAPTER 2

LITERATURE REVIEW

Food colloids are multi-phase systems containing particles or other structures with characteristic spatial dimensions in the colloidal size range i.e. foams, gels, and emulsions [25]. Oil-in-water emulsions are one of the most important classes of food colloids and include products such as for example milk, cream, dressing, mayonnaise, beverage, soups, and sauces [16]. This project utilizes oil-in-water emulsions as dispersant systems for lipid or amphiphilic antimicrobials.

2.1 Emulsions

2.1.1 Definition

Emulsions are fine dispersions of one liquid in a second, partly or completely immiscible liquid in the form of droplets [26]. The droplets typically have mean sizes that are in the colloidal range *e.g.* 10-10,000 nm. [16]. Milk, cream, dressings, mayonnaise, beverages and soups are examples of products that exist in the form of emulsions. Oil-in-water (O/W) emulsions generally consist of oil droplets dispersed in an aqueous phase. In contrast, margarine and butter consist of water droplets dispersed in an oil phase also known as water-in-oil or W/O emulsion. The droplets are also sometimes referred to as the internal, dispersed or discontinuous phase while the surrounding liquid is referred to as the external or continuous phase [16]. In addition to simple O/W and W/O emulsions, multiple emulsions exist that are composed of dispersions in dispersions such as for example an internal phase dispersed in a second internal phase that is

dispersed in a continuous phase such as water-in-oil-in-water W/O/W and oil-in-water-in-oil O/W/O type emulsions. These emulsions may have improved properties compared to conventional emulsion but they are typically less frequently used in the food industry due to the complexity and higher costs involved in their fabrication.

Food colloids are very complicated systems because of the interactions and molecular properties of the three principal ingredients (oil, water, and emulsifier). Factors influencing emulsion properties include ingredient interaction, surfactant-surfactant, and surfactant-droplet interactions, lipid oxidation, and process condition such as temperature, pressure and mechanical agitation [16, 27]. It can thus be quite challenging to produce stable emulsions. Ultimately emulsion manufacturers need to understand the role that each of the parameters play in the overall property and stability of the emulsions in order to be able to design the desired emulsion system.

2.1.2 Formulation of Food Emulsions

Two separate immiscible liquids can be converted into an emulsion by mixing the two liquids followed by reducing the size of the droplets in the premix using a unit operation called *homogenization*. After homogenization, the oil and water dispersion generally have a thermodynamically driven tendency to phase separate that is the oil droplets merge with each other until the two phases rapidly separate into two distinct phases. This is because the presence of a large interfacial area between these molecules of different polarity is thermodynamically highly unfavorable. Emulsions are thus thermodynamically unstable, but may be kinetically stabilized for some time by using

amphiphilic and surface active molecules that adsorb at the interface and provide repulsive interactions that prevent droplet merging and aggregation [16, 26].

Control of the droplet size is one of the most critical parameters required to produce a desirable food colloidal system since the size of the droplets contributes to the stability, appearance, texture, and taste of the emulsion. The factors which influence the droplet size include emulsifier type and concentration, volumetric energy input during the homogenization, composition of component phases, and temperature. In the homogenizer, two processes occur simultaneously resulting in the generation of small droplets from a premix that contains larger droplets. Firstly, the volumetric energy input (applied pressure) induces droplet deformation and disruption generating smaller droplets with new interfaces. Generally, the larger the volumetric energy input the smaller the droplets. The droplet disruption also depends on the viscosity ratio between the two phases which may depend on the temperature of the system. Secondly, surfactants rapidly absorb at the new interfaces creating an interfacial membrane or layer that induces repulsive interactions and prevent re-coalescence. The droplet size thus depends on the molecular properties of the emulsifiers governing the rate of surface adsorption and the degree of induced repulsive and attractive colloids interactions *e.g.* van der Waals, steric, electrostatic [16]. In food emulsions, two classes of surface-active species are predominantly used: (i) small-molecule surfactants such as monoglycerides, sucrose ester, and others and (ii) macro-molecular emulsifiers such as protein or modified starches [25]. In this project, we are predominantly focusing on using small molecule surfactants since we hypothesize that these may form mixed layers with one of the target antimicrobials (lauric arginate).

2.1.3 Surfactants and Interfacial Properties

In food emulsions, surfactants are used to stabilize the dispersion against breakdown [28]. Surfactants are amphiphilic molecules, i.e. molecules that consist of both a hydrophilic and a hydrophobic part. These molecules have thus a tendency to accumulate at surfaces where they may interact simultaneously with both the lipophilic and hydrophilic phases. The main functions of a surfactant are to (i) lower the surface tension thereby reducing the energy required to generate new surfaces and allowing for easier generation of small droplets in the emulsification step and (ii) to provide for long-term stability of the emulsion after their preparation by providing a barrier to coalescence via repulsive interactions between the adsorbed layers of droplets [29].

The hydrophobic and polar groups of surfactants determine their physical and chemical behavior [30]. Understanding their properties is critical to using them in a wide range of application such as detergents, emulsifiers, adhesives, and lubricants [31]. One parameter that has traditionally been used to determine whether surfactants preferentially reside in the water or oil phase is the so-called hydrophilic-lipophilic balance number (HLB) which has also been used to predict the type of emulsion that can be formed by a surfactant [16, 32]. HLB values are calculated from the ratio of the hydrophilic head group to the lipophilic tail group. Surfactants with HLB values in the order of 3-6 typically stabilize water-in-oil emulsions while surfactants with HLB values of 8-15 are more suitable to stabilize oil-in-water emulsions. Surfactant can also be classified by the charge of the polar group, e.g. anionic, cationic, zwitterionic and nonionic. Anionic surfactants (e.g. fatty acid salt, stearyl) are surfactants that have at least one negatively charged group attached to the hydrophobic tail. Examples of anionic head

groups include $–CO_2^-$, $-SO_3^-$, PO_3^{-2}, $–OSO_3^-$. Conversely, cationic surfactants contain positively charged groups (typically nitrogen-based groups). The nitrogen group can be protonated or unprotonated. Therefore, the charge of cationic surfactant and for that matter the charge of some negatively charged surfactants may be influenced by the system pH. Finally, zwitterionic surfactants (e.g. lecithin) may carry both positive and negative charges which may yield a complex pH-dependent charge behavior. Polyelectrolytic molecules such as proteins for example, may be have a net negative charged above their isoelectric point, but be fully protonated and positively charged below their isoelectric point. Finally, the hydrophilic group may not carry any charges, in which case the emulsifier is referred to as a nonionic surfactant (e.g. monoglycerides, Tweens, Polysorbates). Nonionic surfactants are the most widely used group of surfactants used in food applications. These surfactants typically have polyether or polyhydroxyl units that consist of ethyoxylated alcohol and alkylphenols [30]. Below we will now briefly discuss the principal surfactant characteristics that are of importance in the formation of emulsions.

Surface tension. Surface tension has been defined as the increase in the overall free energy of a system with increasing surface area at constant temperature and pressure.

$$\sigma = \gamma = \left(\frac{dG}{dA}\right)_{p,T=const} \tag{2.1}$$

where G is the free energy of the system and A is the surface area. The ability of a surfactant to lower the interfacial or surface tension is one of the critical parameters and indicates an overall lowering of the unfavorable interactions between the dispersed and continuous phase molecules upon adsorption of the surfactant [33].

Adsorption isotherm. The amount of a surfactant that can adsorb at an interface is of importance to the proper formulation of stable emulsions. The concentrations of a surfactant at the interface can be thermodynamically derived from the measurement of the change in surface tension with surfactant concentration in the bulk:

$$\Gamma = \frac{1}{RT}\left(\frac{d\gamma}{dlnc}\right) \tag{2.2}$$

where Γ is the surface coverage in mol/m^2, γ is the interfacial tension, c the concentration of surfactant in the bulk, R the universal gas constant and T the absolute temperature of the system. It should be noted that while thermodynamics predicts equilibrium conditions, mass transport processes continue to occur even in the equilibrium state, i.e. surfactant molecules are repeatedly exchanged between the bulk phase and the interface. Equation 2.2 predicts that in general, as more surfactant is added to the system, more surfactant molecules will adsorb at the interface. This is however only true up to a specific concentration (the critical micellar concentration). Above this critical concentration, excess surfactant will remain in the bulk and self-assemble to form a variety of structures [30, 34].

Adsorption kinetic. Another characteristic of surfactants is that their adsorption at newly formed interfaces doesn't occur instantaneously; rather the transport requires a certain amount of time to complete. During this time, the surface tension will continue to decrease as more and more surfactant molecules are located at the interface until the concentration at the interface has reached an equilibrium as determined by the bulk surfactant concentration:

$$\Gamma(t) = 2c_0\sqrt{\frac{Dt}{\pi}} \tag{2.3}$$

$$\gamma(t) = \gamma_\infty - 2RTc_0\sqrt{\frac{Dt}{\pi}} \tag{2.4}$$

Where D is the diffusion coefficient and c_o is the concentration of surfactant in the bulk. More information can be obtained from other sources [34-37]. The adsorb kinetic may play a role in the emulsification process, since slowly absorbing surfactants may not be able to sufficiently stabilize newly generated surfaces from re-coalescence during the homogenization.

Micelle Formation. The tendency of surfactants to adsorb at oil-water interfaces is due to the fact that the presence the hydrophobic part of the surfactant in either a polar solvent is thermodynamically unfavorable. The system thus seeks to remove this group from contact with the solvent molecules. This can be achieved to a certain point by adsorbing the surfactant at an interface. However, eventually, the interface will be fully covered and no more space is available for additional surfactant molecules. Instead, the surfactant molecules that are now in excess in the bulk will begin to self aggregate into a variety of colloids structures known as micelles that have morphologies that may range from small spheres, disks, ellipsoids, to long cylinders [30, 38]. The critical concentration above which this self-assembly process begins is known as the critical micelle concentration or CMC. The CMC is a key characteristic of surfactants and depends on the length of the hydrophobic tail, the nature of the head group, the valency of counterions in the solution (in case of charged surfactants), and generally the solution environment [34, 39, 40]. For example, with decreasing number of carbons in the hydrocarbon chain, the CMC decreases logarithmically for nonionic surfactants. In the

case of oil-in-water systems, the driving force for the formation of aggregates is the reduction of unfavorable water-surfactant tail interactions. Thus, micellization largely depends on the molecular properties of the hydrophobic tail and the influence of the molecular properties of the head group (size and charge) is thus typically quite small [41]. Nevertheless, the CMC of nonionic surfactants is generally 10 fold lower than that of ionic surfactant [34]. More importantly, the CMC of ionic surfactants may change with pH and presence of ions. This is because the electrostatic charge of the ionic headgroup may be altered which influences the molecular configuration of surfactants in the colloidal aggregate [42, 43]

The role of surfactants in food emulsions. As mentioned, surfactants are used to stabilize emulsions and aid in their production. The choice of a surfactant is thus critical to producing food emulsions with appropriate particle size distributions [44-46]. The mean droplet size generated during a homogenization process is governed by the dynamic equilibrium between two phenomena, droplet breakup and coalescence. Formulation and processing may affect either one or both of these mechanisms and thus influence droplet size. For instance, formulating emulsions with surfactants that are more effective in reducing the interfacial tension (e.g. ultralow tension surfactants) can noticeably improve the droplet disruption process [31, 47]. On the other hand, the headgroup properties of surfactant e.g. nonionic, anionic, and cationic can affect coalescence due to greater or lesser repulsive interactions. [48-50]. Surfactants have also been reported to influence the rate of Ostwald ripening, that is the process whereby larger emulsion droplets grow at the expense of smaller one [51, 52]. Generally, increasing the surfactant concentrations up to the CMC will lead to decreases in the droplet size of the emulsion [31].

The stabilization effect of emulsion droplets by colloid is due to the induction of repulsive interaction forces. In general, nonionic surfactants stabilize the system by steric interaction while ionic surfactants stabilize the system by both steric and electrostatic interactions [16]. The effectiveness of charged surfactants to prevent droplet coalescence is thus influenced by the particle charge. The concentration of cationic or anionic surfactants at oil–water interface may increase when other charged fine solids are present in the aqueous phase [24]. For example, the presence of bacteria and the nature of their cell wall may affect the stability of emulsions containing ionic surfactants while emulsions stabilized by nonionic surfactants are typically not affected by the presence of bacterial cells [19, 53].

Finally, the characteristic of surfactants may play a role in the emulsification process. For example, the presence of micelles has a profound effect on interfacial processing such as foaming, wetting, emulsification and solubilization. Surfactant micelles exist in a dynamic equilibrium with surfactant monomers in the bulk solution. With increasing numbers of micelles the flux of monomers may decrease which translates into less foaming, larger bubble sizes, larger emulsion droplet sizes and a more rapid solubilization of oil [54, 55]. Using binary surfactant mixtures, the mean emulsion droplet size obtained by a mixture of anionic sodium dodecyl sulfate (SDS) and nonionic hexa(ethyleneglycol) mono *n*-dodecylether ($C_{12}E_{16}$) was found to be lower than that using a mixture of cationic dodecyl pyridinium chloride (DPC) with $C_{12}E_{16}$ at the equivalent mole fraction of ionic surfactant at interface. This was attributed to the stronger interactions between sulphate and polyoxyethylene head groups at the interface [56]. Therefore, choice of the surfactant type, concentration including the use of mixed

surfactant systems such as ionic/ionic, ionic/nonionic, or nonionic/nonionic binary mixtures may allow for a better performance in the desired technological application [54, 57].

2.1.4 Emulsion Stability

Emulsions are thermodynamically unstable systems because the presence of large interfacial contact areas between polar and nonpolar molecules greatly increases the free energy of the system. Since any systems has the tendency to decrease the free energy, (second law of thermodynamics) [58, 59], emulsion droplets will merge and eventually phase separate to reduce the contract area between hydrophobic and hydrophilic molecules. This process requires time and the technical stability of an emulsion is thus determined by the thermodynamic driving forces and the ongoing mass transport processes. From a thermodynamic point of view, the formation of new interfaces results in a change in free energy that can be described as:

$$\Delta G_{formation} = \gamma \Delta A - T \Delta S_{config} \quad (2.5)$$

where TDS is the configurational entropy of the system. Upon emulsification, both the contact area and the entropy term increases since droplets are smaller but more evenly distributed through the system. In general though, the loss in the interfacial free energy ($\gamma\Delta A$) is typically much larger than the gain in the configurational entropy ($T\Delta S_{config}$) making this process thermodynamically unfavorable [60].

From a kinetic perspective, the stability of the emulsion is affected by their Brownian motion, gravity, or various interaction forces. The emulsion remains kinetically

stable if it has an activation energy ΔG* large enough to prevent the system from changing to its thermodynamically favorable state. To create a sufficient high activation energy ΔG*, one can use appropriate surfactants or texture modifier [16]. The destabilization of food colloids may occur by a variety of different mechanisms that include creaming or sedimentation, flocculation, coalescence, partial coalescence, phase inversion, and Ostwald ripening **(Figure 2.1)**.

Creaming or Sedimentation. Creaming is induced by gravitational forces if the density of the droplets is less than that of the continuous phase. The density differences causes and accelerating force that results in droplets moving opposite to the direction of the gravitational force, a process known as *creaming*. If the density of droplets is higher than that of the continuous phase, droplets will tend to move in the direction of the gravitational field, a process known as *sedimentation.* Gravitational separation strongly affects on appearance and texture of food emulsions often resulting in unacceptable product qualities. Moreover, the process may promote flocculation or coalescence since average distances between droplets are reduced as the droplet move to the bottom or top of the storage container. The Stokes' law equation describes the dependence of the creaming rate of particles on emulsion properties

$$v_{Stokes} = \frac{-2gr^2(\rho_2-\rho_1)}{9\eta} \tag{2.6}$$

where v_{Stokes} is the creaming velocity. η is the shear viscosity. $\rho_2 - \rho_1$ is the different density between of the continuous and dispersed phase and r is the radius of droplets. From the Stokes' law, one can infer a number of effective approaches to improve the stability of emulsions. For example, one can minimize the gravitational

separation by reducing the density difference between the two phases. This may be achieved by changing the type of oil or adding a so-called weighting agent to the oil. Creaming rates can also be reduced by decreasing the droplet size and modifying the continuous phase viscosity. Viscosity of the continuous phase can be increased by adding thickening agents, or increasing the droplet concentration, and altering the degree of droplet flocculation by altering the nature of the colloidal interactions between droplets [16, 58].

Flocculation. Flocculation is the process whereby two or more droplets associate with each other but maintain their individual integrities. This process depends on the composition of the emulsions since the composition may influence droplet-droplet interactions and continuous phase viscosities. The nature of the generated flocs varies greatly from small to large sizes and open to compact structures that affect the overall properties of emulsion e.g. stability, appearance, and sensory. The presence of flocculation in emulsion induces a shear thinning, i.e. as the shear rate increase, the floc structures are deformed and/or disrupted thereby decreasing their effective volume fraction resulting in less friction upon flow. The rate of flocculation depends on two factors; (1) the frequency of collisions between the droplets and (2) the fraction of collisions that lead to aggregation. The collision frequency depends on the movement of droplets which may be induced by Brownian motion, gravitational forces, or other applied mechanical forces during production, storage, and transport. The tendency of droplets to remain aggregated after collision on the other hand depends on the hydrodynamic and colloidal interactions between droplets.

Consequently, droplet flocculation may be retarded by preventing or decelerating droplet movements, by decreasing the droplet concentration, decreasing the disperse phase volume fraction, decreasing the density difference between the oil and aqueous phase, decreasing shear rate, increasing droplet size, increasing the viscosity of the continuous phase, or narrowing the droplet size distribution. The practical way to govern the colloidal interactions between droplets is building significant greater the repulsive than attractive interaction by regulating the type of ingredients present, the microstructure of the emulsion, and the prevailing environment conditions [16].

Coalescence. Droplet coalescence is the process where two or more droplets come into close contact and then fuse into a single larger droplet. This process is irreversible and results in a growth in the mean droplet diameter and may eventually lead to complete separation of the oil and aqueous phase. The physical processes associated with coalescence are droplet deformation and film rupture. The nature of the emulsifier in the system is a key to this process. For droplet coalescence to occur, after the droplets have come into close contact, the surfactant film must first thin and then rupture. This film stretching, and tearing causes the two interfacial membranes to merge and the interfacial tension will then rapidly result in formation of a spherical larger particle. Coalescence can be prevented by adding a thickening agent, ensuring that strong repulsion forces exist between droplets, preventing flocculation/creaming and using an interfacial layer that is resistant to thinning and rupture (e.g. by crosslinking the film to improve its rheological properties). The probability for coalescence increases as the frequency of droplet collisions increases, e.g. as the oil phase fraction increases [29].

Partial coalescence. If oil droplets are composed of partially crystalline lipid, there is an increased probability that emulsion droplets will aggregate. This is because lipid crystals protruding from the surface of the droplet prefer to be surrounded by oil molecules rather than solvent molecules. The factors that affect this process are contact time, collision frequency, droplet separation, colloidal and hydrodynamic interactions, interfacial tension, and membrane viscoelasticity. In addition, factors affecting the droplet morphology such as crystal concentration, structure, and location of fat crystal in the droplets influence partial coalescence. The degree of crystallization depends on the time-temperature profiles that the emulsion has been exposed to as well as the composition of the system. While partial coalescence is often undesirable, there are products where partial coalescence is required for their formation. For example, partial coalescence is necessary to produce ice-cream, whipping toppings, butter, and margarine. The texture and stability of these products are obtained from the partial fat network created through the aggregation of partially crystalline droplets giving the network mechanical strength and rigidity [16, 61].

Phase inversion. Phase inversion is the process whereby the two phases of an emulsion invert e.g. the emulsion changes from an O/W emulsion to a W/O emulsion or vice versa. . The system may be kinetic stable or unstable before and after the inversion. Phase inversion typically occurs if the composition or environmental conditions of a colloidal system are altered, for example, disperse phase volume fraction, emulsifier type, emulsifier concentration, solvent conditions, temperature, or mechanical agitation. Moreover, phase inversion may be induced if the functionality of the surfactant is altered e.g. by changing the ionic strength or adding additional surfactant that may alter the

interfacial composition of the droplets. Products such as butter and margarine in fact require a controlled phase inversion to yield a product with desirable appearance, texture, stability, and taste. To create butter and margarine, the emulsion (milk/cream) is cooled down to induce fat crystallization followed by shearing. The shearing cause the dispersed fat crystals to eventually phase invert to form a continuous crystal network that entraps water within it [16].

Ostwald ripening. Oswald ripening is the process whereby larger droplets grow at the expense of smaller ones. It is driven by the difference in LaPlace pressure between droplets of different sizes [52, 62-64] The rate of Ostwald ripening depends on the mean droplet size, e.g. the smaller the droplet size, the higher the Ostwald ripening rate. Ostwald ripening is driven by a molecular transport process of lipid through the continuous phase (typically water). Therefore the solubility of the lipid on water is of critical importance. For example, the higher the solubility, the faster the destabilization [65-67]. Using a disperse phase which has a low solubility in the continuous phase can retard this process. One of the few means to decrease Ostwald ripening is by adding insoluble, hydrophobic carrier oils. In addition, emulsifier such as biopolymers which may resist the decreases in droplet size as smaller droplet shrink and larger ones grow, may help reduce Ostwald ripening [68-70]. Adding more surfactants to systems prone to Ostwald ripening typically worsens the situation as the excess surfactant micelles can act as carriers for the lipid [68, 71, 72]

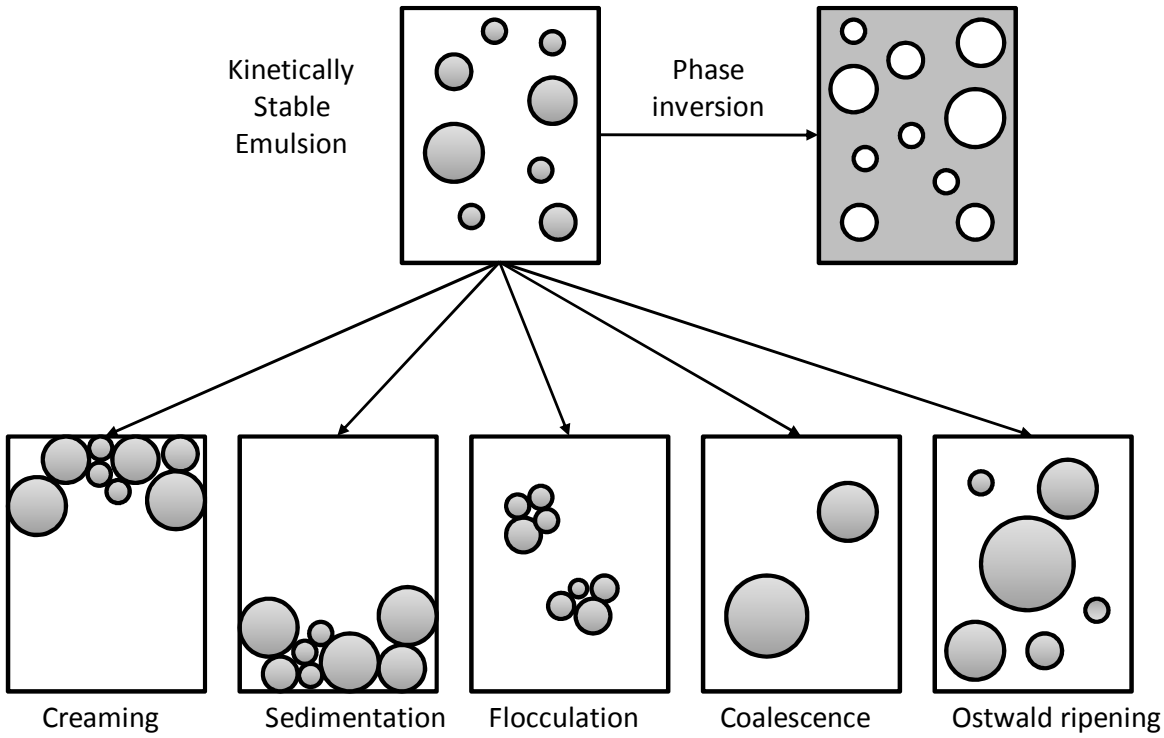


Figure 2.1. Overview over mechanisms that induce emulsions instability [16].

2.2 Food Antimicrobials

Foodborne illnesses have caused tremendous public health concerns for years. These diseases are the direct results of the indigestion of contaminated food with pathogenic bacteria and/or their toxins. Controlling growth of pathogenic bacteria can reduce the number of outbreak and assure consumers having a safe food supply. Consequently, there exists a need to develop new and improved methodologies to control the growth of pathogens in food systems. Food safety may be ensured by mechanically removing bacteria from food, inactivating bacteria through preservation techniques such as heat treatment, ozone treatment, high-intensity ultrasound, high pressure and others, preventing entry of food pathogens in the food product (packaging) or by adding compounds that can inhibit or inactivate food antimicrobials [73].

Antimicrobial agents have been defined as "…chemical compounds present in or added to foods, food packaging, food contact surfaces, or food processing environments that inhibit the growth of, or inactivate pathogenic or spoilage microorganism" [5]. They can be classified into two different types of antimicrobials; chemically-derived (synthetic) and naturally-occurring compounds. Examples of the former include weak-acid organic (acetic, lactic, benzoic, and sorbic acid), hydroperoxide, and chelator while the latter compounds include small organic biomolecules from spices and herbs such as their essential oils (menthol, carvacol, thymol, eugenol, etc.) [73, 74].

To inhibition of pathogens by antimicrobial agents is through to occur by a variety of mechanisms of action. The two principal mechanisms of antimicrobial include inhibition of proton transfer dynamics and the perturbation of membranes. Inhibitors of proton transfer are for example organic acids. In their undissociated state, the molecules may diffuse across the cell wall into the bacteria. The internal pH in the bacterial cell may then cause the compounds to lose their proton resulting in an accumulation of anions and protons inside the cells [73, 75, 76]. Antimicrobials that act as membrane pertubators must insert into the cell membrane and subsequently disrupt the membrane structure. As a consequence, the membrane may lose the ability to controlling the exchange of molecules from the inside to the outside of the cell and vice versa. In the present work, we use two antimicrobial agents that are both expected to be membrane pertubators: eugenol (an simple phenolic essential oil compound) and the amino acid-based surfactant Nα-Lauroyl-*L*-arginine ethyl ester monohydrochloride (lauric arginate).

2.2.1 Essential Oils and Their Components (EOs)

Essential oils are aromatic oily liquids obtained from plant materials such as flowers, seeds, buds, herbs, spices and roots. Compounds present in essential oils include phenylpropanes, terpenes, and aliphatic compounds such as alkanes, alcohols, aldehydes, ketones, esters, and acids [77]. EOs and their components are typically soluble in ethanol or ether but less than 0.01% soluble in water. For generations, humans have been using herbs and spices as food and to treat ailments. Remarkably herbs and spices have shown strong antibacterial properties including chemo preventive properties [74, 78]. The dominant antimicrobial compounds present in essential oils [77] are phytochemicals, such as phenolics, phenolic acid, quinones, flavonoids, and terpenoids. These compounds are synthesized by the plant as a defense against microorganism, insects, and herbivores [74, 78]. Currently more than 300 EOs in the market are used in food as flavorings, perfumes, and pharmaceutical industries [79]. Due to the constantly ongoing discovery of new substances in essential oils and the emergence of new previously underutilized herbs and spices, research on the antimicrobial activity and application of EOs and their components continues to increase.

A number of effective antimicrobial EO components such as carvacrol, thymol, eugenol, peril aldehyde and cinnamic aldehyde are highly effective agents to inhibit growth of target pathogens with minimum inhibitory concentration (MICs) ranging from as little as 0.05-5 $\mu l.ml^{-1}$. In the case of phytophenolics, their activity has been attributed to the hydrophobic part of the molecule facilitating access to the lipids of the cell membrane and mitochondria, leading to leakage of cell content while the hydrophilic parts result in orientation within the membrane and induce some water solubility [74].

Eugenol. Eugenol is an essential oil compound found in clove (bud), allspice, cinnamon and sage [78]. Eugenol has a simple phenolic ring that is monhydroxylated **(Figure 2.2)**. The hydroxyl group has been related to the antimicrobial action of the compound [80]. Eugenol has been reported to affect the growth of *Listeria monocytogenes* [9], *E. coli* O157:H7 [9, 81], *Salmonella Typhimurium* [81, 82], *Staphylococuus aureus, Vibrio parahaemolyticus* [82], and *Clostridium botulinum* [83].

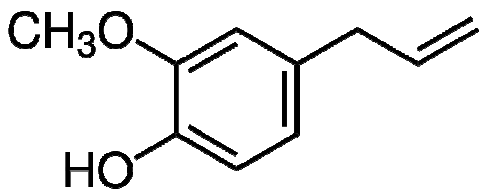


Figure 2.2. Chemical structure of eugenol ($C_{10}H_{12}O_2$).

2.2.2 Amino Acid-Based Antimicrobials

Amino acid-based surfactants have increasingly found industrial applications due to their low toxicity and good biodegradability [84]. Interestingly, some of these surfactants have exhibited very strong antimicrobial activities. Amino-acid based surfactants are composed of polar amino acids/peptides that act as the hydrophilic moieties and non-polar carbon chains such as fatty acids that form the hydrophobic moieties of the surfactants. A wide variety of amino acid and small peptides as well as fatty acids are available to synthesize surfactants with structural diversity and different physicochemical and biological properties [84, 85]..

From a structural point of view, three principal structures of amino acid-based surfactants exists: (i) linear, monomeric or single chain surfactants, (ii) dimeric or Gemini

type surfactants and (iii) glycerolipid-like structures [86] **(Figure 2.3**). The linear-structured amino-acid surfactants consist of an amino acid that joined with a single hydrophobic tail. Gemini or dimeric-type surfactants are composed of two identical or varying linear-structured amino-acid based surfactants that are linked together at or near the head groups by spacer group [87]. Glycerolipid-like structures are analogues of mono-, diglycerides and phospholipids and consist of one polar headgroup and one or two hydrophobic moieties linked together via a glycerol skeleton. [86].

Of these amino-based surfactants, the cationic lipo-amino acids have been found to be the most antimicrobially active compounds [86]. These compounds are able to bind to the surface of lipopolysaccharides of gram-negative bacteria resulting in potassium leakage followed by cell death [88]. Derivatives of amine-based surfactants with arginine and lysine are of particular interest as food antimicrobials to combat foodborne pathogens. This is because these derivatives are in fact produced by a number of microorganisms to protect themselves from infections by pathogenic microbes. For example, nisin and ε-poly-L-lysin commercially produced by fermentation using *Steptomyces albulus* has been approved as a food preservative in Japan [89]. Both of them demonstrated strong antibacterial activity against *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Salmonella typhimurium, Shigella sonnei, Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* [15, 88-91].

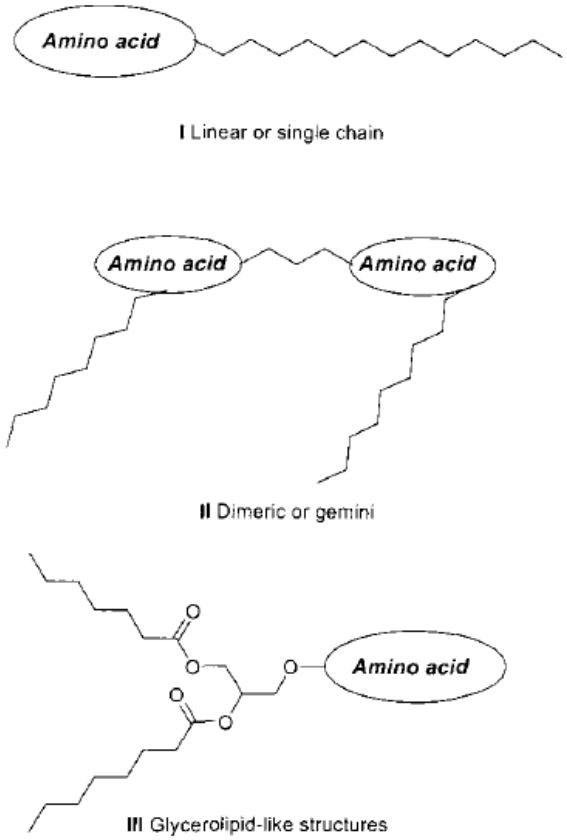


Figure 2.3 Structure of amino acid-based surfactants. The amino acid constitutes the polar head of surfactant [86].

Infante and coauthors studied the structure-function relationship of this group of surfactants focusing on a comparison of single chain and dimeric structure of arginine-based surfactant in terms of their self-assembly properties and biological activity. The critical micellar concentration (CMC) was found to depend on the length of the linear alkyl chain and the nature of the hydrophilic headgroup of the surfactant. In single chain surfactants, the increase in number of hydrophobic methylene groups in the alkyl chain led to a decrease in the CMC. In comparison, the dimeric amino-acid base surfactants had a lower CMC than the single chain surfactants because the dimeric structures was twice as hydrophobic but had a similar water-solubility compared to that of their monomeric

counterparts. Reductions in CMC upon linking two linear surfactants via a spacer molecule has previously been reported for Gemini-type surfactants [30]. Because of these characteristics, a dimeric structure was found to be more effective in terms of adsorption and diffusion into the cell wall making these compounds antimicrobially more effective [84, 86, 87, 92].

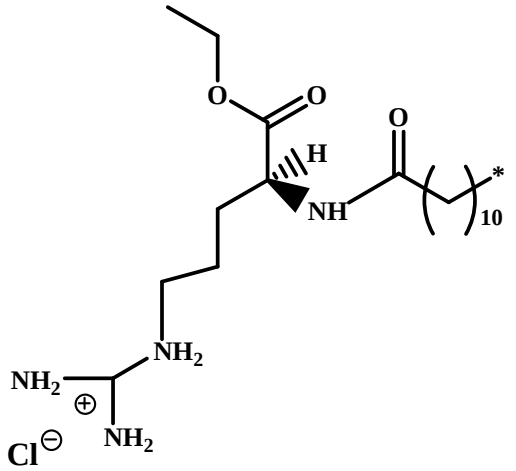


Figure 2.4 LAE, N-α-Lauroyl-L-arginine ethyl ester monohydrochloride, the active compound in Mirenat-N® (patented by Laboratorios Miret S.A. 'LAMIRSA Group', Spain)

N-α-Lauroyl-L-arginine ethyl ester monohydrochloride (LAE). Mirenat®-N is the commercially available non-purified form LAE dispersed at 10.5% w/w in propylene glycol with a density of 1.04 +/- 0.02 g/cm^3. LAE ($C_{20}H_{41}N_4O_3Cl$), a derivative of lauric acid, L-arginine and ethanol, is a cationic amino acid-based surfactant [86]. LAE has been approved by the FDA as Generally Recognized as Safe (GRAS) for use as an antimicrobial in several categories at levels up to 200 ppm on 1st September 2005. While LAE is a synthetic compound, the nature of the ester bond and the relatively simple

chemical structure results in a rapid degrading into natural compounds in the human digestion system (**Figure 2.5**). More specifically, LAE was shown to be rapidly hydrolyzed into L-arginine and lauric acid [12, 13]. L-arginine is then further hydrolyzed into urea and CO_2. L-arginine is an amino acid which can be produced in the kidney and to a lesser extent, in the liver. In the human metabolic system, it is used to synthesize compounds such creatine, L-glutamate, and L-proline, or it can be converted to glucose and glycogen if needed. Lauric acid, which is a natural saturated fatty acid, is commonly found in coconut, palm oil, and human milk. Lauric acid has shown to exhibit some antimicrobial activity against various pathogenic bacteria such as *Staphylococcus aureus* [93], *Clostridium perfringens* [94], *Listeria monocytogenes*, and *Pseudomonas spp.* [95].

As mentioned above, due to the cationic character of LAE and the presence of lauric acid, LAE exhibits strong antimicrobial efficacies against a variety of organisms [96]. For example, LAE is effective against both gram-negative and gram-positive bacteria. It can act as a cell membrane solubilizer causing a loss of membrane potential and leaking of critical cell constituents thereby causing physical damage to the overall cellular structure [15]. Another advantage of Mirenat®-N or LAE is its surface activity. As such, it could be used to stabilize emulsions and form a variety of self-assembled colloidal structures such as micelles that may serve as carriers for other lipophilic compounds to form so-called microemulsions.

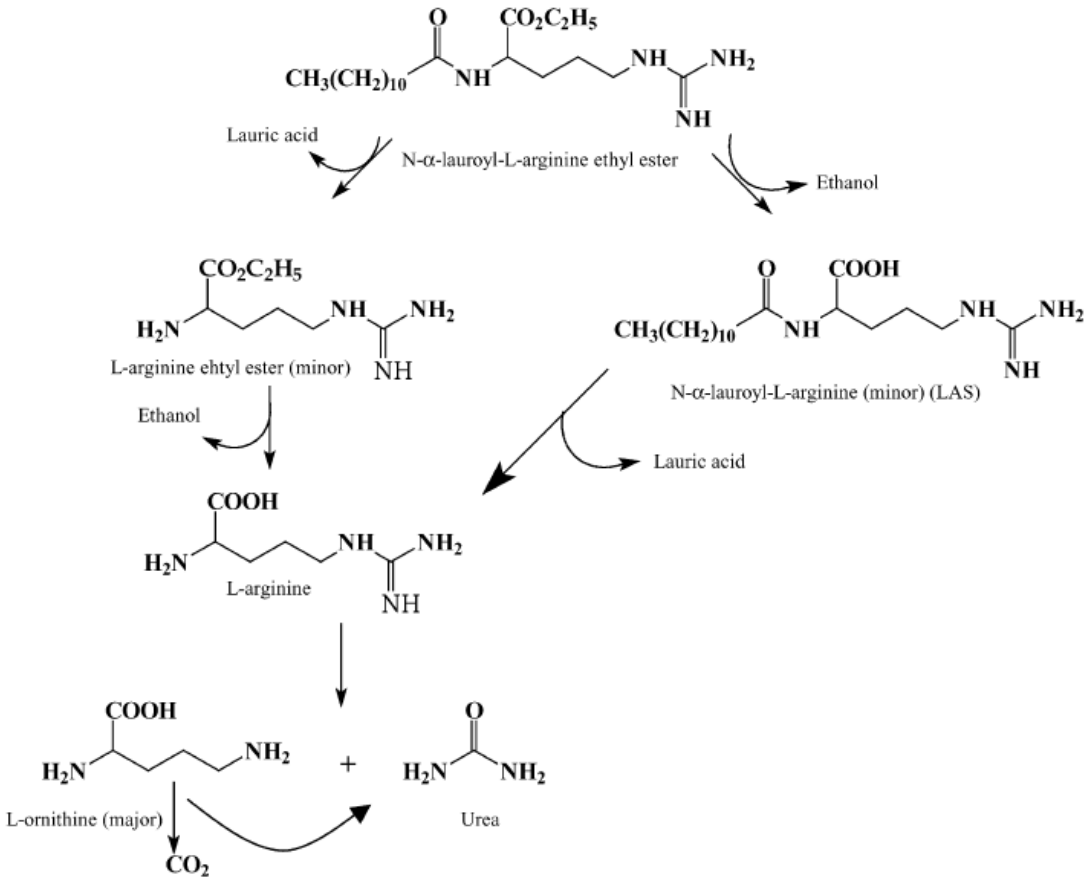


Figure 2.5 Proposed biotransformation pathway of LAE in rats based on in vitro and in vivo studies, following the radio labeled ^{14}C of arginine [97].

2.3 Target Organisms

Microorganisms are necessary for the production of some food products such as cheese, sour cream, yogurt, and sausages; products that are produced by microbial fermentation. On the other hand, uncontrolled and unwanted microbial growth in food can severely degrade food quality such as taste, appearance, and nutrients and in turn cause economic loss for the producer [98]. Most importantly from a food safety point of view, foodborne diseases can lead to severe illnesses and even death. Microbial foodborne diseases are caused by pathogenic viruses, bacteria, fungi, parasites, marine

phytoplankton, and cyanobacteria. Foodborne diseases are classified into three categories. Firstly, food infections may be caused by invasion and penetration of pathogenic organisms into the intestinal mucosa. Secondly, pathogenic microorganisms may have produced toxins that even after destruction of the microorganisms by e.g. heat or radiation may remain active and upon ingestion of the food severely threaten health. Thirdly, pathogenic toxins may be produced by pathogens after the ingestion of food.

Food pathogenic bacteria are generally classified into gram-negative and gram-positive organisms. Gram-negative bacteria contain an outer membrane called the lipopolysaccharide layer or LPS layer [98]. Because of this additional membrane, gram-negative bacteria cells interact much differently with other substances such as salts, acid-base, surfactants, preservative, *etc.* than gram-positive bacteria [19, 53, 99]. In this study, we focus on the activity of target antimicrobials against two select pathogenic bacteria; *Escherichia coli* O157:H7 as a representative of gram-negative pathogens and *Listeria monocytogenes* as a representative of gram-positive bacteria.

2.3.1 *Escherichia coli* O157:H7

E. coli O157LH7 is one of the most dangerous infectious pathogens. It differs from other serotypes of *E. coli* in the presence of the surface antigens O (somatic) and H (flagella), and their pathogenicities. The major symptom of and *E. coli* O157:H7 infection is hemorrhagic colitis (bloody diarrhea). At risk groups include particularly the young and elderly people. The high pathogenicity of this organism is due to the production of two types of shiga toxin (stx1 and stx2). In addition, the organism is able to closely attach to the intestinal cell lining via a Type 3 secretion system. The infectious

dose of *E.coli* I157:H7 may be as low as a 100 cells. Outbreaks of *E. coli* O157:H7 have occurred in ground or emulsified meat products such as hamburgers and sausages and juices such as apple cider and carrot juice. In many cases, outbreaks were linked to *E. coli* being present in irrigation water serving as a reservoir for the organism.

2.3.2 *Listeria monocytogenes*

L. monocytogenes is a unique organism amongst foodborne pathogens. It can grow at refrigeration temperatures, at relatively low pH, and at low water activities. Moreover, it is able to enter the host cells, grow inside the cells, and pass across cellular barriers to nearby cells. Listeriosis results in septicemia often leading to meningitis especially in immunocompromised individuals. In addition, *L. monocytogenes* can cause stillbirth or abortion in pregnant women. The relative ease of transmission of *L. monocytogenes* is due to its ability to survive and grow in soil and water and it being carried asymptomatically in the feces of humans. Foods that have been associated with listeriosis are cheese, fish, raw milk, deli salad, hot dogs, ice cream, cook-and-chill chicken, salted mushroom, and others. Ready-to-eat foods in the USA have a zero tolerance level for *L. monocytogenes.* A particular problem is that even though *L. monocytogenes* can be controlled by pasteurization, contamination often occurs post-processing due to the ability of the organism to reside in processing equipment resisting traditional remediation procedures by forming biofilms.

2.4 Emulsions as Carrier for Functional Compounds.

2.4.1 General Overview

In recent years, emulsions have received much attention as delivery systems for functional components such as antioxidants, flavors and nutraceuticals to ensure high quality and provide good nutrition. Ingredients that have been successfully incorporated in emulsions include vitamins, probiotics, bioactive peptides, antioxidants, and bioactive lipids. While not a lot of research has been conducted to incorporate antimicrobials in emulsions, the antimicrobial activity of some preservatives incorporated within emulsion systems were reported to be greater than that of free preservative added simply to the water phase [21, 100]. Generally, the effectiveness of functional compounds may be limited by their ability to maintain their activity under conditions of processing, storage, and digestion [101, 102]. Emulsions may allow these compounds to be delivered in a controlled fashion and maintain their activity during processing and over a prolonged period of storage [101, 103]

Based on the previously mentioned structural diversity of emulsions, emulsions can be engineered to carry and deliver a wide variety of diverse compounds. Here, the characteristics of the colloidal systems play a key role in determining the functionality of the emulsion as a carrier system. Typically, in O/W emulsions, the functional component is included in the hydrophobic dispersed phase, which allows the compound to be homogeneously distributed through the aqueous phase of the food system. In addition to incorporation on conventional emulsions, the design of multiple emulsions (Oil-in-Water-in-Oil O/W/O, and Water-in-Oil-in-Water W/O/W), multilayer emulsions, solid lipid

(nano)particles, and filled hydrogel particles offers renewed opportunities to engineer the release of encapsulated compounds and improve their stability in complex food systems [101, 103]. Ultimately, the goal of this study is to design emulsion to be appropriate carrier systems for lipid based or amphiphilic antimicrobials.

2.4.2 Emulsions with Functional Components in the Dispersed Phase

In many cases, functional components are mixed with the dispersed phase to improve the dispersability of the typically water-insoluble functional components in a water phase. For example, O/W emulsions are able to encapsulate hydrophobic functional compounds in the oil droplets using the interfacial layer as a protective barrier. . For instance, ω-3fatty acid have been successfully encapsulated and incorporated in O/W food emulsions including ice cream, and meat patties [104, 105]. On the other hand, hydrophilic compounds could be encapsulated in water droplets of W/O emulsions. The lipid nature of the continuous phase could lead to a reduction in the availability of reactive oxygen species since the solubility of oxygen in oil is comparatively low. For hydrophilic functional compounds such as minerals, vitamins, enzymes, and peptides, W/O/W systems have shown to be promising candidates to entrap these components in the inner water phase and thereby creative an effective protection system. Multiple emulsions also allow for fine control of release aroma and flavor compounds [106, 107], β-carotene [108], ω-3 fatty acids [109], and vitamin B [110, 111]. Unfortunately, the production of these multiple emulsions remains difficult as multiple instability mechanisms such as creaming, flocculation, coalescence, and Ostwald ripening may occur in each of the dispersion phases. Thus a key point that remains under investigation

is the ability to prepare W/O/W emulsion that have a prolonged stability [112, 113]. Finally, emulsions have also been used as base systems for the production of powders containing functional components with the convenience of ease of transport and storage.

2.4.3 Emulsions with Functional Components in the Interface

In addition to delivering functional components in the lipid phase, functional components may also be incorporated in the interfacial layer of an emulsions [25, 103]. While the total concentration of components may be low in this case, the components are present at the interface where reactions and mass transport processes occur. Thus they may have enhanced functionalities when adsorbed at interfaces. More recently, multilayer systems have been created that consists of multiple layers of components adsorbed to the surface of the emulsion droplets [25, 103]. These systems can be created by choosing appropriate emulsifiers to form single and mixed surfactant layers followed by addition of compounds that adsorb on the surfactant layer (e.g. via electrostatic attractive interactions). This allows for droplet-droplet interactions to be engineered and for release of compounds to be controlled. Research has shown that this approach is for example suitable to improve the stability of emulsions to lipid oxidation by controlling the concentration of transition metals such as iron near the lipid phase. For example, positively charged interfacial layers may exhibit sufficient repulsive interactions to prevent the transition metals from approaching the lipid phase and catalyzing the lipid oxidation [114]. An example of suitable emulsifiers to achieve this are the use of proteins such as β-lactoglubulin at a pH below that of the isoelectric point of the protein where the protein contains predominantly positive charges and may repel the iron from

the surface of the oil droplets [115, 116]. Other potential applications of multilayer formation (beyond the use in emulsions) includes the incorporation of active cultures in yogurt by immobilization probiotic cultures in whey protein-based spray-dried capsules that are able to better survive the passage through gastric and intestinal juices compared to the ability of free cells [117].

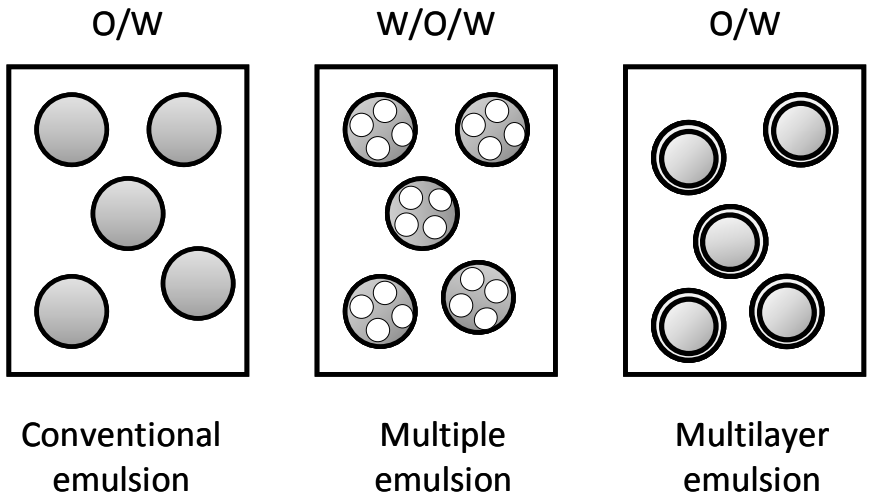


Figure 2.6 Illustration of the principal types of emulsions that can be fabricated.

2.4.3 Control of Functionality of Emulsions as Carrier Systems

The ability of emulsions to act as carriers for functional components depends on the emulsion physicochemical properties. Of these droplet concentration, loading and droplet size are of key importance. For example, encapsulation of lipophilic nutraceuticals in small emulsions droplets has shown to enhance the absorption of the nutraceuticals either by adsorbing to the gastro-intestinal walls thereby prolonging residence time or by improving the uptake by the intestinal epithelium [101, 118].

2.5 Conclusions

In conclusion, emulsions are extremely promising systems to deliver functional ingredients. As such, they may have substantial potential as carriers for food antimicrobials. In this case, the challenge is to create delivery systems which efficiently allow for optimal interaction with target pathogens. The objective of this study is therefore addresses this research need. We hypothesize that we can influence the ability of the emulsion to control the growth of pathogens by incorporating antimicrobials in both the lipid phase and the interfacial layer and by carefully engineering the physicochemical properties of the emulsion droplets (i.e. surface charge and composition, droplet size, concentration and loading).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Mirenat-N, a cationic surfactant (10.5% of N-α-lauroyl-L-arginine ethyl ester monohydrochloride, LAE in propylene glycol) was obtained from Group Lamirsa (Terrassa Spain). Eugenol (4-allyl-2-methoxyphenol), Tween20; a nonionic surfactant (polyoxyethylene 20 sorbitan monolaurate) and corn oil were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions were prepared with distilled and deionized water. Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), and Yeast extract (YE) were purchased from Difco Laboratories, Sparks, MD. Five different *L. monocytogenes* strains were obtained from Martin Weidmann culture collection at Cornell University (J1-225, J2-020, J1-031, C1-115 and J1-1177). Four strains of *E. coli* O157:H7 were purchased from ATCC (ATCC35150, ATCC4385, ATCC51658, and ATCC700599). Bacterial cultures were stored at -75ºc in TSB for *E. coli* and TSB-YE for *L. monocytogenes*. with 5% glycerol.

3.2. Methods

3.2.1 Formation of Emulsions

All stock emulsions were produced by blending aqueous surfactant solution with oil in a high-speed blender 30 seconds. The premix was then further homogenized by passing it through a microfluidizer at a homogenization pressure of 9.5 kPa. (Microfluidics 110 L, Microfluidics Corp., Newton, MA, USA). The pH was adjusted

using HCl or NaOH. All emulsions were produced at room temperature and stored at 20º or 32ºc.

3.2.2 Physicochemical Characterization

Surface/Interfacial Tensiometry. To characterize the interfacial properties of emulsion droplets, the surface tension was measured at the oil-water interface upon addition of the antimicrobial and stabilizing surfactants (e.g. Tween 20). A drop shape analysis tensiometer (Model DSA-G10 MK2, Kruss USA, Charlotte, NC) was used to determine surface tension as a function of surfactant concentration of either the single surfactants or the binary mixtures. The tensiometer determines the surface tension from the shape of pendant drops through numerical analysis. Experimentally obtained surface tension versus concentration data was used to compare CMC results to existing models that predict the CMC as a function of the concentration of surfactants in the binary surfactant mixture.

Particle Size Distribution Analysis **(Figure 3.1).** The z-average particle diameter and polydispersity index was determined using a Dynamic Light Scattering (DLS) technique (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, UK). The instruments incorporates a non-invasive back scatter (NIBS) optics that measures the scattered light at an angle of 173° to maximize the detection of scattered light while maintaining signal quality. The hydrodynamic radius of particles is calculated from the diffusion coefficient of particles that is determined from the interference pattern of scattered light.

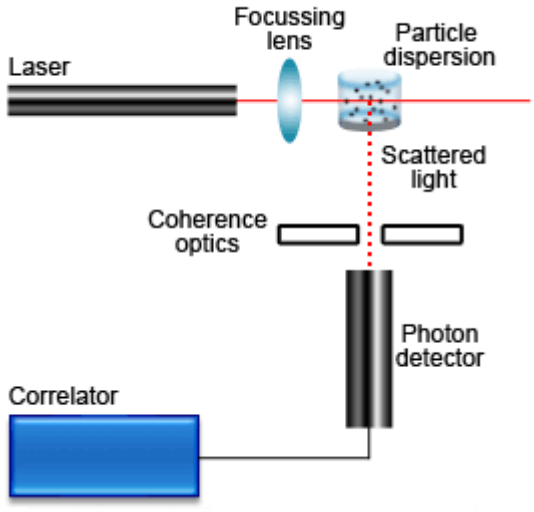


Figure 3.1. Schematic diagram of a conventional 90º dynamic light scattering instrument used for particle size analysis [119]

ζ-Potential Measurement. The electrical charge (Zeta potential) on the surface of emulsion droplets was determined using an electrophoretic technique with a special folded capillary cell (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, UK). A small amount of sample was diluted into buffer at the appropriate pH. An oscillating electric field was applied across the dispersion and the zeta-potential was then determined from the velocity of the droplets migrating toward the oppositely charged electrode (**Figure 3.2**). Generally, the surface charge of droplets mainly depends on the charge of the adsorbed emulsifier molecules as well as the pH and presence of electrolytes in the aqueous phase. It is one of the key factors that determined the nature of the colloidal interactions between the emulsion droplets and other charged species and has a major effect on the stability of droplets and the rheological behavior of the emulsion.

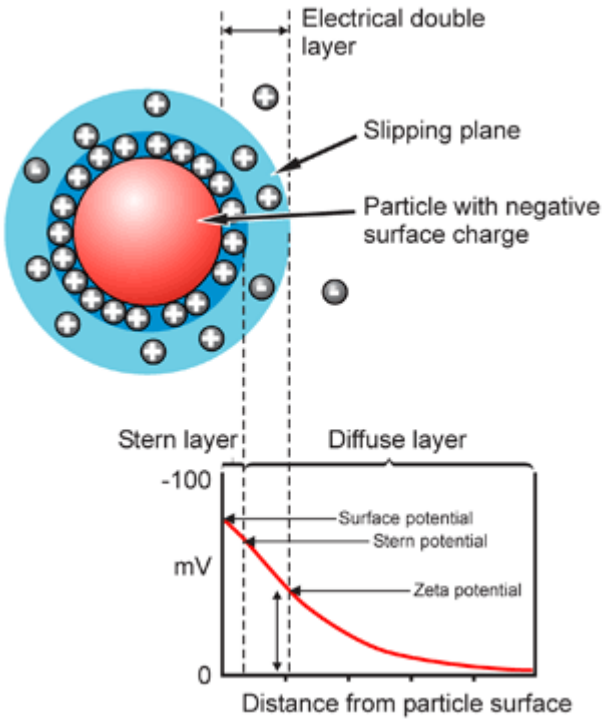


Figure 3.2. Schematic representation of zeta-potential [120]

Creaming velocity measurements **(Figure 3.3).** Emulsion samples were transferred into a test tube (with a 15 mm internal diameter). The total height of the emulsions in the tubes was 7.00 cm. The test tubes were tightly sealed with a plastic cap, and then left to stand at 32ºC or at room temperature. The creaming stability of emulsion was monitored by measuring the backscattering of monochromatic light (λ = 850 nm.) from an emulsion as function of its height (Turbiscan Classic MA 2000, Formulaction, France). The backscattering of light from the emulsion was measured as a function of height at different days.

*Optical microscopy***.** To better understand the behavior of emulsion droplets, emulsion samples were observed using a conventional optical microscope (Nikon microscope Eclipse E-400, Nikon Corporation, Japan). The images of the droplets were

acquired using a CCD camera (CCD-300T-RC, Dag-MTI., Michigan City, IN) connected to Digital Image Processing Software (MVI/MicroVideo Instruments Inc., Avon, MA) installed on computer. Emulsions were agitated in a test tube before analysis. A drop of emulsion was placed between a microscope slide and cover slip and images were taken.

Figure 3.3 Photograph of Turbiscan and Schematic scan mode. The Turbiscan MA 2000 reads transmission and backscattering data every 40µm while reading head move along the cell height [121].

3.2.3 Microbial Protocols

Bacterial cultures. Bacterial cultures were stored at-75 ºC in Tryptic Soy Broth-TSB with 5% glycerol. Working cultures were maintained on slants TSA for *E. coli* and TSA-YE for *L. monocytogenes* stored at 4ºC. Prior to each experiment, Two cycles of a loopful of the *E. coli* slant culture was transferred to TSB (*L. monocytogenes* to TSB-YE, added 0.6% yeast extract), incubated at 32ºC for 24h.

Spot Inoculation Assay. Petri dishes were prepared by adding 10 ml of the emulsion diluted to the desired antimicrobial concentration in 10 ml of 45-50°C double

strain TSA-YE. Control plates with water were prepared. The Petri dishes were dried and added 10µl drops 24h. growth with a final concentration of 10^5 CFU/ml. The Petri dishes were incubated at 32ºC for 24 h. All experiments were duplicated.

Growth curves. Bacterial cultures were maintained on slants stored at 4ºC. A loopful of the culture was transferred to Tryptic Soy Broth (TSB) for *E. coli* O157:H7 and Tryptic Soy Broth supplemented with 0.6% of yeast extract for *L. monocytogenes*, and incubated at 32ºC for 24 h. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 24 h. Based on the MICs determined previously by the above described spot inoculation assay, the growth inhibition in the presence of a specific concentration of antimicrobial compounds in their emulsified form was tested. Emulsions with different ratios of antimicrobial agents were prepared and diluted to obtain concentrations of the antimicrobial at the previously measured MIC in the 9 ml broth. Culture dilution and plating was performed using a spiral plater (Spiral Biotech. Norwood, Mass. Positive controls consisted of plating in the absence of antimicrobial. Plates were enumerated at 0, 3, 6, 12, 24, and 48 h. All experiments were conducted with duplicate samples of each treatment and the entire study was replicated. Spiral plating is a convenient method to determine the bacterial growth by depositing decreasing cell number concentrations on a rotating agar plate (Archimedes spiral). After the sample has been incubated, different colony densities are apparent on the surface of the plate. A modified counting grid is used to relate the area of the plate to the volume of sample. By counting an appropriate area on the plate, the number of bacteria growing can be estimated [122].

CHAPTER 4

EMULSIONS AS ANTIMICROBIAL DELIVERY SYSTEMS: PART I. THE ANTIMICROBIAL AS PART OF THE LIPID PHASE

4.1 Abstract

The stability and antimicrobial efficacy of oil-in-water emulsions formulated with the phytophenol eugenol and other lipids was evaluated. Eugenol and lipids (hexadecane, dodecane, tetradecane or corn oil) were mixed at eugenol: lipid ratios varying from 0:1 to 1:0. Oil-in-water emulsions were prepared by homogenizing 5 wt% of the lipid mixture with 95 wt% of a 0.5 wt% aqueous solution of Tween 20. The particle size distribution was measured after 0, 1, 3, 6, 12, 24, 48, 96, 168, 264, 336, and 504 h. Partitioning of eugenol in lipids was determined spectrophotometrically by measuring the absorbance at 282 nm. Stable emulsions were tested for their antimicrobial activity against four strains of *E. coli* O157:H7 and *L. monocytogenes* using a spot inoculation test and growth curves by plate counting. Formulation of oil-in-water emulsions was found to be challenging as emulsions composed of phytophenols above a critical loading ratio broke down in less than 1 hour. The appreciable water solubility of the antimicrobials promoted rapid breakdown by Ostwald ripening. Below this critical concentration, rate of emulsion particle size increase varied depending on type of carrier lipid and concentration of eugenol. Corn-oil emulsions loaded with eugenol were inhibitory against *E. coli* O157:H7 and *L. monocytogenes* strains depending on loading ratio. Direct addition of eugenol to the aqueous at concentrations similar to the lowest loaded emulsions was inhibitory against all strains of both pathogens. Results suggest that activity of eugenol-loaded emulsions depends on partitioning of eugenol in the water phase rather than direct

interaction between emulsion droplets and pathogens. Results have important implications for formulation of antimicrobial emulsions or partitioning of hydrophobic antimicrobials into available lipid phases in foods.

4.2 Introduction

Phytophenols such as eugenol, carvacrol, and thymol are phenolic components of essential oils that may exhibit antimicrobial activity [123]. Eugenol is a partially hydrophobic compound of low molecular weight that is able to reduce the growth of pathogens in food systems thereby improving microbial safety of the product [124]. Emulsions are an important class of food products that are defined as dispersions of two or more partially or completely immiscible liquids in each other *e.g.* oil-in-water [125]. Emulsions have been used as carrier systems for flavors, antioxidants and other functional ingredients. Emulsions could therefore be a promising carrier system for phytophenols such as eugenol, thymol, carvarol, cinnamic aldehyde and other essential oil components. They may simultaneously provide an insight into the fundamental mechanisms that govern the activity of phytophenols in lipid-containing foods.

Emulsions are thermodynamically unstable systems that tend to break down over time [126],[125]. Destabilization of emulsions is driven by the tendency of the system to minimize the interfacial area between the dispersed and the continuous medium in order to decrease the unfavorable interactions between the different molecules [127]. Several different mechanisms may be involved in the break-down of emulsions including flocculation, gravitational separation, coalescence, creaming, phase inversion, and Ostwald ripening [126], [128]. Ostwald ripening is defined as the gradual growth of

larger particle at the expense of small ones and is of particular importance for emulsions containing lipids with appreciable water solubility (e.g. flavors) [127], [128], [129]. These often small molecular weight compounds rapidly partition into different phases that may be present in the food matrix depending on their affinities toward these phases [130], [131].

In oil-in-water emulsions, the kinetics of Ostwald ripening depends on the diffusion of the oil molecules across the aqueous phase separating droplets. Thus, the Ostwald ripening rate generally increases with increasing solubility of the oil phase in the water phase, [132], [126], [133], [134], [135]. Ostwald ripening has also shown to increase in emulsions that contain excess surfactant, were micelles can serve as carrier vehicles. One On the other hand, emulsions possessing a thick interfacial membrane resistant to molecular transport and deformation [134], [136], [135] have shown to exhibit lower rates of Ostwald ripening. The properties of the interfacial layer surrounding the oil droplet can be manipulated by changing the composition, thickness, rheology and electrical charge of the emulsifier membrane [135]. More importantly, rates of increase in droplet size due to Ostwald ripening may be substantially reduced if emulsions are formulated with a second (carrier) lipid [137]. In this case, compositional ripening may occur rather than Ostwald ripening resulting in a change in composition of the individual droplets rather than their size.

Little is known about the antimicrobial activity of emulsions containing different concentrations of phytophenols. Marinating is a technique that has been used in the meat industry to enrich flavor, increase moisture and tenderness and preserve color has also been known to increase shelf life of products [138],[139] Marinades are typically

emulsions containing salt, sugars, spices, stabilizers (gums) and antimicrobial agents (sorbate or benzoates). The pH is usually acidic, less than 5 [139]. In many cases, spices and their extracts are added to the marinate not just to impart flavor but also as a preservation technique contributing to the long term microbial stability of different meat products such as jerky [140], [141]. Amount of marinades are usually added on meat product at different concentrations varying from 20 to 30 wt%[139].

We hypothesize that emulsions composed of antimicrobially-active essential oil components may rapidly increase in size because of appreciable water solubility of antimicrobials. Consequently, formulation of such emulsions could be challenging. Combination with less water-soluble lipids may help stabilize these emulsions. Secondly, we hypothesize that the antimicrobial activity of phytophenol loaded emulsions may depend on partitioning of the phytophenol in the solvent phase. Thus emulsions with increasing loading ratios may exhibit higher antimicrobial activity. This study was designed to test these two hypotheses by investigating the conditions required to formulate stable eugenol-containing oil-in-water emulsions and investigating their antimicrobial activity against selected food pathogens.

4.3 Materials and Methods

4.3.1 Materials

Eugenol (4-allyl-2-methoxyphenol), hexadecane (<99%), tetradecane (99+%), n-dodecane, corn oil and Tween 20 (polyoxyethylene 20 sorbitan monolaurate) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further

purification. All solutions were prepared with distilled and deionized water. Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), and Yeast extract (YE) were purchased from Difco Laboratories (Sparks, MD).

4.3.2 Bacterial Cultures

Four different *L. monocytogenes* strains were obtained from the Martin Weidmann culture collection at Cornell University (J1-225, J2-020, C1-115, and J1-030). In addition, four strains of *E. coli* O157:H7 were purchased from ATCC® (ATCC 35150, ATCC 43895, ATCC 51658, and ATCC 700559). Bacterial cultures were stored at -75 ºC in 5% glycerol with Tryptic Soy Broth (TSB) for *E. coli* O157:H7 and Tryptic Soy Broth Yeast Extract (TSBYE) for *L. monocytogenes* (Difco Laboratories, Sparks, MD). Working cultures were maintained on slants stored at 4°C. A loopful of the culture was transferred to liquid media and incubated at 32°C for 24 hr. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 24 hr.

4.3.3 Physicochemical Characterization

Preparation of Emulsions Containing Eugenol. Tween 20 was dispersed in water at 0.5 % v/v to prepare the aqueous phase of the emulsion. The lipid phase consisted of 5 % v/v of hydrocarbon (hexadecane, dodecane, or tetradecane) in the absence or presence of eugenol at different ratios (0:10, 10:90, 20:80, 30:70, 40:60, 50:50). 5 %v/v of the lipid phase was mixed with 95 %v/v of the aqueous phase and homogenized in a high-speed blender for 30 seconds to form an emulsion premix. The emulsion premix was further homogenized by passing the emulsion three times through a microfluidizer at 9 kPa

(Microfluidics 110L, Microfluidics Corp., Newton, MA, USA) to further reduce the droplet size.

Particle Size Determination. A static light scattering technique (Horiba LA-9000, Horiba Instruments Inc., Irving, CA) was used to measure the droplet size distribution of emulsions. This technique measures the angular dependence of laser light scattered by the droplets in an emulsion. A relative refractive index of 1.1 was used by the instrument to calculate the droplet-size distributions. The mean droplet diameter of the emulsions is reported as the mean diameter: $d_{10} = \Sigma n_i d_i / \Sigma n_i$, where n_i is the number of droplets with diameter d_i. The light scattering instrument used in this study could detect particles with diameters between 0.04 to 262 μm, and was therefore not sensitive to the presence of surfactant micelles (which have diameters of about 10 nm) [142].

Partitioning Coefficient. The partitioning coefficient of eugenol between water and a lipid phase (stripped corn oil, hexadecane, tetradecane and dodecane) was determined spectrophotometrically (Shimitzu UV-2101PC, Shimitdzu Scientific Intrument Inc., Columbia, MD, USA) by measuring the absorbance in the respective phase at 282 nm after equilibration for a minimum of 48 hours. Due to presence of interfering compounds in commercial corn oil (e.g. tocopherols), corn oil was first stripped of its minor polar components using column chromatography with alumina (Al_2O_3; modified from Wang et al., *48*). The partition coefficient *K* of the lipophilic antimicrobial was determined by dividing the equilibrium concentration of the compound in the oil phase C_o by the equilibrium concentration of the compound in the water phase C_w.

$$K = \frac{C_o}{C_w} \qquad (1)$$

4.3.4 Antimicrobial Test Protocols

Emulsion Preparation for Antimicrobial Assays. A 10% oil-in-water emulsion was prepared by homogenizing 90% of a 1% aqueous surfactant solution with 10 %v/v of a lipid mixture containing corn oil or corn oil mixed with eugenol at different ratios (9:1, 7:3, and 5:5). As previously described, the lipid-surfactant solution mixtures was blended in high-speed blender for 30 seconds to form a premix and the emulsion premix was then further homogenized by passing the emulsion three times through a microfluidizer at 9 kPa (Microfluidics 110L, Microfluidics Corp., Newton, MA, USA) to further reduce the particle size. Emulsions were filtered sterilized using a 0.45 μm polyethersulfone membrane filters (Puradisc™ 25, Whatman® Inc. Florham Park, NJ). After filter sterilization, emulsions were diluted with sterile water to obtain a series of emulsions containing a final concentration of eugenol of 0.01, 0.025, 0.05, 0.075, 0.1, and 0.5 % in the plate. To verify that no contamination occurred in the process of emulsions formation, samples were incubated at 32°C for 24h. Emulsions prepared under sterile conditions showed no growth.

Spot Inoculation Assay. Bacterial cultures were maintained on slants stored at 4°C. A loopful of the culture was transferred to Tryptic Soy Broth (TSB) for *E. coli* O157:H7 and Tryptic Soy Broth Yeast Extract (TSB-YE) for *L. monocytogenes*, and incubated at 32°C for 24 hr. Prior to exposure to antimicrobials, each strain was sub-cultured in liquid media for 24 hr. Petri dishes were prepared by adding 10ml of the

emulsion previously diluted to the desired antimicrobial concentration in 10ml at 50°C of double strain Tryptic Soy Agar with 0.6% of yeast extract. Control plates with only the surfactant and without both surfactant and eugenol were prepared. The Petri dishes containing were dried over night at 32°C and 10 µl drops of the inoculums were plated to give a final concentration of 10^5 CFU/ml. Eugenol and pure micelles were used as control. The Petri dishes were incubated at32°C for 24h. All experiments were duplicated and replicated.

Growth Curves. Bacterial cultures were maintained on slants stored at 4°C. A loopful of the culture was transferred to Tryptic Soy Broth (TSB) for *E. coli* O157:H7 and Tryptic Soy Broth supplemented with 0.6% of yeast extract for *L. monocytogenes*, and incubated at 32°C for 24h. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 24 hr. After MIC determination by spot inoculation (see above), inhibition of growth in the presence of a specific concentration of eugenol (0.01%) present in emulsions was tested. Emulsions were prepared as describe above. Emulsions with different ratios of corn oil and eugenol (9:1, 7:3 and 5:5) were prepared and diluted to obtain a eugenol concentration of 0.01% in the plate. Culture dilution and plating was performed using a spiral plater (Spiral Biotech. Norwood, Mass). Controls consisted of eugenol simply dispersed in water, corn oil emulsions without eugenol, and eugenol dispersed in 1% of ethanol. All experiments were conducted with duplicate samples of each treatment and the entire study was replicated.

4.4 Results

Partition Coefficient. The partition coefficient of eugenol was determined in the various lipids by analyzing their concentration in the oil and the water phases after equilibrium was reached. The partition coefficients between oil and water were calculated as 15 ± 1 in hexadecane/water, 11 ± 3 in tetradecane/water, 31 ± 5 in dodecane/water,. Partitioning experiment of eugenol in hexadecane indicated that for every 20 molecules of eugenol in the lipid there was one in water. The results confirm that eugenol has an appreciable affinity towards water that may contribute to destabilization of pure eugenol emulsions by Ostwald ripening (see below). Partition coefficient of eugenol in stripped corn oil was 51.11 ± 2.

Particle Diameter and Ostwald Ripening Rates. Particle size distributions of emulsions composed of the different lipids containing various concentrations of eugenol were measured after 0, 1, 3, 6, 12, 24, 48, 96, 168, 264, 336, and 504 h. Increase of the emulsion particle size varied depending on type of lipid and concentration of eugenol. Particle size of an emulsion without eugenol at time zero using hexadecane (0.246 μm), tetradecane (0.17 μm), dodecane (0.16 μm), and corn oil (0.28 μm) increased to 0.26 μm, 0.30 μm, 0.60 μm, and 0.29 μm after 504 hours (21 days) of storage at 20 ± 2 °C, respectively. Ostwald ripening rates were calculated from the mean droplet diameter data as a function of time as

$$\omega = \frac{\left(d - d_0\right)^3}{t} \qquad (2)$$

Table 4.1 shows the particle size of a 1:1 lipid-eugenol emulsions as a function of storage time compared to the pure lipid at 0, 24, 168, 336 and 504 hours. Ostwald ripening rates, i.e. the rate of increase of the cube of the droplet diameter with time decreased with increasing alkane chain, e.g. dodecane > tetradecane > hexadecane, corn oil. Interestingly, loading emulsions with eugenol below a critical concentration reduced Ostwald ripening rates for emulsions composed of alkanes with smaller alkane chain lengths such as tetradecane and dodecane. For example, Ostwald ripening rates of dodecane and tetradecane emulsions decreased by nearly two logs as eugenol was introduced into these emulsions. Mean droplet size of emulsions containing corn oil, hexadecane remained unchanged over 21 days of storage. However the initial droplet diameter of emulsions manufactured under the same homogenization conditions containing eugenol and a carrier lipid was larger than the droplet diameter of emulsions composed of only the carrier lipid.

Above a certain critical loading ratio, the emulsion became rapidly (within seconds) unstable and phase separation occurred. The specific ratio of eugenol and lipid at which destabilization occurred was referred to as the critical loading ratio or CR i.e. emulsions carrying less eugenol than the CR were stable while emulsions carrying eugenol above the CR were unstable. Emulsions composed of eugenol only could not be manufactured at all. **Table 4.2** shows the CRs for the different oils and ratios.

Depending on the hydrocarbon solubility, the critical ratio varied. Dodecane and hexadecane were stable up to a ratio of 60:40 (eugenol: hydrocarbon) **Figure 4.1:** 1A, 1B, 1C and 1D shows the particle size distribution over time. In figure 1 all alkanes and corn oil, are mixed with eugenol at a ratio 40:60 alkane: eugenol. Figure 1A, shows

hexadecane and eugenol used to demonstrate a slow Ostwald ripening rate after 264h (11 days). The size changes from 0.48 μm at time zero to 9.6 μm after 264h. Figure 1B shows tetradecane with eugenol to illustrate an example of instant emulsion breakdown after time zero where larger particle formed. In figure 1C, dodecane and eugenol are shown. In this case Ostwald ripening destabilization can be seen after 48h (2days). The particle size change was of 5.87μm after time zero to 12.7 μm after 48h. Figure 1D, shows corn oil and eugenol emulsions where no Ostwald ripening occurs after 504h (21days) having a particle size of 0.5μm after time zero and 0.517μm after 21 days..

Antimicrobial Efficiency. The antimicrobial efficacy of emulsions composed of corn oil loaded with eugenol at three different loading ratios was tested using spot inoculation. Each emulsion was diluted to get obtain a range of effective eugenol concentration in the overall system (0.01, 0.025, 0.05, 0.1, and 0.5%). Table 4.3 shows the eugenol concentration in the respective emulsion that inhibited growth of *E. coli* O157:H7 and *L. monocytogenes* strains. Emulsions formulated with high corn oil concentrations and required higher overall concentrations of eugenol in the system than emulsions carrying higher concentrations of eugenol. For example, corn oil emulsions formulated at a 9:1 ratio of corn oil: eugenol did not inhibit growth at any of the eugenol concentrations tested (0.01-0.5%). However, as the relative concentration of eugenol in corn oil was increased, *E. coli* O157:H7 strains were inhibited. Form the spot inoculation assay, all *L. monocytogenes* strains were more resistant toward the emulsions than *E. coli* O157:H7 strains. Eugenol simply dispersed in water at a concentration of 0.5% inhibited growth of all organism and all strains except *E. coli* O157:H7 ATCC® 700599 and *L. monocytogenes* J1031 which were inhibited at 0.1% of eugenol. For comparison, the

effectiveness of eugenol in the presence of a solubilizer such as ethanol was tested as well (data not shown). Eugenol dispersed in 1% ethanol inhibited growth of *E. coli* O157:H7 and *L. monocytogenes* at 0.1%. It should be noted that the solubilizer (1% ethanol) did not contribute to the inhibition, i.e. ethanol did not inhibit growth of pathogens at the 1% level.

Based on the eugenol spot inoculation results, a concentration of 0.1% of eugenol in corn oil emulsions was selected for growth over time studies. The emulsions were prepared at the three different loading ratios of corn oil: eugenol and diluted until a final concentration of eugenol of 0.1% was obtained. **Figure 4.2:** 2 A and 2 B shows the growth curves of the most resistant (ATCC® 35150) and most sensitive (ATCC® 700599) strain of *E. coli* O157:H7 while **Figure 4.3:** 3A and 3B show the growth curves of the most resistant (J1225) and most sensitive (J1031) strain of *L. monocytogenes*. *E. coli* O157:H7 was grown in TSB and *L. monocytogenes* was grown in TSB YE as positive controls. Emulsions without inoculation were used as controls to assure that the emulsions were not contaminated after preparation and filtration. For instance, at a corn oil: eugenol ratio of 9:1, the corn oil concentration was 0.9% while the eugenol concentration was 0.1%. At a corn oil: eugenol ratio of 7:3, the corn oil concentration was 0.233% and eugenol concentration was 0.1%, and for a ratio of 5:5 or 1:1, the concentrations for both were 0.1%. **Figure 4.2 and 4.3** demonstrate that after 48h eugenol dispersed in water had a bactericidal effect against both strains of *E. coli* O157:H7and *L. monocytogenes*. *E. coli* O157:H7 ATCC® 35150 was reduced below detectable levels after 6h. **Figure4.2:** 2A and 2B indicate that the amount of corn oil in the emulsions plays a critical role in the antimicrobial efficacy of the emulsions against

E. coli O157:H7. The more corn oil is present in the system, the less active the eugenol becomes. The 1:1 corn oil: eugenol emulsions were more active followed by the emulsions composed of a 7:3 ratio. The corn oil emulsions composed of a 9:1 ratio did not show any antimicrobial activity. Emulsions were least effective against *L. monocytogenes* showing almost no antimicrobial activity (**Figure 4.3**). However, the lag phase of *L. monocytogenes* J1031 (Figure 3B) was delayed. This effect was dependent on the corn oil concentration in the emulsion. As the corn oil concentration was decreased, the lag phase was increased. In general, *E. coli* O157:H7 was more sensitive against emulsions and eugenol than *L. monocytogenes.*

4.5 DISCUSSION

Emulsion Stability. The objective of this research was to create stable phytophenol-containing emulsions that exhibit antimicrobial activity. Eugenol has shown to exhibit strong antimicrobial activity against foodborne pathogens such as *L. monocytogenes* and *E. coli* O157:H7. However, eugenol is a small molecular weight, partially hydrophilic essential oil component with appreciable water solubility (0.01% (Merk Index). Formulation of emulsions with flavor compounds of similar solubility and molecular weight as eugenol has previously been shown to be challenging as Ostwald ripening, the growth of larger droplets at the expense of smaller ones, may proceed very rapidly and lead to a breakdown of the emulsions[129, 135, 143], [144, 145]. Similarly in this study we found that homogenization of eugenol with Tween 20 did not result on formation of a stable emulsions. Different methodologies have been suggested to decrease Ostwald ripening rates. These include for example coating the emulsion droplet with a thicker layer by electrostatically depositioning a secondary biopolymer around the

emulsion droplet [135], changing the emulsifier to form a thicker layer surrounding the droplet [131], and eliminating solubility-enhancing compounds such as ethanol [128] or micelles [125], [146]

In this study, emulsions were instead prepared by adding a carrier lipid to eugenol to decrease the rate of Ostwald ripening. It has been suggested that in binary lipid mixtures compositional ripening rather than Ostwald ripening occurs. Compositional ripening is the gradual change in composition of droplets with different sizes. For example, the compound with the higher water solubility tends to migrate through the aqueous phase from smaller into larger droplets [132]:

$$\omega = \varphi_1 / \omega_1 + \varphi_2 / \omega_2 \quad (3)$$

where ω is the Ostwald ripening rate of component 1 and 2 and φ is the molar fraction of component 1 and 2.

In binary lipid emulsions, growth of droplets by Ostwald ripening is governed by the compound with the lowest water solubility. The bulk lipids used in this study as carrier lipid consisted of alkanes with varying chain lengths (C-12, C14 and C16) and corn oil. Addition of any of these lipids to eugenol above a critical concentration resulted in emulsions with a mean particle diameter that remained virtually unchanged over the 504 h of storage. Interestingly, growth of dodecane-eugenol emulsions was also slower than growth of dodecane emulsions.

Above a critical concentration, emulsions formulated with the carrier lipid and eugenol destabilized within a matter of minutes. This critical concentration depended on

the carrier lipid used to formulate the emulsions. We hypothesize that the partitioning coefficient of eugenol between the aqueous phase and the carrier lipid influence this process. This is because the concentration of eugenol in the aqueous phase, which governs Ostwald ripening, depends on the partitioning of eugenol from the emulsion droplet into the aqueous phase unless that concentration exceeds the solubility of eugenol in which case the concentration is equal to the solubility. However, for compounds that are substantially water soluble, the solubility in the aqueous phase may not have been reached and the effective concentration driving Ostwald ripening is thus a function of the initial concentration of eugenol used to formulate the emulsions and the partitioning coefficient.

We therefore determined the partition coefficients between the lipid and water phase. Partitioning coefficients of eugenol between lipids and the water phase ranged between 11 and 31. Huang et al. (1997) [130] reported the partitioning coefficients of several hydrophilic and lipophilic antioxidants. Partition coefficients between oil (corn oil) and water were reported as 23.4 for methyl carnosate, 15.9 carnosol, and 10.2 for carnosic acid.

Our results also show that the higher molecular weights of the oil, the more stable the emulsion became with corn oil being the most stable emulsions followed by hexadecane, tetradecane and dodecane. Egger and McGrath (2006) [125] follow the destabilization of oil-in-water emulsions prepared with water/n-alkane/Triton X-100 as a function of time to determine the influence of the oil molecular weight on the mechanism of destabilization. By increasing the chain length of the oil from hexane to tetradecane,

they found an almost five orders of magnitude increase in emulsion stability while maintaining all other chemical and physical aspects of the emulsions.

Emulsions are required to have a long shelf life. Our results suggest that stable emulsions can be produced that carry a phytophenolic antimicrobial as part of the lipid droplet. Oswald ripening, the major problem of destabilization of o/w emulsions composed of aromatic compounds can be avoided by formulating the emulsions with a water-insoluble lipid carrier. However, the affinity of the carrier lipid for the phytophenol will influence the maximum concentration of phytophenol that can be incorporated into the emulsion.

Antimicrobial activity of emulsions. The most stable emulsion was the one prepared with corn oil. Moreover, corn oil is a good example of a food grade emulsion. Hence, corn oil emulsions were selected to evaluate the antimicrobial efficacy at different loading ratios. Eugenol, an essential oil component extracted from clove [147], [148]. has been shown to inhibit *L. monocytogenes* [149] and *E. coli* O157:H7 [149], [150] at concentrations ranging from 0.05-0.1% [10]. Since eugenol is sparingly soluble in water, researchers regularly use ethanol [147] or emulsifiers [151] to disperse them. For food products, ethanol is not an additive that can be easily added to a wide variety of foods due to regulatory hurdles. Thus, emulsifiers are often used instead. In some cases, at sufficiently high emulsifier concentrations, inclusion of eugenol in micelles may occur which aids in the transport mechanism of these compounds through the water phase quite to the bacterial surface [143]. To date, only a few studies have been conducted using emulsions as essential oil component delivery systems. A number of studies demonstrated the antimicrobial efficiency of a variety of marinades in meat products

[139], [140], [138]. For example, the antimicrobial efficiency of pimento leaf oil and clove oleroresins in marinades was reported. Authors stated that with 0.5 wt% of pimento leaf oil or clove oleoresin, growth of *Pseudomonas* was inhibited and yeast counts were significantly reduced [138]. In our study, we found that eugenol emulsions with 0.1% of eugenol were more efficient against *E. coli* O157:H7 than *L. monocytogenes.*

Results indicate that the level of corn oil in the oil phase influences the activity of emulsions. This suggests that antimicrobial efficiency of emulsions is influenced by the partitioning coefficient. While it is generally assumed that essential oil components have a low solubility in water and have a high preference for hydrophobic phases or interfaces [152], partitioning coefficients between 11 and 30 indicate that eugenol has an appreciable affinity for the water phase. Consequently, with higher concentrations of lipid in the emulsion, the concentration of eugenol (while constant in the overall system) will decrease in the aqueous phase. The results also suggest that the mechanism of action of eugenol in emulsions is governed by the transport of eugenol through the aqueous phase to the bacterial surface rather than a direct interaction of emulsion droplets with pathogens. In case of a direct interaction, the concentration of eugenol per droplet should be the determining factor. Clearly more studies need to be done to elucidate the precise mechanism of action. Inhibition studies using a membrane system with the emulsion on one side of a membrane and the pathogens on the other side of the membrane could give more insights into the process.

Eugenol at 0.5% inhibited the growth of all strains tested of *E. coli* O157:H7 and *L. monocytogenes.* Since eugenol is not soluble in water, many research groups disperse it in ethanol [153], [154]. In this study we dispersed eugenol in TSB and eugenol in 1%

of ethanol and the added to TSB. Our results showed that mixing eugenol with ethanol decrease its activity. This could be due increased evaporation of eugenol when mixed with ethanol, during incubation.

In summary, the stability and antimicrobial activity of eugenol emulsions were evaluated. Eugenol-containing emulsions were formed by mixing the eugenol with other hydrocarbons in the oil phase of the emulsion. Stability of emulsions was improved when the alkanes chain length or the molecular weight increased [143]. Free eugenol inactivated or inhibited bacteria after 24h of exposure but at 48 h inactivation occurred. In general, *L. monocytogenes* was more resistant than *E. coli* O157:H7. Emulsions with higher ratios of corn oil were less efficient than emulsions with a 1:1 corn oil: eugenol ratio. Results yield important new insights into the processes that occur when eugenol is used in the presence of another lipid phase. If emulsions are initially loaded with antimicrobial, the concentration in the aqueous after partitioning will govern the overall activity. If eugenol is in the aqueous phase and lipid emulsions are introduced, eugenol may partition into the lipid phase and activity would again be determined by the equilibrium concentration of remaining eugenol in the water phase. This study highlights the importance of understanding fundamental physicochemical processes when developing a preservation system in compositionally complex foods.

Table 4.1. Mean particle diameter of emulsions without eugenol and emulsions loaded at a ratio of 1:1 and Ostwald ripening rates of emulsions.

Lipid	Mean Particle Diameter d_{10} (μm)						$\omega=\Delta d^3/t$ ($\mu m^3/h$)
	t=0h	t=24h	t=48h	t=168h	t=336h	t=504h	
Corn oil-eugenol	0.42	0.42	0.43	0.40	0.39	0.42	2.05E-09
Corn oil	0.28	0.29	0.29	0.27	0.28	0.29	1.28E-09
Hexadecane-eugenol	0.33	0.34	0.33	0.33	0.34	0.34	1.59E-09
Hexadecane	0.24	0.24	0.24	0.25	0.26	0.26	1.06E-08
Tetradecane-eugenol	0.50	0.50	0.50	0.52	0.53	0.53	6.32E-08
Tetradecane	0.17	0.18	0.24	0.27	0.29	0.30	4.54E-06
Dodecane-eugenol	0.53	0.48	0.50	0.56	0.63	0.66	4.37E-06
Dodecane	0.16	0.30	0.32	0.53	0.54	0.60	1.70E-04

Table 4.2. Critical loading ratios for emulsions containing eugenol and a carrier lipid

	Critical Loading Ratio			
	E:C	**E:H**	**E:T**	**E:D**
Ratio	1:0.43	1:0.66	1:1	1:0.66
Concentration	70:30	60:40	50:50	60:40
Mean Droplet Size (μm)	0.50	0.49	0.50	0.49

E= Eugenol, H= Hexadecane, T= Tetradecane, D= Dodecane, C= Corn oil.

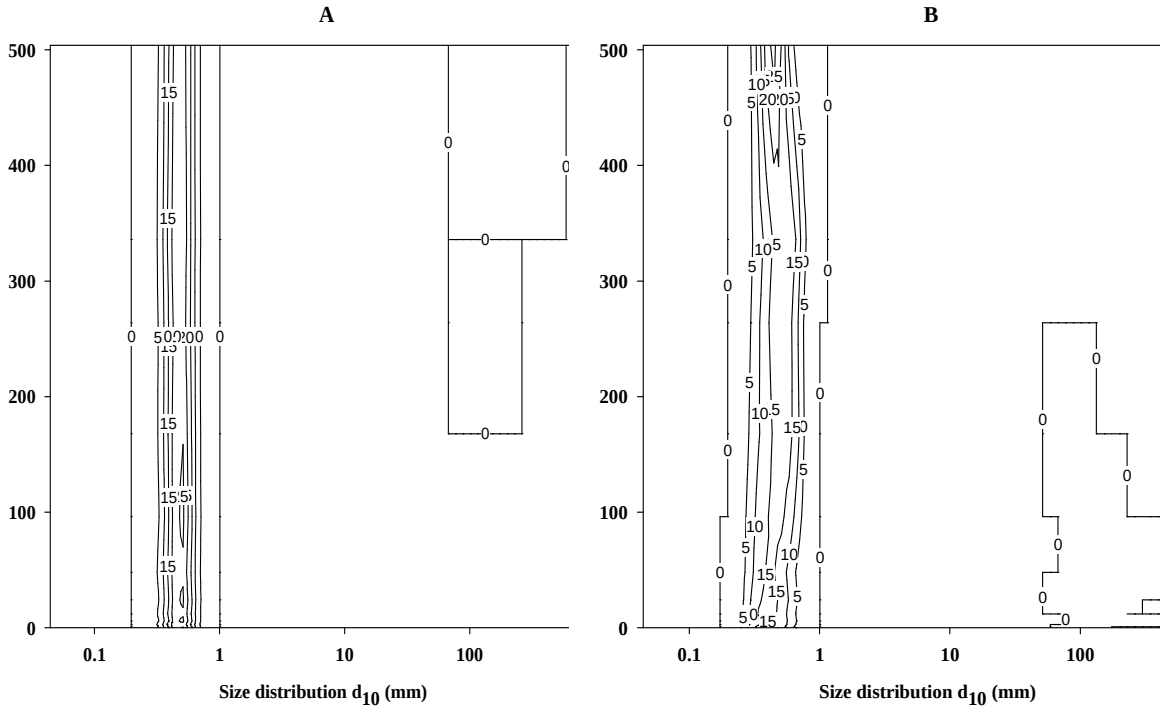
Table 4.3. Inhibition of four strains of *E. coli* O157:H7 and *L. monocytogenes* against eugenol –corn oil containing emulsions after incubation for 24h at 32° C.

		Emulsion Ratio (CO:E)						MIC (%V/V) of 1 % Eugenol in Water	MIC (%V/V) of 1% Eugenol in 1% Ethanol
		9:1		7:3		5:5			
		MIC (%v/v)	%v/v droplet concentration	MIC (%v/v)	%v/v droplet concentration	MIC (%v/v)	%v/v droplet concentration		
***E. coli* O157:H7**	**ATCC® 35150**	>0.500	> 5.000	0.100	0.333	0.100	0.200	0.500	0.100
	ATCC® 4385	>0.500	>5.000	0.100	0.333	0.100	0.200	0.500	0.100
	ATCC® 516858	>0.500	>5.000	0.100	0.333	0.100	0.200	0.500	0.100
	ATCC® 700599	>0.500	>5.000	0.100	0.333	0.100	0.200	0.100	0.100
L. monocytogenes	**J1225**	>0.500	>5.000	0.200	0.667	0.200	0.400	0.500	0.100
	J2020	>0.500	>5.000	0.200	0.667	0.200	0.400	0.100	0.100
	J1031	>0.500	>5.000	0.200	0.667	0.200	0.400	0.500	0.100
	C1115	>0.500	>5.000	0.200	0.667	0.200	0.400	0.500	0.100

Growth of the organisms was observed on all of the control and ethanol plates. When >0.5% v/v no inhibition was observed at the concentration tested.

CO=corn oil and E= eugenol.

Figure 4.1. Figure 1A, 1B, 1Cand 1D shows the particle size distribution over time. In figure 1 all alkanes and corn oil, are mixed with eugenol at a ratio 40:60 alkane: eugenol. Figure 1A, shows hexadecane and eugenol used to demonstrate a slow Ostwald ripening rate after 264h (11 days). Figure 1B shows tetradecane with eugenol to illustrate an example of instant emulsion breakdown after time zero. In figure 1C, dodecane and eugenol are shown. In this case Ostwald ripening destabilization can be seen after 48h (2days). Figure 1D, shows corn oil and eugenol emulsions where no Ostwald ripening occurs after 504h (21days).


A
B
500
400
300
200
100
0
0.1
1
10
100
Size distribution d_{10} (mm)

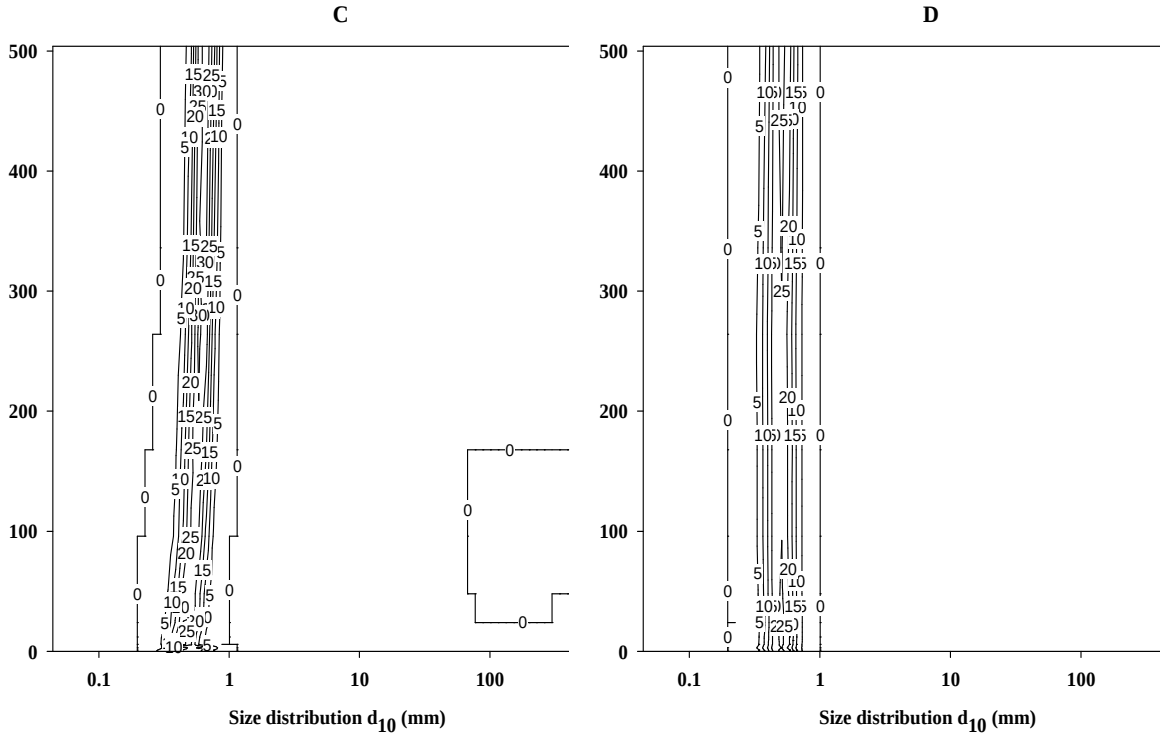

C
D
500
400
300
200
100
0
0.1
1
10
100
Size distribution d_{10} (mm)

Figure 4.2. Figure 2A shows the growth curve of the resistant strain (ATCC® 35150) while Figure 2B shows the most sensitive strain (ATCC® 700599) of *E. coli* O157:H7 against emulsions at different corn oil –eugenol ratios diluted to 0.1% of eugenol in the system.

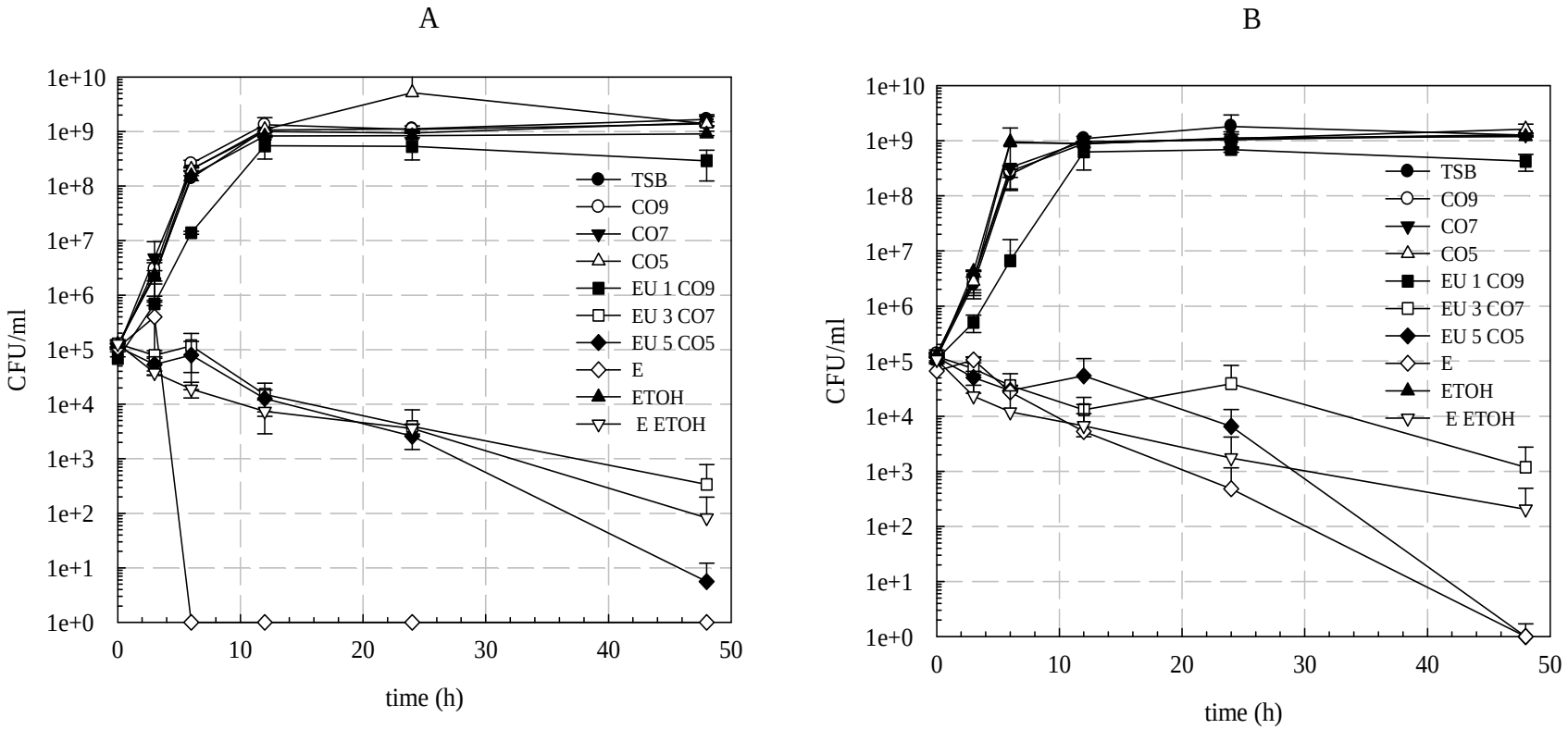
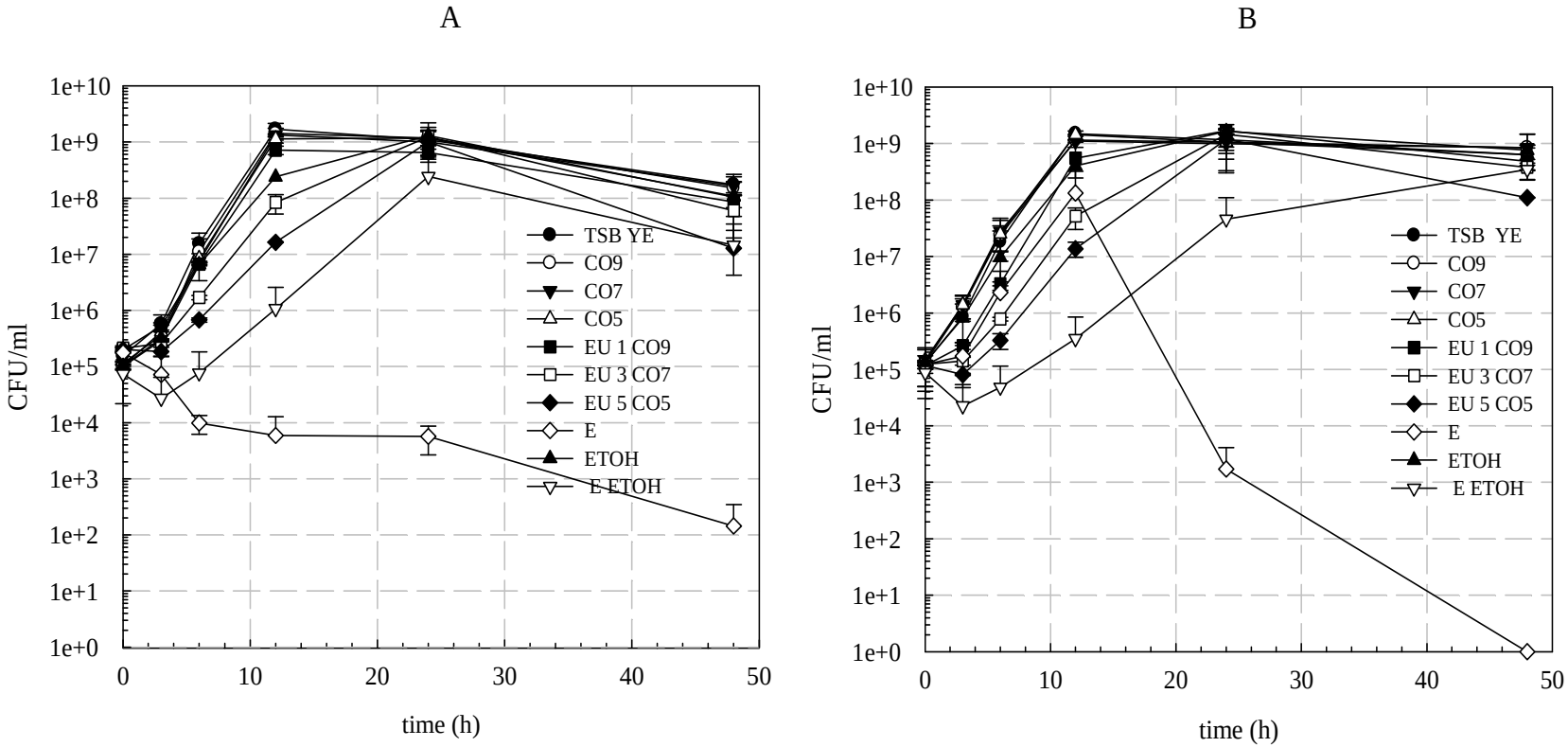


Figure 4.3. Figure 3A shows the growth curve of the resistant strain (J1225) while Figure 3B shows the most sensitive strain (J1031) of *L. monocytogenes* against emulsions at different corn oil –eugenol ratios diluted to 0.1% of eugenol in the system.



CHAPTER 5
EMULSIONS AS ANTIMICROBIAL DELIVERY SYSTEMS: PART I. THE ANTIMICROBIAL AS PART OF THE INTERFACIAL PHASE

5.1 Abstract

Physical stability and antimicrobial activity of corn oil-in-water emulsion formulated with lauric arginate, a cationic antimicrobial surfactant, was evaluated. Emulsions were produced by homogenizing corn oil with lauric arginate and/or Tween 20 using a microfluidizer. Emulsions were formulated at pH values ranging from 4 to 7. Particle size analysis, creaming index determination and optical microscopy was used to assess stability of emulsions during storage. Minimum inhibitory concentrations (MIC) of emulsions against strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes* was determined using a spot inoculation assay. Control emulsions formulated with Tween 20 as controls were stable and neither flocculation nor change in average droplet size was observed during storage. Emulsions formulated with only lauric arginate more rapidly broke down after manufacturing at pH>5 i.e. the corn oil phase separated from the emulsion: Emulsions formulated with both lauric arginate and Tween 20 at a 1:1 ratio were stable to aggregation and coalescence even at pH 7, *e.g.* the droplet size of the emulsion stabilized by the binary surfactant mixture remained 165 nm. ζ-potential measurements suggested that the interfacial layer consisted predominantly of lauric arginate. Emulsions containing lauric arginate were both highly effective in inhibiting growth of both *E. coli* O157:H7 and *L. monocytogenes*, *e.g.* the MIC of lauric arginate against *E.coli* was 0.005%v/v and 0.oo25%v/v for *L. monocytogenes*.

5.2 Introduction

Antimicrobials are often added to foods to improve their quality and microbial safety. Antimicrobials are compounds that when added to a food inhibit or inactivate spoilage or pathogenic microorganisms [155]. Antimicrobials vary greatly in their chemical properties, e.g. they may be hydrophilic, partially lipophilic and completely hydrophobic [78, 156]. In many applications, the antimicrobials are directly dispersed in the target system (sometimes with the help of a dispersing agents such as alcohol or a surfactants), with the result that the antimicrobial efficacies often dramatically decrease compared to those observed in microbiological model system. This is because the antimicrobials may partition from the water phase into hydrophobic phases or interact with interfering compounds reducing the interaction with target microorganisms [91, 157]. In general, there is still a considerable lack of knowledge as to the role that mass transport processes resulting in an altered non-homogeneous spatial distribution plays in the activity of antimicrobials although recent research has suggested that design of encapsulation systems that provide for a more homogeneous distribution in the aqueous phase of foods can improve the efficiency of antimicrobials. The goal of this study is to evaluate the feasibility of using emulsions as potential carrier systems for antimicrobials.

Food emulsions are an important class of products that are widely used in the food industry, e.g. to formulate products such as mayonnaise, salad dressings, spreads, soups and others. Emulsions are liquid-liquid dispersions with one liquid being dispersed in the other in the form of droplets. The properties of emulsions are generally governed by both the properties of the droplets such as droplet concentration, droplet size and droplet interfacial composition and the properties of the continuous phase such as viscosity, ionic

strength and pH [45]. Of these properties, the droplet interface plays a major role in determining many bulk physicochemical and organoleptic properties of food emulsions [158]. For example, droplet-droplet interactions of importance to the physical and chemical stability of the emulsion depend on the composition and structure of the interfacial membrane which in turn is controlled by the type and concentration of the surface-active species present in the system [16, 29, 45, 159]. While emulsions could potentially be formulated as antimicrobial carrier systems by (a) including the antimicrobial in the droplets or (b) using an antimicrobial that may adsorb at the interface of droplets, in the context of this study, we are focusing on formulating emulsions with an antimicrobial at the interface.

Lauric arginate (N-α-lauroyl-L-arginine ethyl ester monohydrochloride; LAE), commercially available as Mirenat-N®, is a novel antimicrobial that has been Generally Recognized as Safe (GRAS) by the FDA for use in a variety of foods, due to it being easily metabolized in the human body [14]. Lauric arginate has shown to be highly effective against a great variety of microorganisms including both gram-positive and gram-negative bacteria such as *Staphylococuus aureus* [93], *Clostridium perfringens* [94], *Listeria monocytogenes,* and *Pseudomonas spp.* [95]. Its antimicrobial activity has been attributed to its ability to insert and disrupt the integrity of bacterial cell walls resulting in the inability of microorganisms to maintain homeostasis [15]. Most importantly, lauric arginate is a cationic surfactant, i.e. it has shown to lower surface and interfacial tension upon addition and it self-assembles into a variety of structures such as micelles above a critical concentration. As such, we hypothesize that lauric arginate could

be used to stabilize emulsions and simultaneously induce antimicrobial activity in the emulsion.

The objective of this study was to test this hypothesis by fabricating oil-in-water emulsions stabilized by lauric arginate and to test their stability and antimicrobial efficacy. Based on previous studies conducted in our lab, we also used Tween 20 - lauric arginate mixtures to stabilize emulsions, since studies on the physical stability of dispersions of lauric arginate had shown that the compound may become insoluble at higher pH but remain soluble upon addition of Tween 20.

5.3 Materials and Methods

5.3.1 Materials

Mirenat-N® (10.5% of N-α-lauroyl-L-arginine ethyl ester monohydrochloride in propylene glycol) was obtained from Grupo Lamirsa (Barcelona, Terrassa, Spain). Tween 20 (polyoxyethylene 20 sorbitan monolaurate) and corn oil were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions were prepared with distilled and deionized water. Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), and Yeast extract (YE) were purchased from Difco Laboratories (Sparks, MD).

5.3.2 Bacterial Cultures

Four different *L. monocytogenes* strains were obtained from the Martin Weidmann culture collection at Cornell University (J1-225, J2-020, J1-031, and J1-1177). Four strains of *E.coli* O157:H7 were purchased from ATCC (ATCC35150,

ATCC4385, ATCC51658, and ATCC700599). Bacterial cultures were stored at -75ºC in TSB for *E. coli* O157:H7 and TSBYE for *L. monocytogenes* with 5% glycerol. Working cultures were maintained on slants stored at 4°C. A loopful of the culture was transferred to liquid media and incubated at 32°C for 24 hr. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 24 hr.

5.3.3 Physicochemical Characterization

Emulsion preparation. First, solutions of single and binary surfactant systems were prepared by dispersing the single or the two surfactants in buffer (100 mM acetate buffer) to obtain an overall surfactant concentration of 0.5% (v/v). The binary surfactant system consisted of a mixture of 0.25% of Tween20 and 0.25% of Lauric arginate. The surfactant solution was then blended with 5% (v/v) of corn oil using a high speed blender (Warring, Inc. New Hartford, CT) to produce a coarse emulsion premix. Third, the coarse premix was then passed through a microfluidizer (Microfluidics 110 L, Microfluidics Corp., Newton, MA, USA) at 9 kPa to produce a fine-disperse emulsion with mean particle sizes of 150-200 nm. Finally, the pH of the emulsions was adjusted to between 2 and 7 using HCl or NaOH. The emulsions were then stored at 32ºC and samples withdrawn for analysis in regular intervals.

Droplet size determination. The z-average particle diameter and polydispersity index was determine by using Dynamic Light Scattering (DLS) sizer (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, UK). The instruments incorporates a non-invasive back scatter (NIBS) optics that measures the scattered light at an angle of 90° to maximize the detection of scattered light while maintaining signal quality. The

hydrodynamic radius of particles is calculated from the diffusion coefficient of particles that is determined from the interference pattern of scattered light.

ζ-Potential Measurement. The electrical charge (ζ-potential) on the surface of emulsion droplets was determined using an electrophoretic technique with a special folded capillary cell (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, UK). A small amount of sample was diluted into buffer at the appropriate pH. An oscillating electric field was applied across the dispersion and the zetapotential was then determined from the velocity of the droplets migrating toward the oppositely charged electrode.

Creaming measurements. Emulsion samples were transferred into a test tube (with 15 mm internal diameter). The total height of the emulsions in the tubes was 7 cm. Tubes were tightly sealed with a plastic cap, and then left to stand at 32ºC. The creaming stability of emulsion was monitored by measuring the backscattering of monochromatic light (λ = 850 nm) from the test tube as function of its height (Turbiscan Classic MA 2000, Formulaction, France) after storing the emulsion for up to 71 days.

Optical microscopy. To better understand the behavior of emulsion droplets, emulsion samples were observed using a conventional optical microscope (Nikon microscope Eclipse E-400, Nikon Corporation, Japan). The images of the droplets were acquired using a CCD camera (CCD-300T-RC, Dag-MTI., Michigan City, IN) connected to Digital Image Processing Software (MVI/MicroVideo Instruments Inc., Avon, MA) installed on computer. Emulsions were agitated in a test tube before analysis. A drop of emulsion was placed between a microscope slide and cover slip and images were taken.

Interfacial tensiometry. To characterize the interfacial properties of emulsion droplets, the surface tension was measured at the oil-water interface upon addition of the antimicrobial and stabilizing surfactant (e.g. Tween 20). A drop shape analysis tensiometer (Model DSA-G10 MK2, Kruss USA, Charlotte, NC) was used to determine surface tension as a function of surfactant concentration of either the single surfactants or the binary mixtures. The tensiometer determines the surface tension from the shape of pendant drops through numerical analysis. Experimentally obtained surface tension versus concentration data was used to compare CMC results to existing models that predict the CMC as a function of the concentration of surfactants in the binary surfactant mixture. The density of corn oil at 22°C was 0.9198 g/cm^3.

5.3.3 Antimicrobial Test Protocols

Emulsion preparation for antimicrobial assays. Emulsions were prepared as described above and filtered-sterilized using a 0.20 μm. polyethersulfone membrane filter (Puradisc^{TM} 25, Whatman® Inc. Florham Park, NJ). After filter sterilization, emulsions were diluted with sterile water to obtain a series of emulsions containing oil droplet concentrations of 0.005-0.500 % (v/v) per plate.

Spot Inoculation Assay. Two cycles of a loopful of the *E. coli* slant cultures was transferred to TSB (*L. monocytogenes* to TSB-YE, added 0.6% yeast extract), incubated at 32ºC for 24h before exposure to the antimicrobial emulsion. Petri dishes were prepared by adding 2 ml of the emulsion diluted to the desired antimicrobial concentration in 20 ml of TSAYE. Control plates with water were prepared. The Petri dishes containing were dried over night at 32°C and 10μl drops of the inoculums were plated to give a final

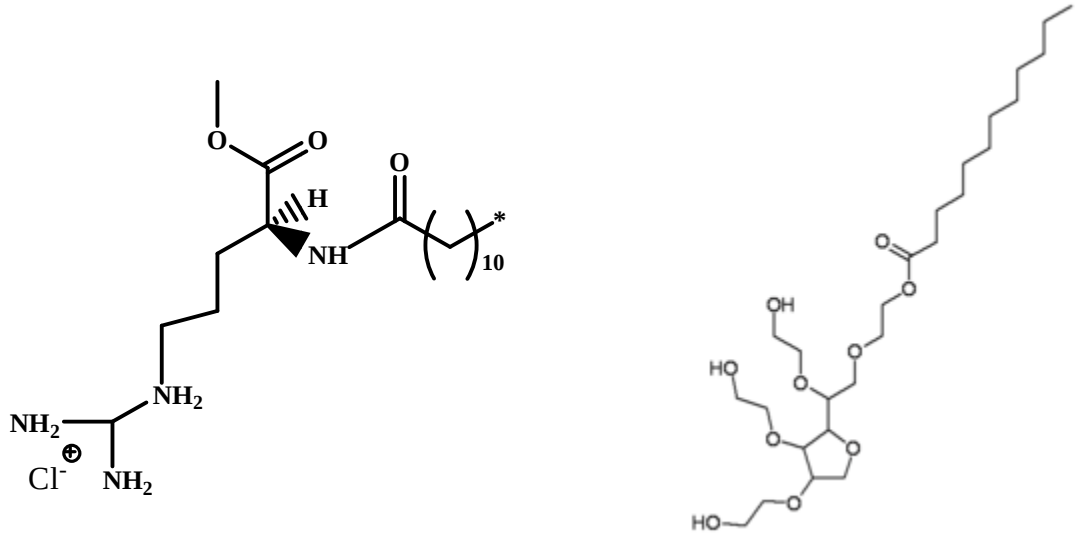
concentration of 10^5 CFU/ml. The Petri dishes were incubated at 32ºC for 24 h. All experiments were replicated and duplicated.

5.4 Result and Discussion

Emulsion droplet size. Corn oil-in-water emulsions were produced with three different surfactant systems that are two single surfactant systems (0.5% v/v LAE and 0.5% v/v Tween 20) and one binary surfactant system consisting of 0.25% v/v LAE and 0.25% v/v Tween 20. Emulsions manufactured with the three different surfactant systems had noticeably differences in z-average droplet diameter as a function of pH when measured within 1 hour after manufacturing (**Figure 5.2**). At pH 2-5, the droplet size of emulsions manufactured with 0.5 % v/v LAE was the lowest with less than 145 nm while emulsions manufactured with Tween 20 or Tween 20 – LAE had slightly larger droplets i.e. $d_{z,Tween20}$~175 nm and $d_{z,Tween20+LAE}$=165 nm. This could be due to LAE (a) inducing higher repulsive interaction forces between newly formed droplets (b) more rapidly covering newly formed droplet interfaces or (c) more strongly reducing the interfacial tension during the homogenization process. Based on the fact that the mixed system has droplet sizes larger than that of pure LAE but smaller than that of pure Tween 20 is a first indication that the droplets are possibly being covered by both surfactants. At pH>5, emulsions formed with LAE in the had large droplet sizes ($d_{z,LAE,pH>5}$ > 500 nm) while emulsions manufactured with Tween 20 system remained small with z-average droplet sizes ranging between 165 and 185 nm.

Interfacial and surface tension. To gain a better insight into the properties of the interfacial layer in the presence of the surfactant systems, the interfacial tension was

measured at a corn oil-water interface and the surface tension was measured at the air-water interface as a function of pH (**Figure 5.3**). LAE had the lowest the surface and interfacial tension suggesting that LAE is possibly more surface active than Tween 20. LAE and Tween 20 differ greatly in their molecular properties. For example, LAE has a molecular weight of approximately 421 g/mol while Tween 20 has a molecular weight of 1227 g/mol. The two molecules do not vary significantly in their hydrocarbon tail length with both tails consisting of lauric acid however they differ greatly in their headgroup composition.



a) b)

Figure 5.1 a) N-α-lauroyl-L-arginine ethyl ester monohydrochloride (LAE) $C_{20}H_{41}N_4O_3Cl$: the active compound in Mirenat N [1], b) polyoxyethylene (20) sorbitan monolaurate (Tween20) $C_{58}H_{114}O_{26}$ [2]

Tween 20 consists of a large headgroup composed of polymerized ethyleneoxides linked to the fatty acid by a sugar alcohol while the lauric arginate headgroup consist of

simply arginine. Typically, surfactants with smaller headgroups but similar hydrophobic chain length will absorb more rapidly at interfaces compared to surfactants with larger headgroup [31]. Both the interfacial and surface tension of Tween 20 containing surfaces or interfaces changed little with pH, but surfaces or interfaces containing only LAE were strongly influenced by the pH. Results suggest that the surface activity of LAE is strongly influenced by pH, making the environmental conditions in the emulsion a key factor for the suitability of LAE as an emulsifier.

ζ-Potential. While the determination of the interfacial and surface tension gives a first insight into the possible configuration at the droplet interface, interfacial tensiometry is not completely representative of the situation encountered in the emulsified system. This is firstly because the total surface area is much larger thus leading to a greater decrease in the amount of surfactant adsorbed at interfaces, secondly, the tensiometry is done on a single, large droplet where curvatures are much smaller than in emulsions composed of fine disperse droplets. Therefore, to further elucidate the interfacial membrane composition in emulsions, the ζ-potential of emulsions stabilized by LAE, Tween 20 or their mixtures was measured as a function of pH (**Figure 5.4**). Droplets stabilized by lauric arginate were strongly positive with the z-potential being 52-63 mV, but the surface charge decreased at pH>7 to below 35 mV. Emulsion droplets stabilized were slightly negative with z-potentials of -4 $\pm$ 1.3 mV, which is good agreement with values reported in the literature. In the mixed surfactant system, the zeta-potential was between the measured for the Tween 20 – stabilized emulsions and the emulsion stabilized by LAE, i.e. z=+10 mV. Moreover, the surface charge of droplets stabilized by the mixed surfactant system did not noticeably vary with pH. The results suggest that the

interface was indeed composed of both surfactants and that the presence of Tween 20 mediated the pH-dependent decrease typically observed in pure LAE emulsions **(Figure 5.5)**

Emulsion Stability. The stability of the emulsions during storage for up to 64 days was determined by measuring the creaming profile of emulsions stored in test tubes and by taking photographic images of test tubes periodically during the storage test (**Figure 5.6).** Emulsions stabilized by Tween 20 were stable to coalescence during 64 days of storage at 32°C, e.g. the backscattering intensity measured in the test tubes containing the emulsion remained constant during the storage period and photographic images of test tubes suggest absence of a coalesced oil layer. However, emulsions stabilized by lauric arginate were unstable at pH 6 and 7, e.g. the emulsion creamed with backscattering intensities being greatly decreased due to the formation of a concentrated emulsion droplet layer and coalesced oil layer on the top of the test tube. Emulsions composed of the surfactant mixture were much more stable to creaming and coalescence then emulsion stabilized by Tween 20 alone, that is backscattering intensities where slightly lower but remained constant during the storage time. It should be noted though that at pH 7 after 64 days of storage, some coalescence at the surface of the test tube was noticeable.

The results further confirm that the ability of LAE to stabilize emulsion is highly pH dependent. At pH above 5, the repulsive interaction forces no longer sufficient to prevent droplet coalescence. As a result, droplets rapidly coalesce and cream. Similar results have previously been reported with ionic emulsifiers [25]. For instance, in emulsions were stabilization occurs predominantly by electrostatic repulsion, the emulsion stability was found to strongly depend on the conditions of the medium e.g. pH,

ionic strength and temperature [25]. Moreover, droplet interfaces stabilized by lauric arginate have lower interfacial tensions (**Figure 5.3**), which makes deformation and merging of the interfaces of two droplets that have come into close contact more likely [27]. **Table 5.1** further shows the increase in droplet size at pH 6 and 7 in the emulsion stabilized by LAE during the course of the storage. Tween 20 and Tween 20 – LAE emulsions however were quite stable throughout the stability experiments. The results demonstrate that addition of Tween 20 can help improve the physical stability of an emulsions containing LAE.

Optical microscopy. **Figure 5.8** shows microscopy images of emulsions that were manufactured with the three different surfactant systems at pH 2 - 7 after 50 days of storage. Emulsion droplets stabilized by only lauric arginate were noticeable affected by pH *e.g.* the droplets grew to significantly larger sizes at pH 5 - 7 compared to all other emulsions. Emulsions stabilized by Tween 20 were little affected by pH that is the droplets did not coalesce into larger ones. Finally, in the binary surfactant system composed of lauric arginate and Tween 20 at pH 5- 7 droplets while being larger than at pH 2 - 4 were noticeably smaller than droplet of the emulsion stabilized by only lauric arginate. This suggests that Tween 20 improved the stability of lauric arginate containing emulsions.

Antimicrobial activity. The antimicrobial activity of the three emulsions was tested against four strains of *E.coli* O 157:H7 and *L. monocytogenes* using a spot inoculation assay. **Table 5.2** shows the minimum inhibitory concentrations (the concentration at which growth was completely inhibited for 24h) obtained from the spot inoculation tests both based in the active ingredient concentration (lauric arginate) and

the droplet concentration. A positive control consisted of cultures grown in the absence of antimicrobial and a negative control consisted of cultures grown in the presence of lauric arginate dispersed in water. All strains of both cultures were completely inhibited in systems that contained lauric arginate with *L. monocytogenes* being more sensitive to the presence of lauric arginate than *E. coli* O157:H7, e.g. $MIC_{E.coli}$ = 0.005%v/v and $MIC_{L.monocytoegens}$= 0.0025%v/v. Because of the presence of the lipopolysaccharide layer, gram-negative bacteria may effectively obstruct the insertion of membrane-pertubating antimicrobial agents thus making them less susceptible. Interestingly, on a active ingredient basis, there was no difference between the MIC of water-dispersed LAE and the MIC of emulsions containing LAE. On a droplet concentration basis however, twice the number of droplets were required for the emulsions stabilized by the mixed surfactant layer than the emulsion stabilized by LAE only. At this point in time, we are not clear about the mechanisms that are involved in the inactivation. The fact that no differences in the activity were observed between the water and the emulsion system could suggest a direct interaction between the emulsion droplets and the bacterial pathogens that is similar to that found for the molecularly-dispersed LAE.

5.5 Conclusion

Results demonstrate that the formulation of antimicrobially active emulsions with lauric arginate is feasible. Adsorption of lauric arginate at the surface of oil droplets appears to have no negative effect on the antimicrobial activity. It should be noted though that emulsion droplets similar to molecularly-dispersed lauric arginate will bind to oppositely charged compounds such as for example carrageenan or pectin or protein above their pKa, which could lead to a reduction in antimicrobial activity. Nevertheless,

results are promising as they suggest that LAE may serve as an effective emulsifier in combination with Tween 20 at pH 2-7. More importantly, the positive charge of LAE containing emulsion droplets could simultaneously repel positively charged ions such as iron that may promote lipid oxidation. Thus lauric arginate stabilized emulsions may offer antimicrobial and antioxidant functionalities, a subject that will be further explored in a subsequent study. Overall results may be of substantial interest to food manufacturers looking towards a novel way to improve the antimicrobial activity of systems containing dispersed lipid phases.

Table 5.1 Z-average droplet diameters of emulsions as a function of storage time and pH formulated using the three different surfactant systems: 0.5%v/v LAE, 0.25%v/v LAE and 0.25% v/v Tween 20, and 0.5%v/v Tween 20.

pH	Z-average Droplet Diameter (nm)										
	Day										
	1	**8**	**15**	**22**	**29**	**36**	**43**	**50**	**57**	**64**	**71**
0.5%LAE											
2	140.8	144.4	142.3	140.9	140.7	141.6	143.4	141.6	139.7	141.2	145.7
3	141.0	141.6	143.1	141.2	140.5	142.3	142.5	143.6	141.3	141.4	141.2
4	146.0	158.2	144.6	138.8	142.1	142.7	142.7	142.8	141.9	144.5	143.5
5	151.0	142.7	142.6	140.9	143.4	142.3	146.4	144.3	145.8	146.1	148.1
6	163.6	143.2	144.6	144.3	156.5	208.9	349.7	618.6	1465.0	-	-
7	322.8	151.9	207.1	199.1	208.2	254.8	356.9	620.3	1539.5	-	-
0.25% LAE + 0.25% Tween 20											
2	150.2	151.3	152.5	148.1	152.2	153.6	153.7	151.9	152.8	151.7	151.5
3	152.6	153.8	154.0	150.3	153.9	153.9	155.5	151.8	152.8	157.7	153.3
4	154.1	149.9	153.7	150.2	152.6	153.5	153.0	151.0	153.2	152.8	156.8
5	166.4	151.7	152.7	149.8	152.4	152.5	153.3	151.5	153.0	153.8	162.5
6	163.4	163.7	176.2	155.6	159.2	173.8	176.7	238.2	235.9	214.1	313.1
7	190.6	185.4	192.3	225.6	196.5	199.1	179.2	180.4	186.0	285.1	149.9
0.5% Tween 20											
2	175.5	172.4	171.0	168.5	172.0	173.0	173.9	175.5	172.4	174.7	175.9
3	172.0	172.2	187.4	178.4	174.1	172.6	183.1	174.6	170.7	170.8	170.0
4	180.8	171.7	178.1	170.9	180.4	173.5	171.9	172.2	170.0	171.5	168.0
5	182.4	174.1	171.1	175.1	171.0	170.7	184.2	170.7	160.6	171.7	168.3
6	177.0	171.7	221.5	170.3	173.5	172.0	172.7	175.3	174.0	178.9	170.0
7	173.2	173.5	194.9	171.1	223.1	172.9	175.4	171.6	173.1	170.6	174.2

Table 5.2 Minimum inhibitory concentration of emulsions in terms of their active concentration against four strains of *E. coli* O157:H7 and *L. monocytogenes* after incubation for 24 h at 32ºC.

		Surfactant System Composition						
		0.5% LAE		**0.25%LAE +0.25%Tween20**		**0.5%Tween20**		**1 % LAE in H_2O**
		MIC (%v/v)	**%v/v droplet concentration**	**MIC (%v/v)**	**%v/v droplet concentration**	**MIC (%v/v)**	**%v/v droplet concentration**	**MIC (%v/v)**
***E. coli* O157:H7**	**ATCC® 35150**	0.005	0.05	0.005	0.100	>0.05	>0.500	0.005
	ATCC® 4385	0.005	0.05	0.005	0.100	>0.05	>0.500	0.005
	ATCC® 516858	0.005	0.05	0.005	0.100	>0.05	>0.500	0.005
	ATCC® 700599	0.005	0.05	0.005	0.100	>0.05	>0.500	0.005
L. monocytogenes	**J1225**	0.0025	0.025	0.0025	0.050	>0.05	>0.500	0.0025
	J2020	0.0025	0.025	0.0025	0.050	>0.05	>0.500	0.0025
	J1031	0.0025	0.025	0.0025	0.050	>0.05	>0.500	0.0025
	J1-1177	0.0025	0.025	0.0025	0.050	>0.05	>0.500	0.0025

Growth of the organisms was observed on all of positive control plates. No inhibition was observed for >0.05.

Figure 5.2 Effect of pH on z-average diameter of 5 % v/v corn oil-in-water emulsions formulated with 0.5 % v/v of surfactant (Tween 20, LAE, and 1:1 Tween 20-LAE).

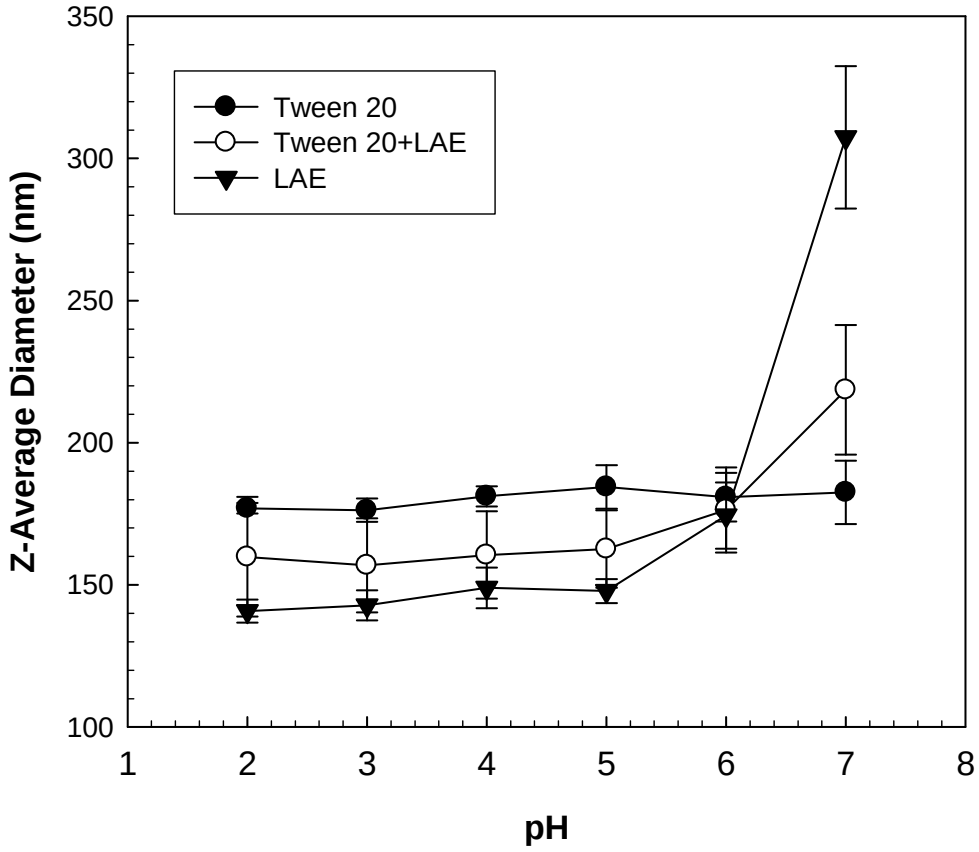


Figure 5.3 (A) Interfacial and (B) surface tension at the corn oil-water interface or air-water surface in the presence of 0.5 % v/v LAE, 0.5 % v/v Tween 20 and 0.25% v/v Tween 20 – 0.25 %v/v LAE as a function of aqueous pH.

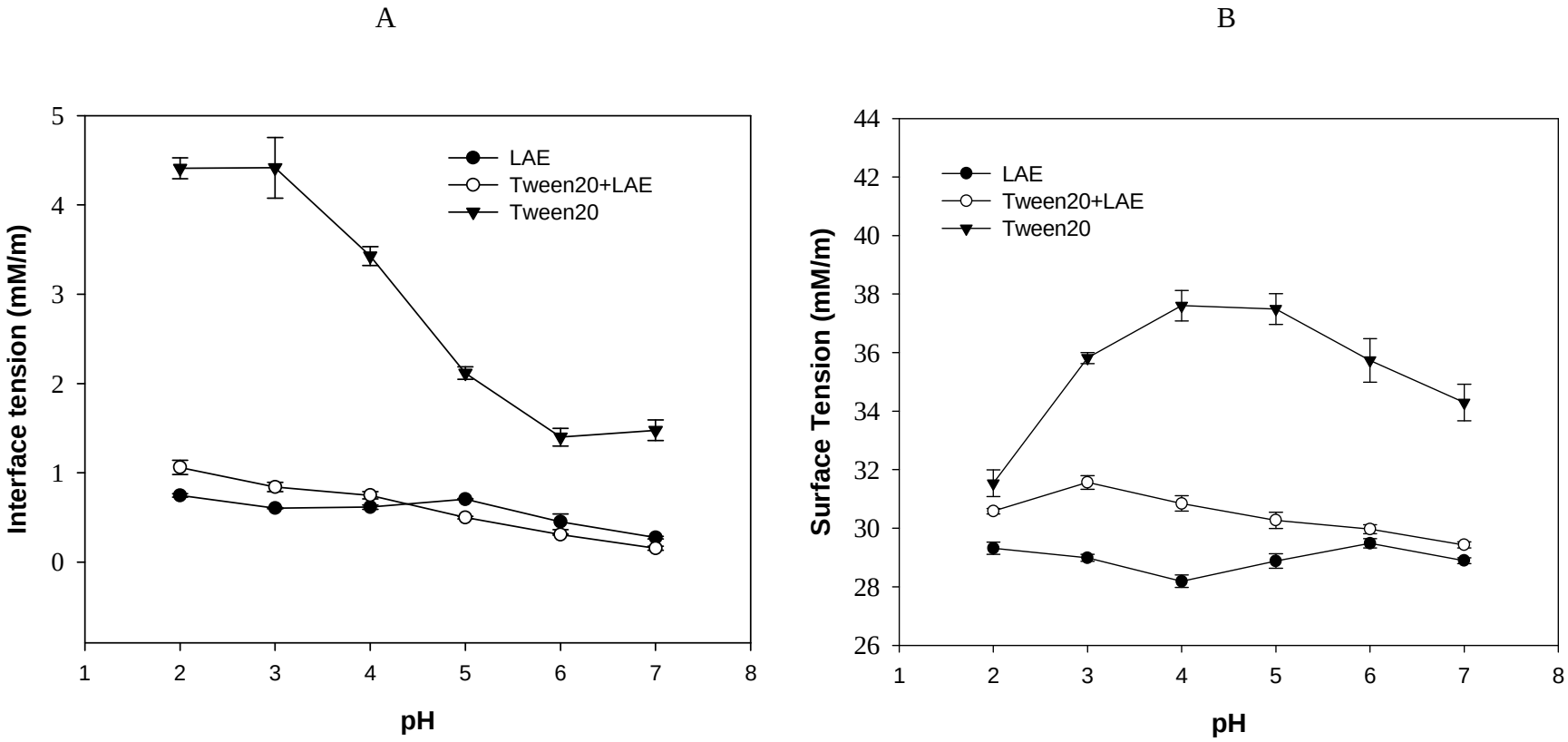


Figure 5.4 Effect of pH on ζ-potential of corn oil-in-water emulsion droplets in the presence of 0.5 %v/v LAE, 0.5 %v/v Tween 20 and 0.25%v/v Tween 20 – 0.25 %v/v LAE.

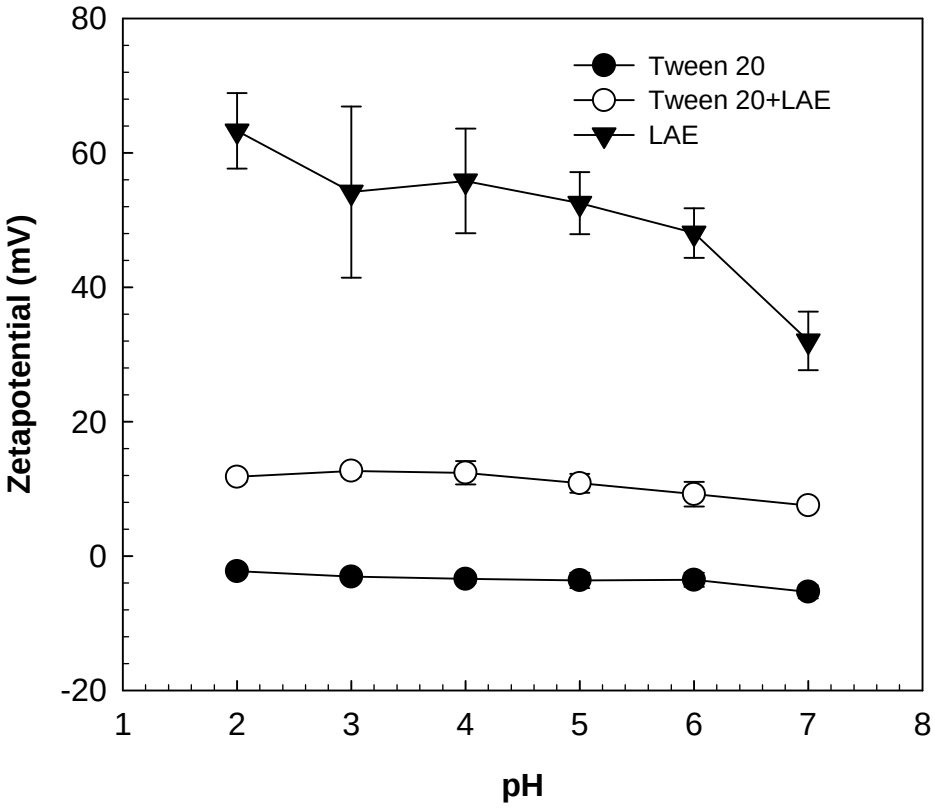


Figure 5.5 Schematic of the interfacial composition of droplets stabilized 0.5 %v/v LAE, 0.5 %v/v Tween 20 and 0.25%v/v Tween 20 – 0.25 %v/v LAE.

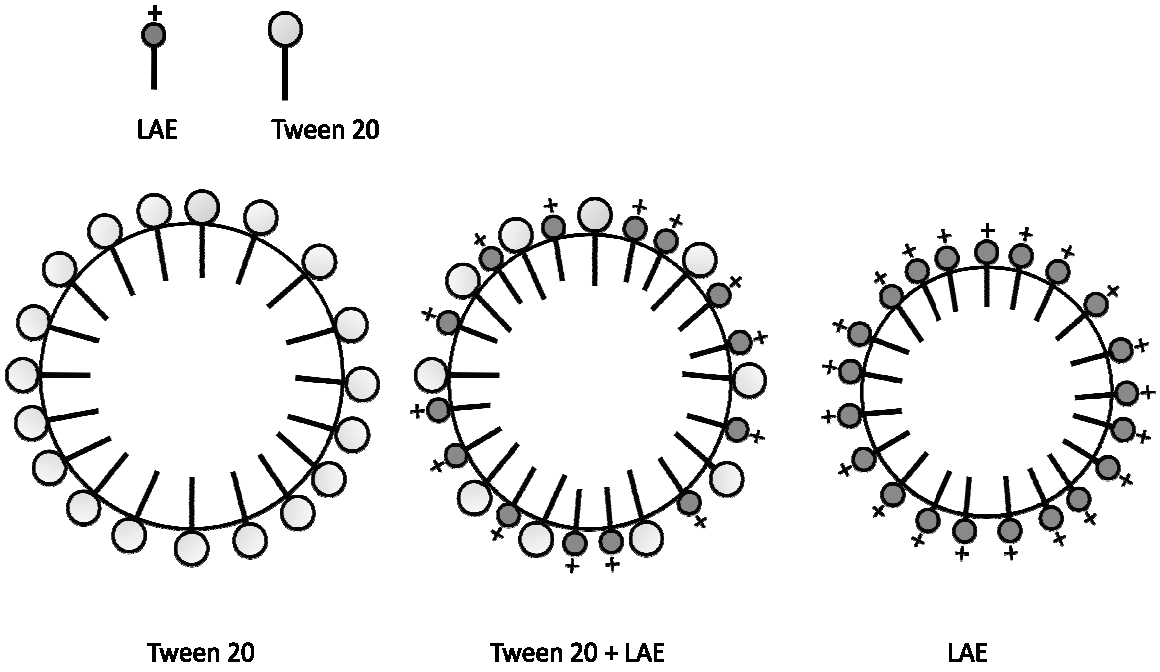


Figure 5.6 Stability of (A) LAE (B) Tween 20 and (C) LAE – Tween 20 stabilized emulsions after 64 days of storage at 32°C as a function of pH as shown by photographic images of the test tubes.

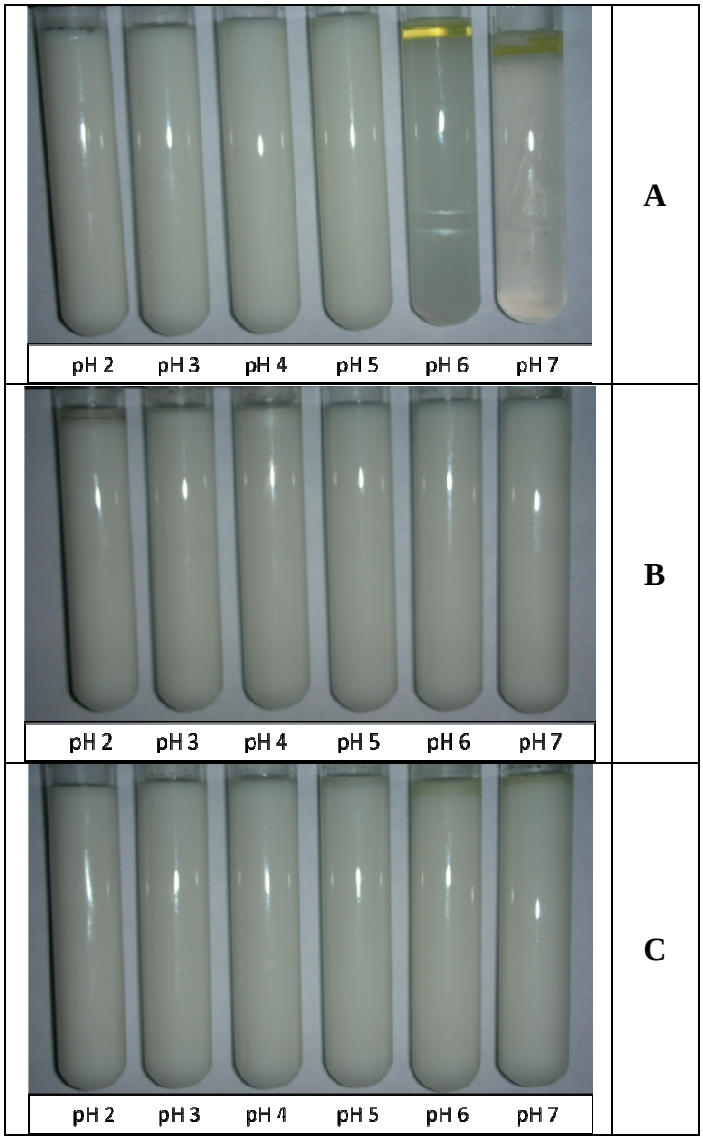


Figure 5.7 Effect of pH on the backscattering intensity of test tubes at a scanning height of 35 mm containing emulsions that were stabilized by lauric arginate, Tween 20 and Tween 20 – lauric arginate.

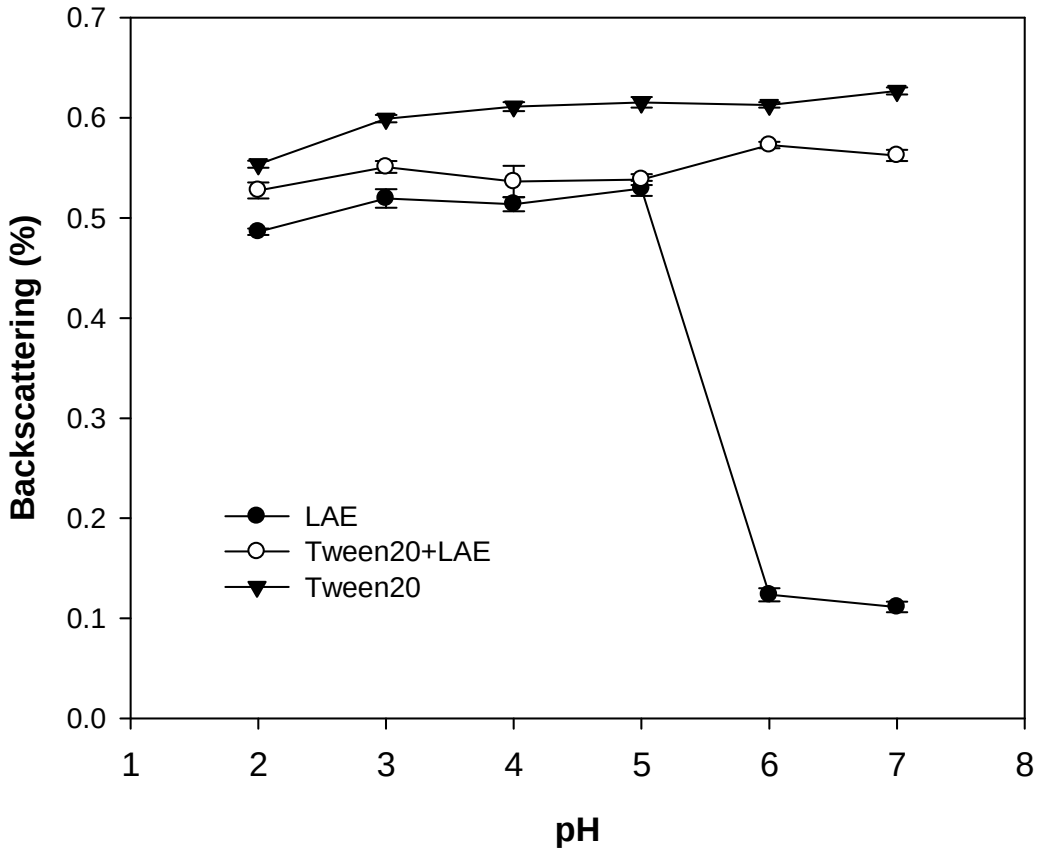
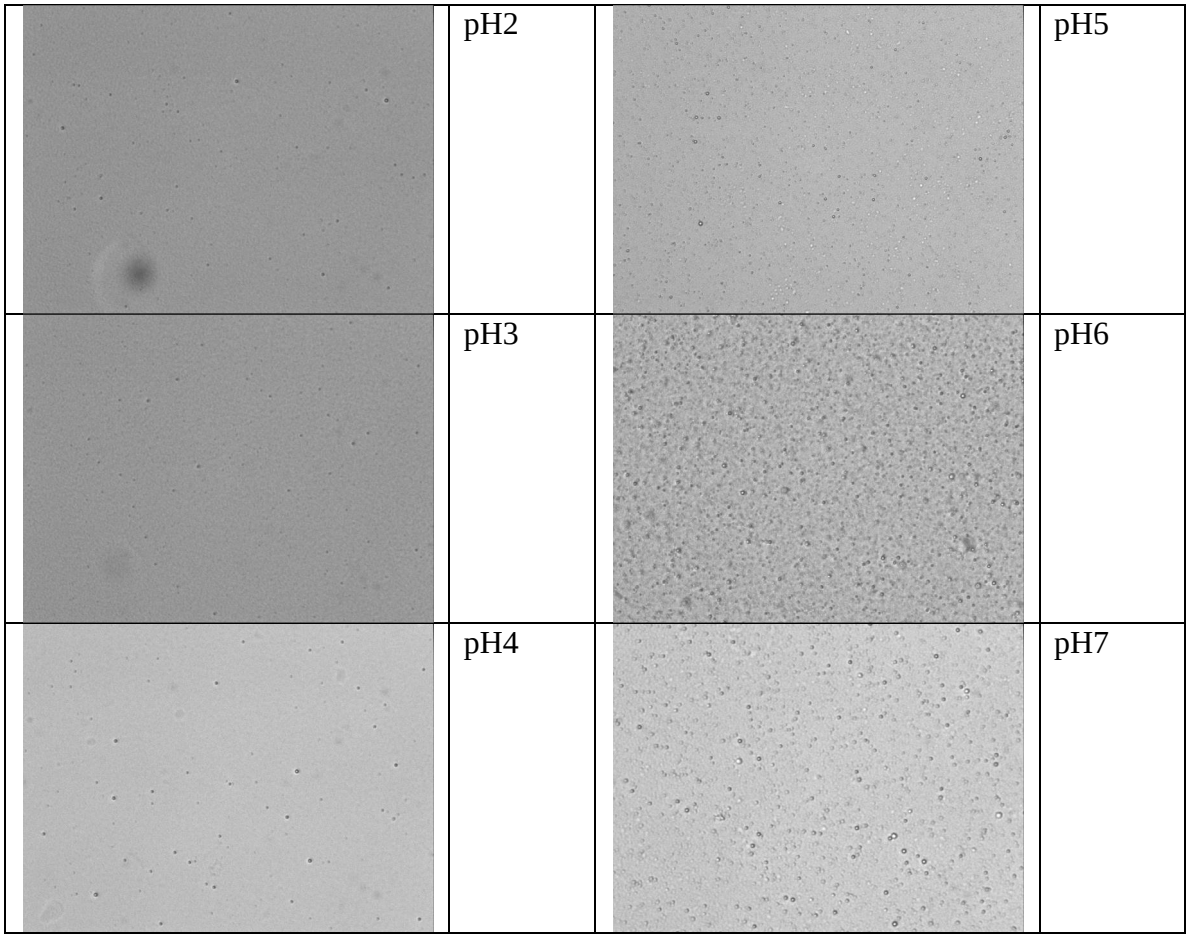
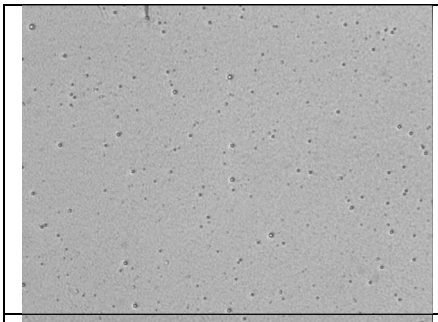
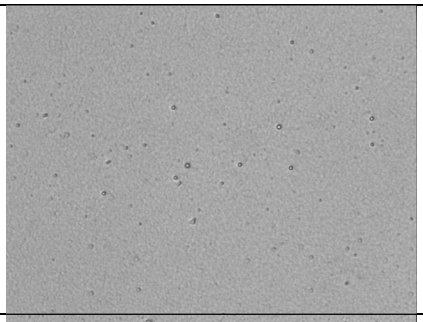
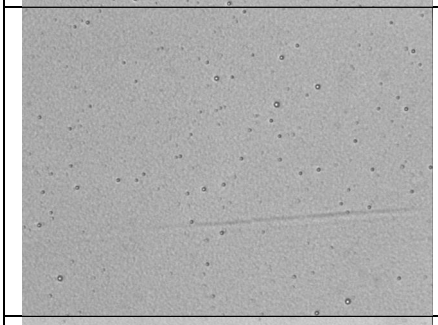
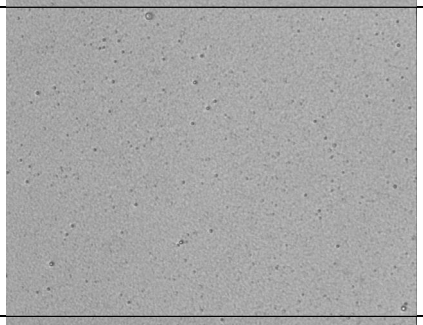
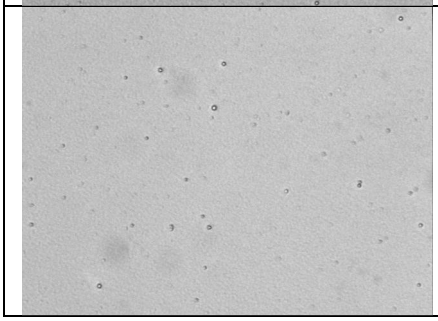
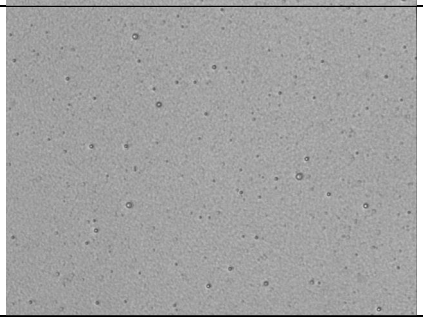


Figure 5.8 Microscopy images (200x magnification) of emulsion droplet stabilized by (A) lauric arginate (B) Tween 20 and (C) lauric arginate – Tween 20 after 50 days of storage at 32°C.

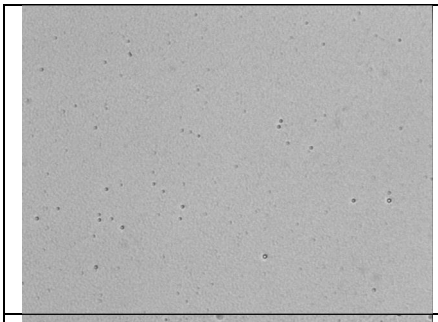
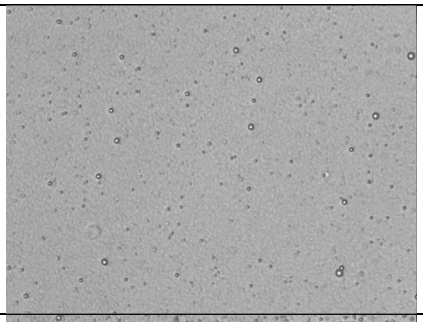
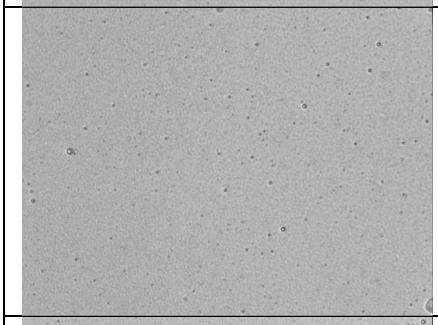
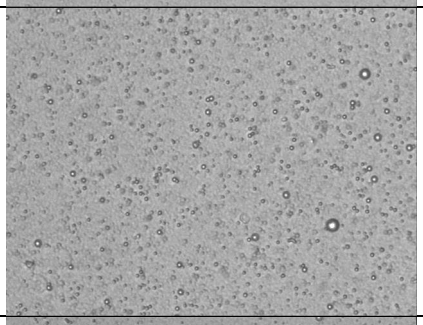
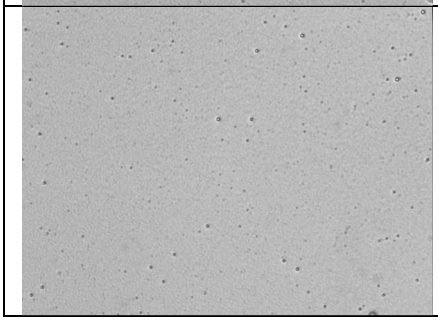
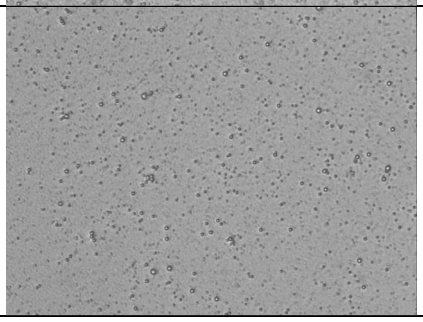
A



B

	pH2		pH5
	pH3		pH6
	pH4		pH7

C

	pH2		pH5
	pH3		pH6
	pH4		pH7

CHAPTER 6

EMULSIONS AS ANTIMICROBIAL DELIVERY SYSTEMS: PART III. THE ANTIMICROBIALS AT THE INTERFACE AND AS PART OF THE LIPID PHASE

6.1 Abstract

Corn oil-in-water emulsions were formulated that were composed simultaneously of two active antimicrobials; one at the interface (lauric arginate) and one in the lipid phase (eugenol). The physicochemical properties of the emulsions (particle size distribution, particle charge, creaming stability) and their antimicrobial activities against two pathogenic microorganisms (*E. coli O157:H7* and *Listeria monocytogenes*) were measured to determine suitable compositions that allow for maximum physical stability while simultaneously exhibiting high antimicrobial activity. We hypothesize that the attractive electrostatic interaction of lauric arginate with microbial surfaces may enhance the delivery of emulsion droplets containing encapsulated eugenol. The results demonstrated that the composition of interface affected the physicochemical and antimicrobial activities of emulsions. Oil-in-water emulsions were prepared using corn oil with or without eugenol as the dispersed phase (5 %v/v) and Tween 20 alone or in the combination with lauric arginate as emulsifiers (0.5 %v/v). Concentrations of Tween 20 to lauric arginate were varied (0.5%:0%, 0.495%:0.005%, 0.454%:0.046%, 0.25%:0.25%, 0.046%:0.456%, 0.005%:0.495% and 0%:0.5%) and droplet size and charge measured. The droplet size in the absence of eugenol was 0.20 - 0.32 µm. Emulsion droplet size increased with addition of lauric arginate possibly due to decreased steric repulsive interactions that prevent recoalescence during the homogenization

process. Increasing the ratio of lauric arginate also increased droplet charge from -2 mV to + 36 mV.

Stability of emulsions formulated with eugenol depended both on the eugenol concentration and the ratio of lauric arginate to Tween 20. Emulsions broke down above critical eugenol loading concentrations. These critical loading concentrations generally increased in emulsions that contained higher concentration of lauric arginate. Surprisingly, at the same loading concentration of eugenol the emulsion with a higher lauric arginate composition had a better stability than emulsions that contain higher Tween 20 concentrations. With addition of eugenol to the emulsion below the critical loading concentrations, the droplet size slightly decreased and the droplet charge didn't changed compared to eugenol-free emulsions. The antimicrobial activities of emulsions were generally high. When LAE was used as the only antimicrobial in the emulsions, $MIC_{E.coli}$ = 0.003-0.007%v/v except at a ratio of 0.046%:0.454% where the $MIC_{E.coli}$ increased to 0.02%v/v and $MIC_{L.monocytogenes}$ = 0.002-0.003%v/v were obtained. When eugenol was the only antimicrobial in the emulsion (e.g. the emulsion was stabilized by 0.5%v/v Tween20), $MIC_{E.coli}$ = 0.02%v/v and $MIC_{L.monocytogenes}$ = 0.03%v/v were obtained. In comparison, the double antimicrobial emulsions containing LAE and eugenol, had the lowest MICs as $MIC_{E.coli}$ of 0.001-0.008%v/v and $MIC_{L.monocytogenes}$ = 0.001-0.002%v/v except at a ratio of 0.046%:0.454% where the $MIC_{E.coli}$ increased to 0.02-0.03%v/v. The MIC of the double antimicrobials was strongly influenced by the concentration of lauric arginate. The results suggest that formulation of a double antimicrobial emulsions leads to a highly effective system that may improve food safety and stability.

6.2 Introduction

Each year foodborne illnesses cause significant economic damages and threaten the health and safety of US consumers. Therefore additional measures to control the growth of foodborne pathogens are urgently required. Bacterial pathogens such as *Campylobacter, Salmonella, Clostridium, Escherichia*, and others are the most commonly identified cause of foodborne illness that occur in a variety of food (FDA/CFSAN 2005). The principle method used to preserve the quality and enhance the safety of food is by thermally inactivating, aseptically processing or mechanically removing microorganisms. Alternatively, antimicrobials, chemical compounds that inhibit the growth of or inactivate microorganisms may be added to foods [73]. A key requirement of antimicrobials is that they fulfill their inhibitory or inactivating function in the target food system without significantly affecting appearance, taste, color and aroma of foods so as to not negatively impact consumer preferences. This aim can be very difficult to fulfill, since antimicrobials may have to be added at high concentrations to be effective. The objective of this study was to evaluate the feasibility of formulating novel double antimicrobial emulsions that have higher efficiencies than their single component counterparts. Double antimicrobial emulsions are defined as emulsions that contain simultaneously two antimicrobial agents, either both in the lipid phase, both at the interface or one at the interface and one in the droplet phase. In this study, we focus on the formulation of an emulsion with an antimicrobial at the interface and one antimicrobial in the lipid phase. This study is based on the results from two previous studies in which we showed that formulation of an emulsion containing high levels of a lipophilic antimicrobial (eugenol or carvacrol) is feasible as long as a critical loading

concentration is not surpassed. In the second study, we demonstrated that lauric arginate, a cationic surfactant antimicrobial, can be used to manufacture and stabilize emulsions without sacrificing antimicrobial activity [160].

Antimicrobials that are emulsifiers: Surfactants are amphiphilic molecules that are able to accumulate at surfaces or interfaces by interacting with both the lipophilic and hydrophilic phases. Their main function is to enable the formation of small drops during the emulsification step and to provide long-term stability after their preparation [31, 46]. Selection of appropriate surfactants is a key step in the preparation of stable emulsions as the presence of emulsifiers will greatly reduce the interfacial tension thereby facilitating droplet disruption. More importantly, emulsifiers form a membrane at the interface between the oil and water phase that provides a barrier to coalescence and flocculation by inducing repulsive interactions between two colliding droplets [29]. The ethyl ester of the lauramide of the arginine monohydrochloride-LAE, Mirenat-N ($C_{20}H_{41}N_4O_3Cl$) is a novel synthetic cationic surfactant (Belton, United States Patent) that was approved as 'Generally Recognized as Safe' (GRAS) by US FDA and USDA. It is rapidly metabolized in the human digestive system to the amino acid arginine and lauroyl amides [14]. Lauric arginate has shown to display high antimicrobial efficacies which has been attributed to its action on the cytoplasmic membranes of microorganism where it alters the membrane potential leading to a loss of homeostasis [15]. Our previous studies showed that formulation of emulsions with LAE alone is not feasible as the emulsions rapidly destabilize and hence an additional emulsifier such as Tween 20 needs to be added.

Antimicrobial that is part of the dispersed phase: Essential oils are generally complex mixtures of a large number of different compounds. Essential oils have shown to exhibit antimicrobial activity, which has been in particular linked to the presence of phytophenols such as eugenol, carvacol, and thymol. Phytophenols insert into the cytoplasmic membrane of microorganisms where they rapidly associate with membrane-bound protein complexes disrupting their functionality leading to a loss of the proton motive force [6, 152, 161, 162]. In this study, we used eugenol as a model antimicrobial compound due to its ability to inhibit both Gram-positive and Gram-negative bacteria [124].

Our previous results suggested that the major problem of forming O/W emulsions with aromatic compounds is destabilization of the emulsions by Ostwald ripening. However, stable emulsions could be produced by including a carrier lipid that is highly insoluble (e.g. corn oil) and combining it with the phytophenolic antimicrobial as part of the lipid droplet. Based on these results, we suggest that emulsions could be formulated that are composed of antimicrobially-active essential oils, such as form example eugenol, as part of oil phase (with corn oil) and an antimicrobial-active surfactant such as LAE to produce an emulsion that can be easily added to a food product to enhance the safety and quality of the product. To this purpose, we investigated the physicochemical properties and antimicrobial activity of emulsions that were composed of these two antimicrobials.

6.3 Materials and Methods

6.3.1 Materials

Mirenat-N® (10.5% of N-α-lauroyl-L-arginine ethyl ester monohydrochloride in propylene glycol) was obtained from Grupo Lamirsa (Barcelona, Terrassa, Spain). Tween 20 (polyoxyethylene 20 sorbitan monolaurate) and corn oil were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions were prepared with distilled and deionized water. Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), and Yeast extract (YE) were purchased from Difco Laboratories (Sparks, MD).

6.3.2 Bacterial Cultures

Four different *L. monocytogenes* strains were obtained from the Martin Weidmann culture collection at Cornell University (J1-225, J2-020, J1-031, and J1-1177). Four strains of *E.coli* O157:H7 were purchased from ATCC (ATCC35150, ATCC4385, ATCC51658, and ATCC700599). Bacterial cultures were stored at -75ºC in TSB for *E. coli* O157:H7 and TSBYE for *L. monocytogenes* with 5% glycerol. Working cultures were maintained on slants stored at 4°C. A loopful of the culture was transferred to liquid media and incubated at 32°C for 24 hr. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 24 hr.

6.3.3 Physicochemical Characterization

Emulsion preparation. Aqueous solutions of mixtures of Tween 20 and LAE were be prepared by dispersing the two surfactants in buffer (100 mM acetate buffer pH7) to

obtain a solution that had an overall surfactant concentration of 0.5% (v/v) but with varying ratios of Tween 20 and LAE [0.5%:0% (100:1), 0.495%:0.005% (100:1), 0.454%:0.046% (10:1) , 0.25%:0.25% (1:1), 0.046%:0.456% (0.1:1),0.005%:0.495% (0.01:1) and 0%:0.5% (0:100)]. Emulsions were then manufactured by blending 5% of a lipid phase that was composed of corn oil and eugenol at various mixing ratios i.e. 5%:0% (100:0), 4.75%:0.25% (95:5) 4.5%:0.5% (90:10), 4.25%:0.75% (85:15), 4%:1% (80:20), 3.75%:1.25% (75:25), and 3.5%:1.5% (70:30) with 95 wt% of solutions of the above described surfactant mixtures (100 mM acetate buffer). A high speed blender was used (Warring, Inc. New Hartford, CT) to produce this coarse emulsion premix. The coarse premix was then passed through a microfluidizer (Microfluidics 110 L, Microfluidics Corp., Newton, MA, USA) at 9 kPa to produce a fine-disperse emulsion. Finally, the pH of the emulsions was adjusted to 7 using HCl or NaOH. The emulsions were stored at 32ºC and samples were withdrawn for analysis in regular intervals.

Droplet size determination. A static light scattering technique (Horiba LA-9000, Horiba Instruments Inc., Irving, CA) was used to measure the droplet size distribution of emulsions. This technique measures the angular dependence of laser light scattered by the droplets in an emulsion. A relative refractive index of 1.1 was used by the instrument to calculate the droplet-size distributions. The mean droplet diameter of the emulsions is reported as the mean diameter: $d_{43} = \Sigma n_i d_i^4 / \Sigma n_i^4$, where n_i is the number of droplets with diameter d_i.

ζ-potential measurement. The electrical charge (ζ-potential) on the surface of emulsion droplets was determined using an electrophoretic technique with a folded

capillary cell (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, UK). A small amount of sample was diluted into acetate buffer at pH 7. An oscillating electric field was applied across the dispersion and the ζ–potential was then determined from the velocity of the droplets migrating toward the oppositely charged electrode.

6.3.4 Antimicrobial Test Protocols

Emulsion preparation for antimicrobial assays. Emulsions were prepared as described above and filtered-sterilized using a 0.45 μm polyethersulfone membrane filter (Puradisc^{TM} 25, Whatman® Inc. Florham Park, NJ). After filter sterilization, emulsions were diluted with sterile water to obtain a series of emulsions containing oil droplet concentrations of 0.00025-2.5%v/v per plate.

Spot Inoculation Assay. Two cycles of a loopful of the *E. coli* slant cultures was transferred to TSB (*L. monocytogenes* to TSB-YE, added 0.6% yeast extract), and incubated at 32ºC for 24h prior to exposure to the antimicrobial emulsion. Petri dishes were prepared by adding 10 ml of the emulsion diluted to the desired antimicrobial concentration into 10 ml of double strength TSA or TSAYE. Control plates consisted of plates where water was added instead of the emulsion. Petri dishes were then dried and 10 μl drops of the inoculums were plated to give a final concentration of 10^5 CFU/ml. The Petri dishes were incubated at 32ºC for 24 h. All experiments were duplicated and replicated.

6.4 Results and Discussion

Emulsion droplet size. Emulsions were produced that were composed of seven different surfactant combinations at an overall surfactant concentration of 0.5% v/v by mixing Tween20 and LAE at ratios of 0.5%:0%, 0.495%:0.005%, 0.454%:0.046%, 0.25%:0.25%, 0.046%:0.456%, 0.005%:0.495% and 0%:0.5%. The droplet size of emulsions immediately after manufacturing was 0.20-0.32 µm (**Table 6.1)**. When LAE was added as a co-surfactant, smaller mean droplet sizes were obtained at surfactant ratios of 0.495%:0.005%, and 0.454%:0.046%. However, at high LAE concentrations in the droplet interface (0.25%:0.25%, 0.046%:0.456%, 0.005%:0.495% and 0%:0.5%) larger droplets were obtained. This is consistent with results of a previous study, in which the ability of LAE to stabilize emulsion droplets was decreased at pH 7 and an additional emulsifier (Tween 20) needed to be added to stabilize the system (**Figure 6.1).** The droplet sizes of emulsions containing eugenol at day 0 were generally smaller than those with no eugenol except the emulsions stabilized by Tween 20: LAE 0.5%:0%, 0.454%:0.046%, and 0.25%:0.25% containing 1.25%, 1.5%, and 1.5% eugenol, respectively (**Table 6.1**). After manufacturing, the emulsions with Tween 20: LAE 0.5%:0%, 0.454%:0.046% and 0.25%:0.25% containing 1.25%, 1.5%, and 1.5% eugenol, respectively, broke down very rapidly indicating that the eugenol concentration was above the critical loading as previous described.

ζ-Potential. The seven emulsions composed of the respective surfactant systems were then manufactured with a disperse phase that contained various ratios of eugenol. The ζ-potential was measured immediately and after one day of incubation at 32°C (**Figure 6.1)**. The ζ-potential on emulsion droplet depended predominantly on the

surfactant composition. When the concentration of LAE was increased in the surfactant mixture, emulsion droplets became increasingly positively charge from -2 mV for pure Tween 20 to +36 mV for emulsions containing pure lauric arginate (**Figure 6.2a)**. The addition of increasing concentration of eugenol had little effect on the charge of emulsion droplets, suggesting that the concentration of Tween 20 and lauric arginate in the interface was not altered by the addition of the phytophenol.

Emulsion stability. The stability of the emulsions during storage for 14 days was determined by measuring the mean droplet diameter **(Figure 6.3)** and by taking photographic images of test tubes periodically during the storage duration (**Figure 6.4).** From the photographic images it can be seen that the emulsions containing various ratio of LAE in the absence of eugenol were increasingly less stable as more LAE was present in the interface, i.e. more coalescence was observed. As previously mentioned, lauric arginate is not as effective a surfactant as Tween 20, particularly at pH 7 where repulsive charges are reduced. Moreover at a surfactant ratio of 0.046%:0.454% of Tween 20 to LAE emulsions rapidly destabilized after storage of as little as 3 days. Interestingly**,** emulsions containing higher concentrations of LAE could be loaded with higher concentrations of eugenol. For example, increasing the LAE concentration in the interface from 0, 0.005, to 0.046, 0.25, 0.454, 0.495, and 0.5 % led to maximum loading concentrations of eugenol of 1, 1, 1.25, 1.5, 1.5, 1.5, and 1.5%, respectively. At higher critical loading ratios of eugenol, the emulsion rapidly destabilized. Below the critical loading concentration, emulsions stabilized by all surfactant systems showed little change with respect to their mean droplet size during 14 days of storage at 32 °C **(Figure 6.3)**. However, photographic images of emulsions composed of Tween20:LAE 0.5%:0% and

0.495%:0.005% and eugenol 0.5, 0.75, and 1% (**Figure 6.4a, b)** show that addition of increasing concentrations of either Tween 20 or eugenol presence led to some oiling off on top of the test tubes even after storage for just one day. A plot of the mean droplet size versus storage time (**Figure 6.5)** further suggests that emulsions formulated with increasing concentrations of LAE after 10 days of storage underwent less increase in mean droplet diameter than emulsions composed of more Tween 20. Hence the result suggests that LAE was more effective in stabilizing emulsions that contain an essential oil component. This is surprising since the destabilization mechanism of emulsions containing eugenol occurs predominantly by Ostwald ripening, which should not be influenced by the surfactant composition and rather only by the solubility of eugenol in the aqueous phase. Moreover, **Figure 6.6** shows that emulsions composed of higher Tween 20 concentrations in the absence of eugenol better maintained their droplet size throughout 14 days of storage than emulsions containing increasing concentrations of LAE (**Fig 6.6 a, c**). Upon addition of 1% v/v eugenol to the emulsions, at higher LAE concentrations in the interface, emulsions were more stable than emulsions containing similar concentrations of eugenol but that were stabilized by predominantly Tween 20 (**Fig 6.6.b, d).**

Antimicrobial activity. The antimicrobial activity of the emulsion was tested against four strains of *E.coli* O157:H7 and *L.*monocytogenes using a spot inoculation assay. **Table 6.2 and 6.3** show the minimum inhibitory concentrations obtained from the spot inoculation assays both based on the active ingredient concentration (LAE) and the droplet concentration. A positive control consisted of cultures grown in the absence of either antimicrobial and a negative control consisted of cultures grown in the presence of

LAE dispersed in 100mM acetate buffer. **Table 6.2 (**no eugenol) demonstrates that all strains of both cultures were completely inhibited by the emulsions containing LAE except at the ratio of Tween20:LAE 0.495%:0.005% and pure Tween 20. Because of their gram-negative cell wall, *E.coli* O157:H7 were more resistant to the presence of LAE than *L.monocytogenes* [88, 163]. Moreover, the MIC of the negative control system (pure LAE, no emulsions) was slightly lower than that of the emulsion based system. At a ratio of 0.046%: 0.454% Tween 20: LAE, the $MIC_{E.coli}$ was 0.02 %v/v which is much higher than any other emulsion and the negative control while the MIC $_{L.monocytogenes}$ with 0.003% v/v was not significantly different. We are not sure what the reason for this decrease in activity is. **Table 6.3** shows the MIC of eugenol: corn oil emulsions at a loading of 1% eugenol. The data indicates that the MIC of both cultures were mainly affected by the concentration of LAE. However, the MIC $_{L.monocytogenes}$ was lower than if eugenol was present (**Table 6.2**). We therefore attribute this increased activity to the presence of eugenol. At surfactant ratios of 0.495%:0.005% and 0.454%:0.046% Tween20: LAE in the presence of eugenol, $MIC_{E.coli}$ and MIC $_{L.monocytogenes}$ were significantly smaller than in the absence of eugenol (**Table 6.2**). There could be multiple reasons for the increased activity. For example, the LAE may have increased attractive interactions with the bacterial surfaces thereby delivering both antimicrobials better to the interface of microorganisms. Secondly, emulsions were more stable which may have contributed to the increased antimicrobial activity. Conversely, in the presence of only eugenol (e.g. Tween 20: LAE 0.5%:0%) the MIC was greatly increased e.g. MIC $_{E.coli}$ and $_{L.monocytogenes}$ > 0.25%v/v.

6.5 Conclusion

Our results demonstrate that double antimicrobial emulsions could be formulated using LAE as emulsifier and eugenol as the lipid phase. Composition of the interface and the droplet phase influenced both physicochemical properties and antimicrobial activity of oil-in-water emulsions. To improve the stability of emulsions that contained the cationic surfactant LAE, incorporation of Tween 20 was required. With increasing concentrations of LAE in the emulsions, the positive charge on the droplet interface increased and the droplet size decreased. Most importantly, the critical loading ratio, that is the concentration of eugenol with which the lipid dispersed phase can be maximally loaded increased with increasing concentration of LAE in the interface. This is an interesting observation for which we generally have no good explanation since the principal breakdown mechanism of emulsions that are loaded with too much eugenol is due to Ostwald ripening. This would indicate that the presence of lauric arginate influences compositional ripening. Antimicrobial activity was higher in double antimicrobial emulsions than in single antimicrobial emulsions. Evaluation of the minimum inhibitory concentrations suggests strong synergistic activities between the two antimicrobials possibly due to an enhanced mass transport of droplets to the surface of microorganisms, where release of eugenol is more effective. Ultimately, more experiments as to the exact mechanism will be needed to exactly elucidate this phenomenon.

Table 6.1 Diameter droplet (µm) of emulsions after manufacturing as the function of the ratio of eugenol for emulsions formulated with seven different surfactant systems.

Corn oil: Eugenol	Surfactant system composition Tween20 : LAE							
	0.5%:0%		0.495%:0.005%		0.454%:0.046%		0.25%:0.25%	
	diameter (µm.)	SD.	diameter (µm.)	SD.	diameter (µm.)	SD.	diameter (µm.)	SD.
100:0	0.305	0.007	0.315	0.007	0.260	0.014	0.300	0.014
95:5	0.285	0.007	0.295	0.007	0.195	0.007	0.215	0.007
90:10	0.240	0.014	0.240	0.014	0.200	0.014	0.180	0.014
85:15	0.185	0.007	0.200	0.014	0.185	0.007	0.190	0.014
80:20	0.185	0.007	0.175	0.007	0.185	0.007	0.190	0.014
75:25	0.230	0.014	0.195	0.007	0.175	0.007	0.175	0.007
70:30					0.935	1.082	0.280	0.028

Corn oil: Eugenol	Surfactant system composition Tween20 : LAE					
	0.046%:0.454%		0.005%:0.495%		0%:0.5%	
	diameter (µm.)	SD.	diameter (µm.)	SD.	diameter (µm.)	SD.
100:0	0.270	0.014	0.260	0.010	0.280	0.000
95:5	0.245	0.007	0.240	0.017	0.220	0.000
90:10	0.200	0.014	0.187	0.006	0.190	0.000
85:15	0.190	0.000	0.183	0.006	0.190	0.000
80:20	0.180	0.000	0.187	0.006	0.190	0.000
75:25	0.190	0.000	0.190	0.010	0.200	0.000
70:30	0.210	0.014	0.180	0.000	0.180	0.000

Table 6.2 Minimum inhibitory concentration of emulsions in the absence of eugenol against four strains of *E. coli* O157:H7 and *L. monocytogenes* after incubation for 24 h at 32°C.

		Surfactant System Composition Tween20:LAE							
		100:0		100:1		10:1		1:1	
		MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration
E. coli O157:H7	ATCC® 35150	>25	>2.50	>0.0025	>2.50	0.006	0.66	0.006	0.12
	ATCC® 4385	>25	>2.50	>0.0025	>2.50	0.005	0.55	0.005	0.10
	ATCC® 516858	>25	>2.50	>0.0025	>2.50	0.005	0.55	0.005	0.10
	ATCC® 700599	>25	>2.50	>0.0025	>2.50	0.005	0.55	0.005	0.10
L. monocytogenes	J1225	>25	>2.50	>0.0025	>2.50	0.003	0.33	0.003	0.06
	J2020	>25	>2.50	>0.0025	>2.50	0.003	0.33	0.003	0.06
	J1031	>25	>2.50	>0.0025	>2.50	0.003	0.33	0.003	0.06
	J1-1177	>25	>2.50	>0.0025	>2.50	0.003	0.33	0.003	0.06

		Surfactant System Composition Tween20:LAE						LAE in 100mM. Acetate buffer
		0.1:1		0.01:1		0:1		
		MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)
E. coli O157:H7	ATCC® 35150	0.020	0.220	0.007	0.07075	0.007	0.07	0.004
	ATCC® 4385	0.020	0.220	0.007	0.07075	0.006	0.06	0.004
	ATCC® 516858	0.020	0.220	0.007	0.07075	0.006	0.06	0.004
	ATCC® 700599	0.020	0.220	0.007	0.07075	0.006	0.06	0.004
L. monocytogenes	J1225	0.003	0.033	0.003	0.03025	0.003	0.03	0.002
	J2020	0.003	0.033	0.003	0.03025	0.003	0.03	0.002
	J1031	0.003	0.033	0.003	0.03025	0.003	0.03	0.002
	J1-1177	0.003	0.033	0.003	0.03025	0.003	0.03	0.002

Table 6.3 Minimum inhibitory concentration of emulsions containing 1% eugenol against four strains of *E.coli* O157:H7 and *L. monocytogenes* after incubation for 24 h at 32°C.

		Surfactant System Composition LAE:Tween20: Loading 80:20 Corn oil : Eugenol							
		100:0		100:1		10:1		1:1	
		MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration
E. coli O157:H7	ATCC® 35150	0.2	1.00	0.001	1.01	0.003	0.33	0.008	0.16
	ATCC® 4385	0.2	1.00	0.001	1.01	0.003	0.33	0.007	0.14
	ATCC® 516858	0.2	1.00	0.001	1.01	0.003	0.33	0.007	0.14
	ATCC® 700599	0.2	1.00	0.001	1.01	0.003	0.33	0.006	0.12
L. monocytogenes	J1225	0.3	1.50	0.001	1.01	0.001	0.11	0.002	0.04
	J2020	0.3	1.50	0.001	1.01	0.001	0.11	0.002	0.04
	J1031	0.3	1.50	0.001	1.01	0.001	0.11	0.002	0.04
	J1-1177	0.3	1.50	0.001	1.01	0.001	0.11	0.002	0.04

		0.1:1		0.01:1		0:1	
		MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration	MIC (% v/v)	%v/v droplet concentration
E. coli O157:H7	ATCC® 35150	0.030	0.330	0.007	0.07075	0.006	0.06
	ATCC® 4385	0.020	0.220	0.006	0.0605	0.006	0.06
	ATCC® 516858	0.020	0.220	0.006	0.0605	0.006	0.06
	ATCC® 700599	0.020	0.220	0.006	0.0605	0.006	0.06
L. monocytogenes	J1225	0.002	0.022	0.002	0.02025	0.002	0.02
	J2020	0.002	0.022	0.002	0.02025	0.002	0.02
	J1031	0.002	0.022	0.002	0.02025	0.002	0.02
	J1-1177	0.002	0.022	0.002	0.02025	0.002	0.02

Figure 6.1 Effect of 0.5%v/v surfactant system on average diameter of 5% v/v corn oil-in-water emulsions on day1 of storage at 32C°.

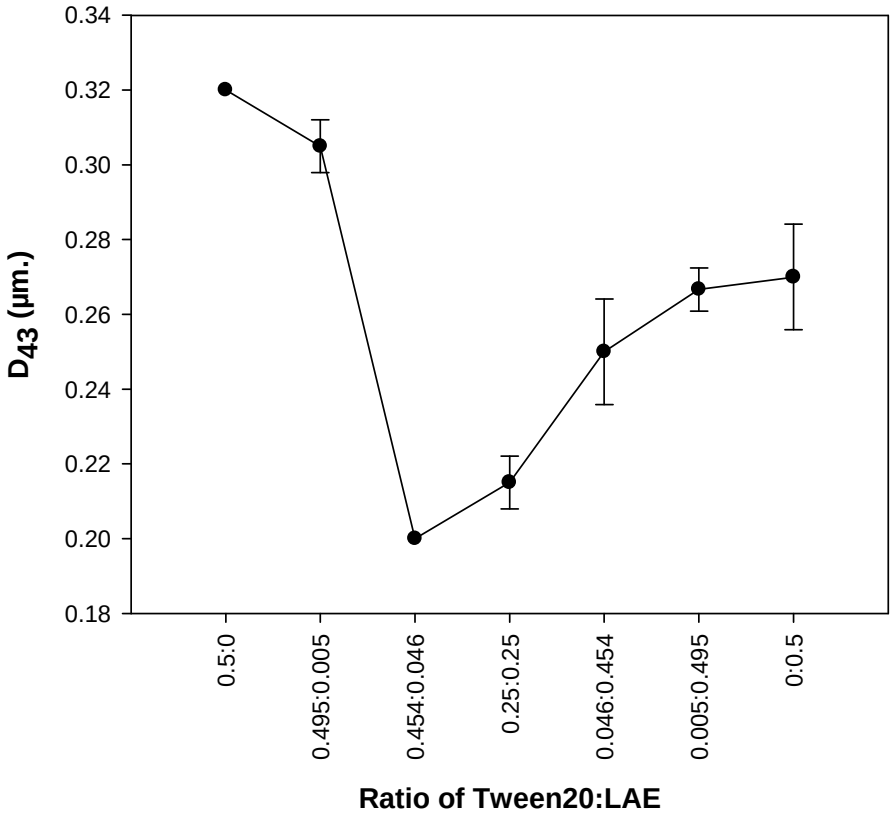
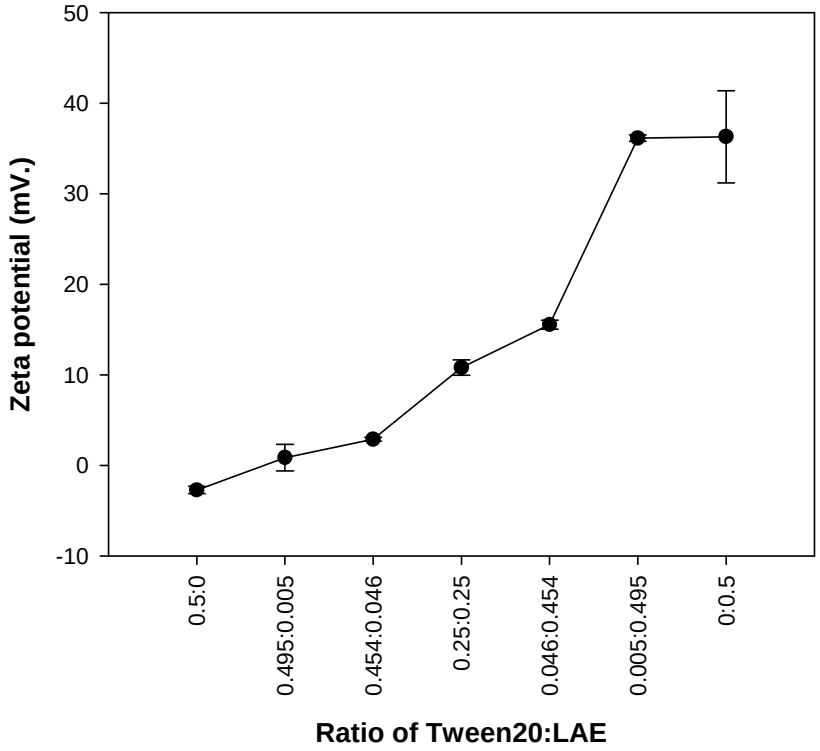


Figure 6.2 Influence of surfactant composition on ζ-potential of 5% v/v oil-in-water emulsion manufactured with Tween 20 and Lauric arginate (LAE)

(A) in the absence of eugenol



(B) in the presence of eugenol at different ratio

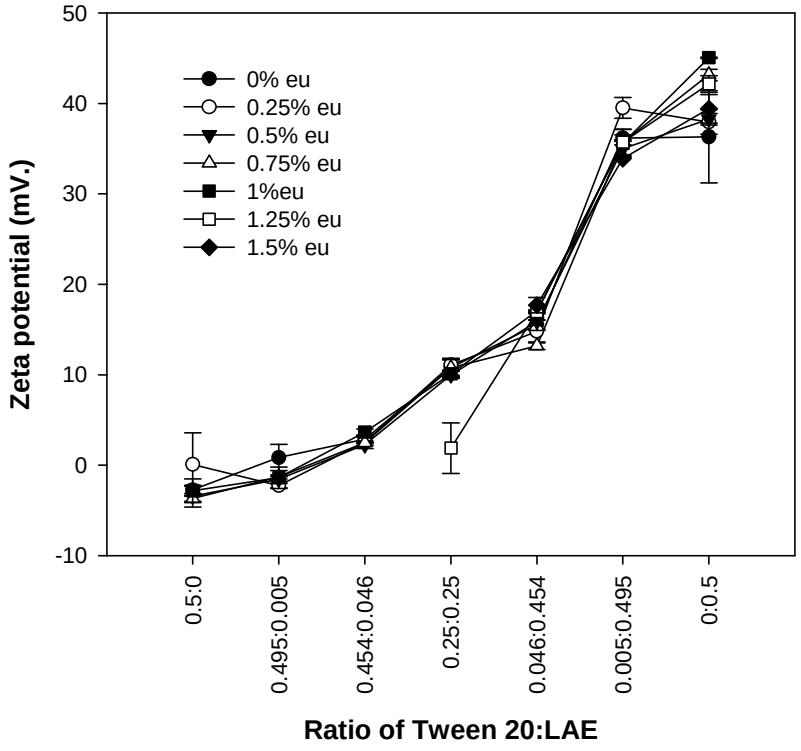


Figure 6.3 Stability of 5%v/v oil-in-water emulsion by influence of surfactant composition (Tween 20 and LAE) on day 0. 0.5, 1, 2, 3, 5, 7, 10, and 14 of storage at 32C° as a function of droplet size d_{43}.

(A) Absence eugenol

(B) Eugenol:Corn oil 05:95

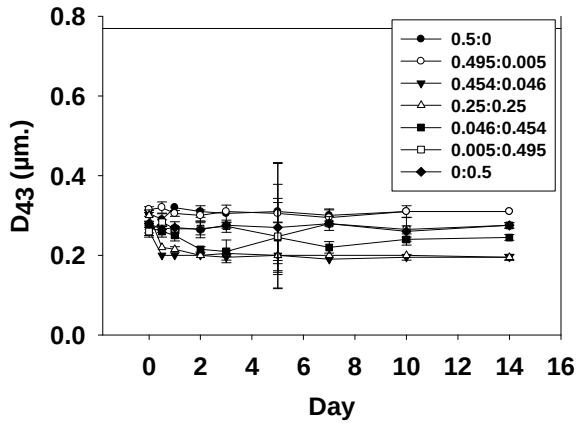
(C) Eugenol:Corn oil 10:90

(D) Eugenol:Corn oil 15:85

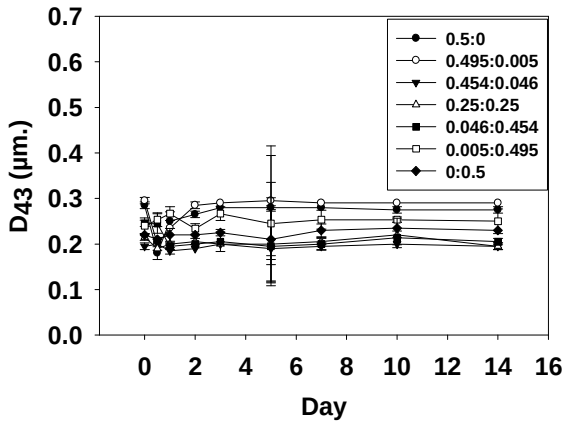
(E) Eugenol:Corn oil 20:80

(F) Eugenol:Corn oil 25:85

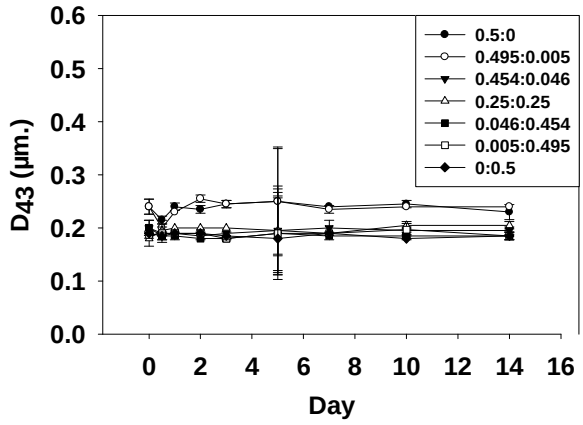
(G) Eugenol:Corn oil 30:70


D43 (µm.)
Day
0.5:0
0.495:0.005
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

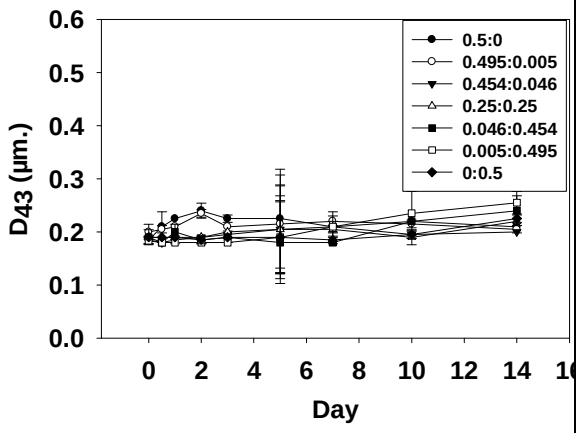
(A)


D43 (µm.)
Day
0.5:0
0.495:0.005
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

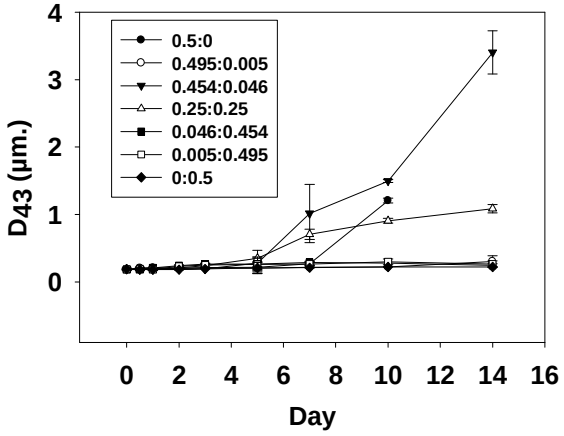
(B)


D43 (µm.)
Day
0.5:0
0.495:0.005
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

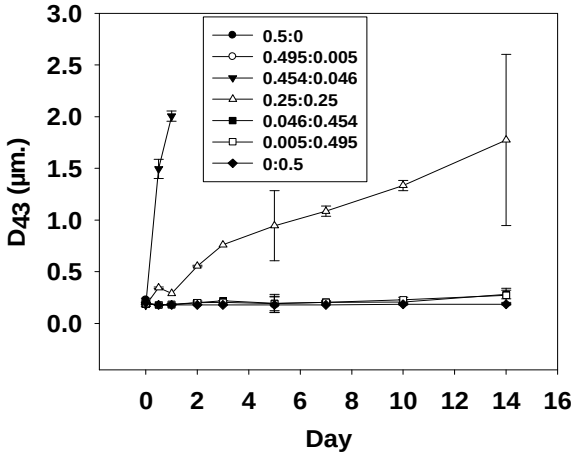
(C)


D43 (µm.)
Day
0.5:0
0.495:0.005
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

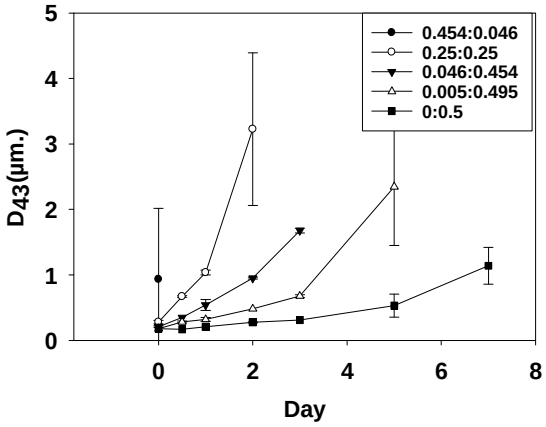
(D)


D43 (µm.)
Day
0.5:0
0.495:0.005
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

(E)


D43 (µm.)
Day
0.5:0
0.495:0.005
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

(F)


D43(µm.)
Day
0.454:0.046
0.25:0.25
0.046:0.454
0.005:0.495
0:0.5

(G)

Figure 6.4 Stability of 5%v/v oil-in-water emulsion by influence of oil composition (Eugenol and Corn oil) on day 1 and 14 of storage at 32C° as a function of 0.5% v/v surfactant composition (Tween20 and LAE) by showing photographic images of the test tubes. The left shows the stability of day1. The right column shows the stability of day14.

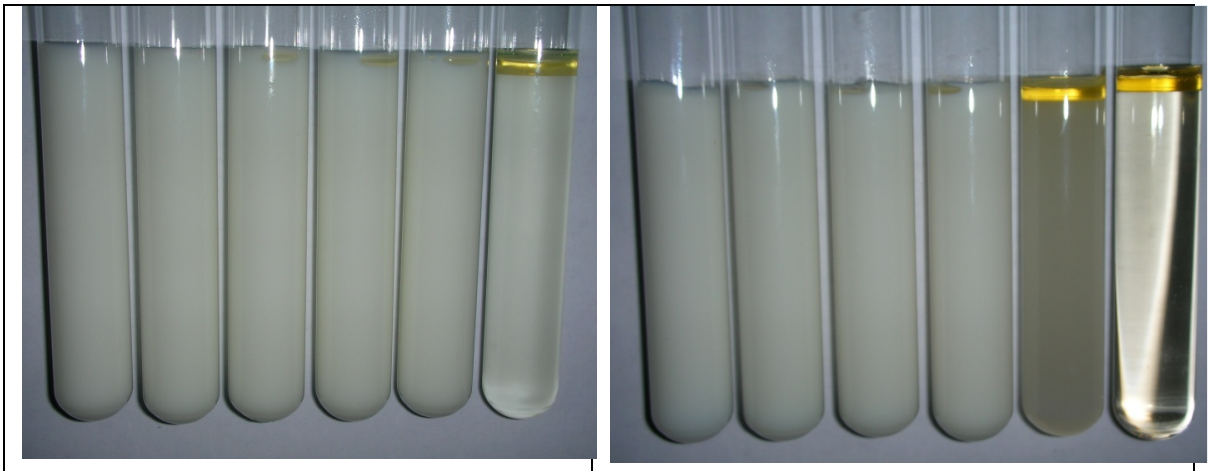
(A) Tween20: LAE 100:0 (B) Tween20: LAE 100:1

(C) Tween20: LAE 10:1 (D) Tween20: LAE 1:1

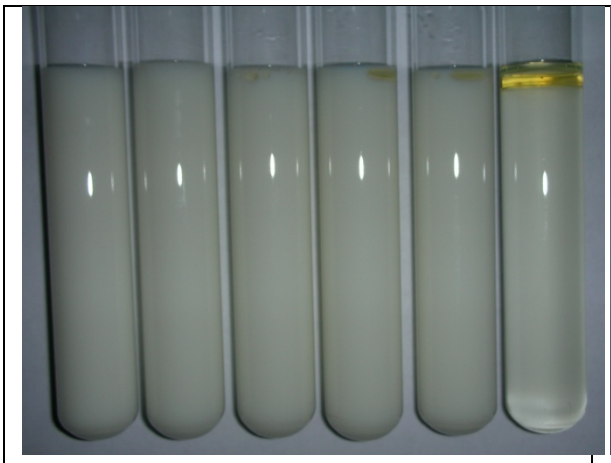
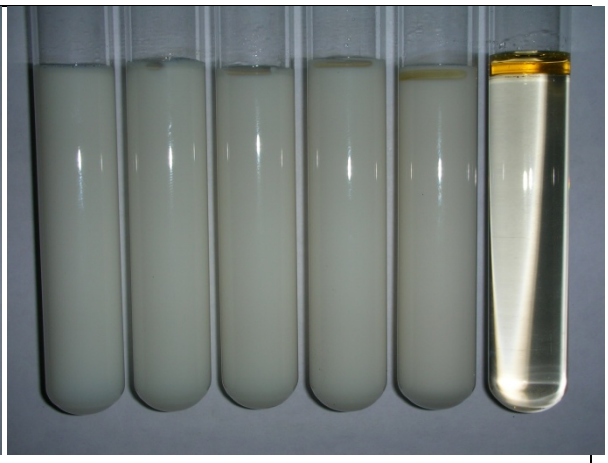
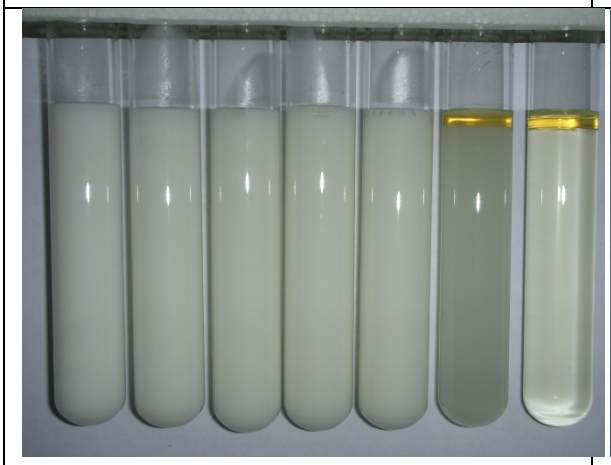
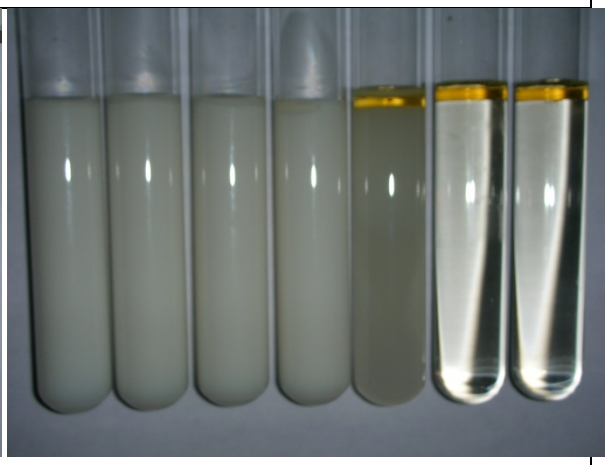
(E) Tween20: LAE 0.1:1 (F) Tween20: LAE 0.01:1

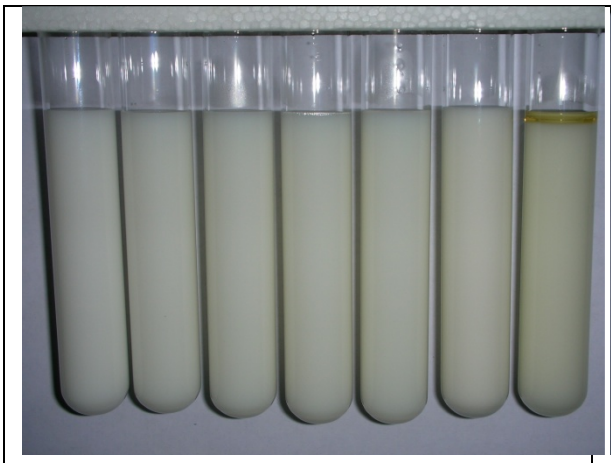
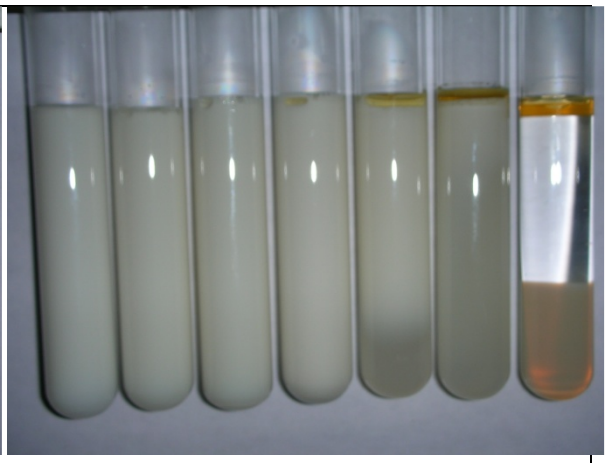
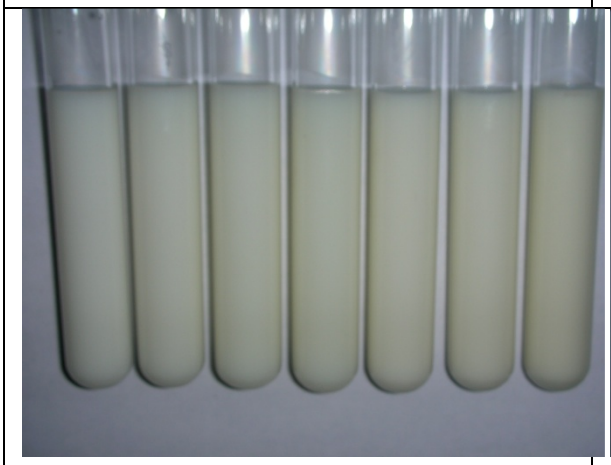
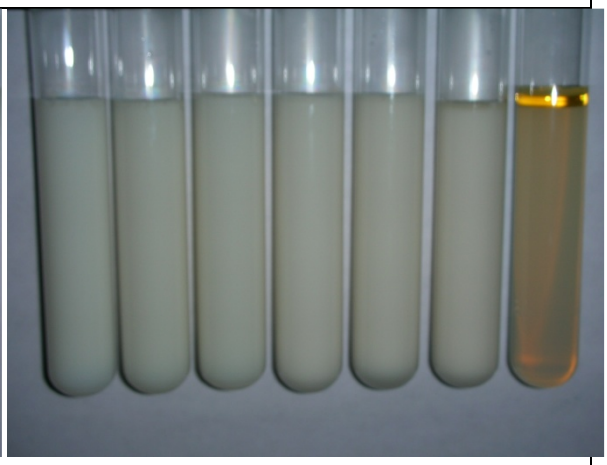
(G) Tween20: LAE 0:100

In each of picture, the test tubes were arranged from left to right by ratio of eugenol: corn oil, 0:100, 5:95, 10:90, 15: 85, *etc.*



(A)

	
(B)	
	
(C)	

	
(D)	
	
(E)	

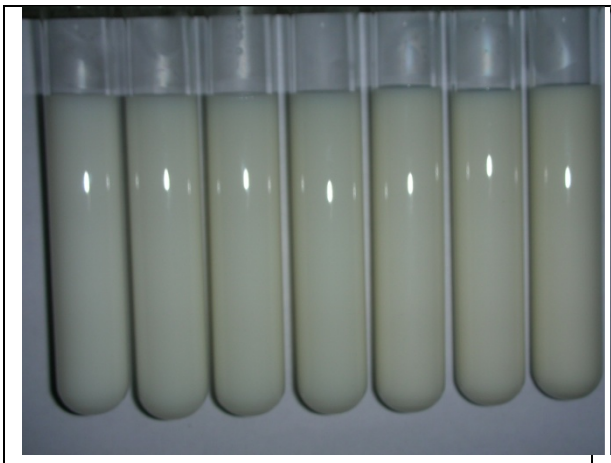
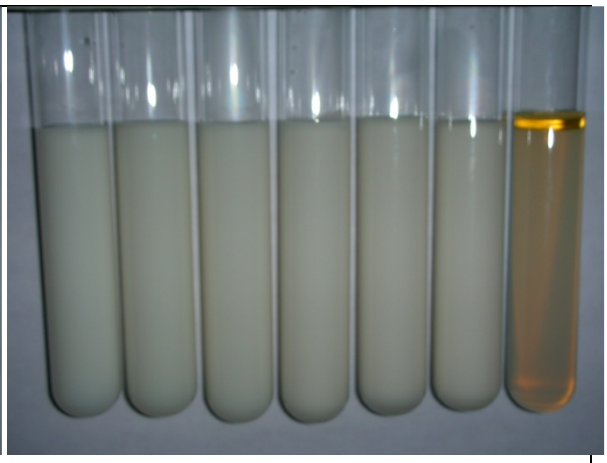
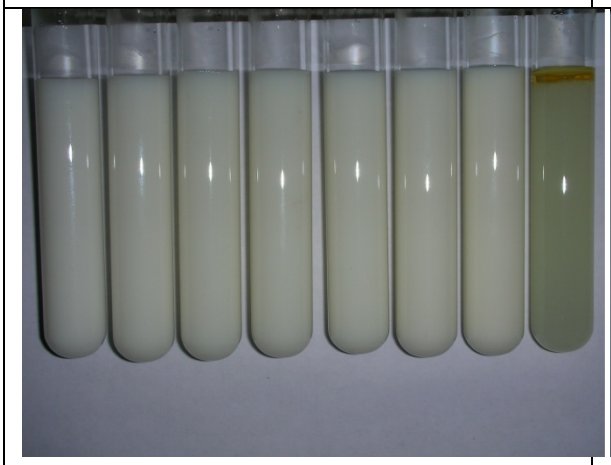
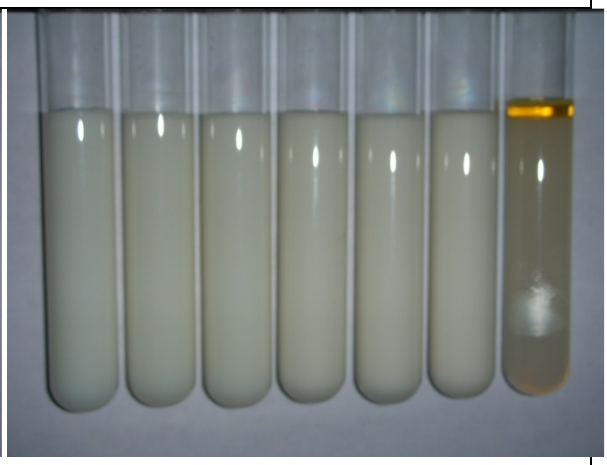
	
(F)	
	
(G)	

Figure 6.5 Particle size distributions of Eugenol: Corn oil 20:80 emulsion prepared with 0.5% v/v surfactant on day 10 of storage at 32C°

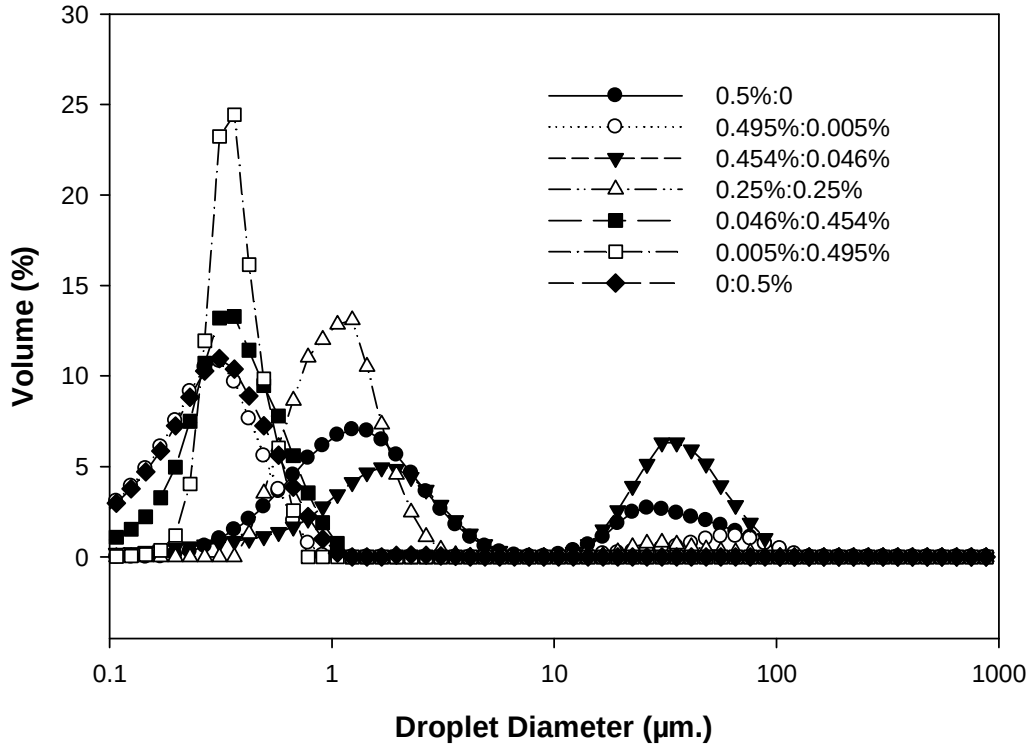


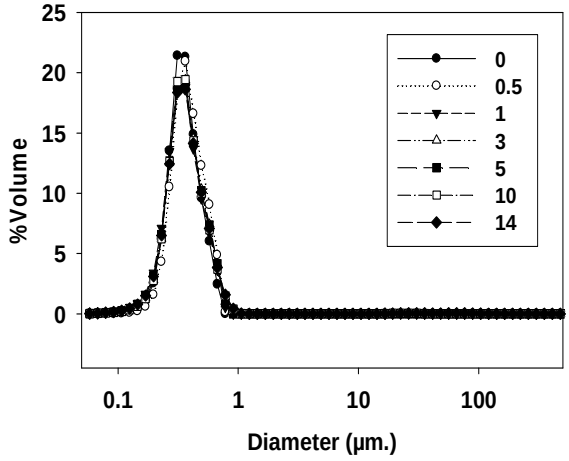
Figure 6.6 Particle size distributions of 5%v/v oil phase (0% and 1% eugenol) and 0.5%v/v surfactant Tween20 : LAE (0.495%:0.005% and 0.046%: 0.454%)

(A) 0.495%:0.005% Tween20:LAE with 0% eugenol (5%Corn oil)

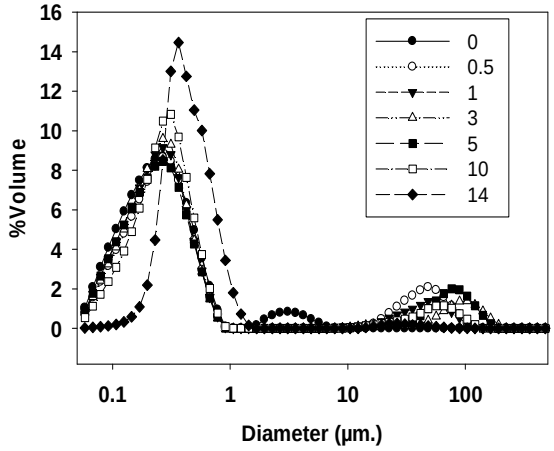
(B) 0.495%:0.005% Tween20:LAE with 1% eugenol (4%Corn oil)

(C) 0.046%:0.454% Tween20:LAE with 0% eugenol (5%Corn oil)

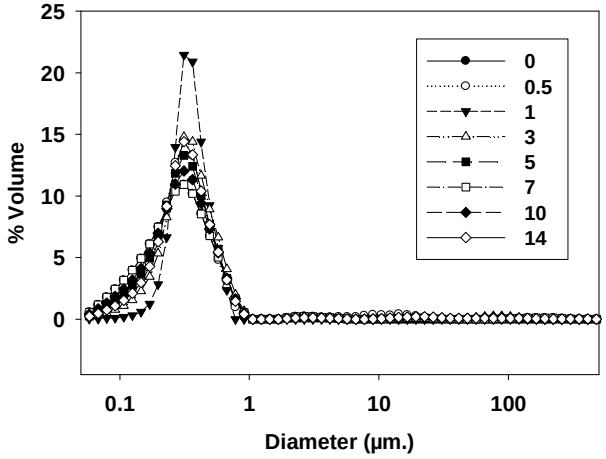
(D) 0.046%:0.454% Tween20:LAE with 1% eugenol (4%Corn oil)


%Volume
Diameter (µm.)
0
0.5
1
3
5
10
14

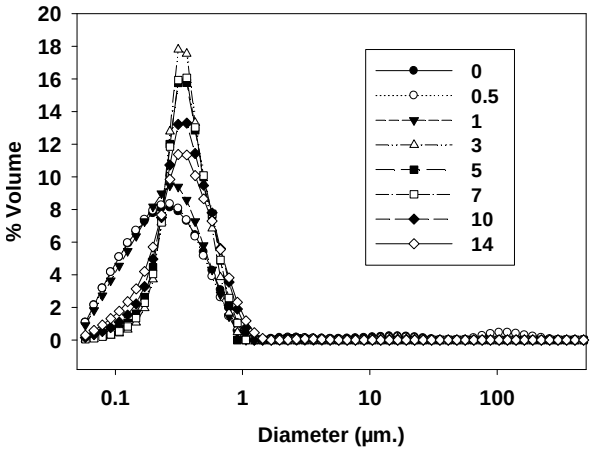
(A)


%Volume
Diameter (µm.)
0
0.5
1
3
5
10
14

(B)


% Volume
Diameter (µm.)
0
0.5
1
3
5
7
10
14

(C)


% Volume
Diameter (µm.)
0
0.5
1
3
5
7
10
14

(D)

CHAPTER 7

CONCLUSIONS

Oil-in-water food emulsions are thermodynamically unstable dispersions of oil droplets in water coated by a monolayer of adsorbed surfactant. This class of products has found widespread use in the food industry as ingredients, precursors to products or actual products. This thesis was designed to evaluate the ability of oil-in-water emulsions to become antimicrobial active to improve the stability and quality of a variety of foods. Two antimicrobials were used as model active compounds to formulate the emulsions: eugenol as a lipid phase, and lauric arginate as an emulsifier. In this thesis we showed that formulation of single antimicrobial emulsions with either eugenol or lauric arginate if feasible, but that particular attention needs to be paid to both the composition of the lipid phase and the interface as formulation of emulsions with only the antimicrobial (e.g. eugenol as lipid phase or lauric arginate as emulsifier) is not feasible. Instead, a lipid carrier such as corn oil needs to be added to the lipid phase to stabilize eugenol-containing emulsions and a nonionic surfactant such as Tween 20 needs to be added to provide sufficient repulsive interactions for lauric arginate containing droplets interfaces. Finally, emulsions containing both antimicrobials need to again be separately formulated as the presence of lauric arginate in the interface influences the maximum concentration of eugenol with which the emulsion can be loaded. However, these double antimicrobial emulsions have superior antimicrobial functionality compared to their single component counterpart, possibly suggesting an improved mass transport process of emulsion droplets to the surface of microorganisms, which may induce the observed synergism. Thus a good knowledge of the structure and biophysical behavior of antimicrobial emulsions is

needed to properly formulate such systems for subsequent application in a wider variety of food systems by the food industry.

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