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**PHYSICOCHEMICAL AND ANTIMICROBIAL
PROPERTIES OF ANTIMICROBIALS ENCAPSULATED IN
SURFACTANT-BASED NANOPARTICLES**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Sylvia Gaysinsky Segura
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Dedication

This thesis is dedicated to my parents, Luisa and Harry, for their support and encouragement to strive higher to reach my goals, to let me fly, with whose unending love and support completing this dissertation and this degree was possible. To my aunt Adriana, that has been my best friend and biggest supporter. Lastly, I would like to thank Andres with whom I have shared every joy, every frustration, and every fear involved in my life and bringing this dissertation to completion. Thank you for enlightening my life.

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Abstract

Phytophenols, components of essential oil extracts, are antimicrobials that may inhibit the growth of foodborne pathogens. However, their antimicrobial activity in food systems is low because of low water solubility and association with other food components. Incorporation of phytophenols in nanoscale surfactant micelles may offer a potential solution to increase solubility and improve activity in food systems. The objectives of this study were to determine the stability and physicochemical and antimicrobial properties of micellar antimicrobial-surfactant systems.

Carvacrol and eugenol containing micelles were prepared by dispersing Surfynol[®] 485W and Surfynol[®] 465 in water at room temperature. Stability was determined by UV-visible spectroscopy, particle size was determined by dynamic light scattering and, structural information about the mixed micellar systems was obtained by nuclear magnetic resonance spectroscopy (NMR). The antimicrobial activity was determined using a microbroth dilution assay.

Incorporation of carvacrol and eugenol to surfactant-based nanoparticles was very rapid. Depending on the surfactant-antimicrobial combination, the particles produced varied in size between 5 to 20 nm. The phytophenolic-based nanoparticles were effective against *Escherichia coli* O157:H7 and *Listeria monocytogenes* at low surfactant and essential oil component concentrations, i.e., 1 % of Surfynol[®] 485W and 0.15 % eugenol was sufficient to inhibit growth of all strains of *E. coli* O157:H7 and three of four strains of *L. monocytogenes* (Scott A, 310, and 108). A fourth strain, *L. monocytogenes* 101 was

inhibited with 2.5 % Surfynol[®] and 0.225 % eugenol. 1 % Surfynol[®] 485W in combination with 0.025 % carvacrol was effective in inhibiting three of four strains of *E. coli* O157:H7. Strain H1730 was the most resistant requiring 0.3 % of carvacrol and 5 % of surfactant for complete inhibition. Growth inhibition of *L. monocytogenes* by combinations of carvacrol and Surfynol[®] 465 varied between 0.15 and 0.35 % and 1 and 3.75 % , respectively. Nanocapsules composed of Surfynol[®] 485W were generally more inhibitory than those containing Surfynol[®] 465. *E. coli* O157:H7 was more sensitive to the antimicrobial system than *L. monocytogenes*.

All nanoparticles were stable over a wide range of pHs; however, temperature and essential oil component concentration influenced the stability of the mixed micelles, i.e., micelles destabilized at lower temperatures with increasing encapsulated essential oil component concentration. For example, 0.9 % of eugenol encapsulated in Surfynol[®] 485W exhibited turbidity (cloud point) at 55°C, while at 0.5 %, 70 °C was required to reach the cloud point. At temperatures optimal for microbial growth, micelles were stable and retained activity.

The study indicated that encapsulation of essential oil components in surfactant-based nanoparticles offers an excellent means to incorporate large amounts of these compounds in an aqueous system and thereby increase antimicrobial activity.

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1 Introduction

Phytophenols such as carvacrol and eugenol are antimicrobial agents that can inhibit growth of foodborne pathogens. However, antimicrobial activity of these compounds in food systems is low because of low water solubility and association with other food components (e.g. lipids, proteins). A potential solution to this problem could be to incorporate these non-polar compounds in self-assembled surfactant micelles (nanoparticles).

The objectives of this study were to solubilize phytophenols in selected surfactant micelles, determine the physicochemical properties and stability of the nanocapsules at different pH and temperatures, and finally to test their antimicrobial efficacy in model microbiological systems. Eugenol and carvacrol, ranging from 0.025-2.13 % (w/w), were solubilized in aqueous Surfynol[®] 465 and Surfynol[®] 485W surfactant solution (1-10 % (w/w)). The maximum additive concentration (MAC) was determined via absorption measurements at 632 nm. Turbidity decreased to zero within 10 mins upon the addition of carvacrol and eugenol to surfactant solutions indicating that nanoparticles rapidly incorporated phytophenols. Solutions were clear and colorless. Nanoparticle size was determined by dynamic light scattering at a scattering angle of 90°. Surfynol[®] 465 and Surfynol[®] 485W, with the incorporation of carvacrol and eugenol, produced particles with a size ranging from 5.7 to 19.7 nm depending on the type of surfactant-antimicrobial combination and concentration of antimicrobial in surfactant micelles. The swollen micelles remained colorless between pH 3-9 with the exception of nanoparticles

containing eugenol. Mixed micelles were stable over the entire range of pH with the exception of Surfynol[®] 485W with carvacrol. This combination demonstrated instability at pH 9 and high carvacrol concentration. The solutions became turbid at lower temperatures indicating demicellization and release of encapsulated component. At lower pH values, at the same carvacrol concentration, higher temperatures were required to initiate breakdown. Thus, Surfynol[®] 485W was generally less thermoresistant at higher pHs whereas, Surfynol[®] 465 was less thermoresistant at low pHs. In addition, at a high pH (> 8), solutions, while clear, were yellow in color.

As expected, temperature and phytophenol concentration played a major role in the stability of mixed micelles. The higher the essential oil component concentration, the lower the cloud point temperature (the temperature at which solutions became turbid). For example, micelles containing 0.9 % of eugenol became turbid at 55 °C, while micelles containing only 0.5 % remained translucent up to 70 °C. Overall, Surfynol[®] 485W was more resistant to changes in temperature, and was stable above 90 °C at low oil concentrations. In contrast, Surfynol[®] 465 was less thermostable, and reached the cloud point at 75 °C even in the absence of phytophenols. Results were attributed to the differences in the hydrophilic polyoxyethylene chain length. Surfynol[®] 485W contains 30 moles of ethyleneoxide while Surfynol[®] 465 contains 15 moles.

Using Nuclear Magnetic Resonance (NMR) spectroscopy, the structure of mixed micelles was elucidated. Based on the results of the NMR study, the shape of the micelles was identified to be a bracket-like structure where phytophenols are located within the

shell of the micelle in direct contact with adjacent surfactant monomers. The hydrophobic tails of the surfactant are oriented towards the core of the micelle and the hydroxyl group faces the water phase.

Eugenol encapsulated in emulsifier-based nanoparticles inhibited both microorganisms tested at pH 5, 6 and 7. At pH 5, there was a direct pH affect on the inhibition of *Listeria monocytogenes*, *i.e.*, inhibition occurred regardless of the presence of antimicrobial. In contrast, *Escherichia coli* O157:H7 was not inhibited at pH 5. Reducing the pH to 6 did not adversely affect the antimicrobial activity of nanoparticles compared to pH 7. The nanoparticles were effective at both temperatures tested (32 °C and 22 °C) indicating that activity was not affected by a reduction in temperature in the range tested. Therefore, neither pH nor temperature eliminated activity of the encapsulated antimicrobials against either microorganism.

Overall, strains of *E. coli* O157:H7 were more sensitive than strains of *L. monocytogenes*. Inhibition of *E. coli* O157:H7 occurred at 0.2 % of eugenol at all pH values while *L. monocytogenes* required 0.5 to 0.9 % for complete inhibition. Eugenol at 0.5 and 0.9 % inhibited both microorganisms at all pHs. This is unusual in that gram-negative bacteria are generally more resistant to essential oil components because of the protective effect of the lipopolysaccharide (LPS) layer that comprises the outer membrane. Results were attributed to a potential disruption of the outer membrane due to partial solubilization in the presence of the non-ionic surfactant (Surfynol® 485W) which may allow the essential oil component to contact and disrupt the cytoplasmic membrane.

In the future, stability of the nanoparticles under typical food processing conditions will need to be determined if these systems are to be successfully applied to foods.

Encapsulation of essential oils components in nanoparticles offers an excellent means to incorporate large amounts of these components into aqueous systems by increasing their solubility in the phase that is key to the growth of the microorganisms. Furthermore, antimicrobial activity of the nanoparticles was maintained regardless of changes in system pH and temperature, which demonstrates their versatility and suitability for a wide variety of food formulations.

2 Literature Review

2.1 Introduction

The Centers for Disease Control and Prevention (CDC) reported approximately 76 million illnesses each year in the United States caused by foodborne pathogens (CDC 2004b). In 2003, 15,600 laboratory-diagnosed cases of infections caused by pathogens were identified. 6,017 cases were attributed to *Salmonella*; 5,215 to *Campylobacter*; 3,021 to *Shigella*; 480 to *Cryptosporidium*; 443 to *Escherichia coli* O157; 161 to *Yersinia*; 138 to *Listeria*; 110 to *Vibrio*; and 15 to *Cyclospora* (CDC 2004b). The illnesses resulted in 325,000 hospitalization and 5,000 deaths. The symptoms of these foodborne pathogens may range from mild gastroenteritis to life-threatening neurological, hepatic, or renal syndromes.

In 1997, the U.S. Department of Agriculture's (USDA) Food Safety and Inspection Services (FSIS) implemented the Pathogen Reduction/Hazard Analysis and Critical Control Point (HACCP) system in meat and poultry slaughter and processing plants that resulted in a decline of human *E. coli* O157 infections in 2003. However, no substantial changes were observed in the incidence of illnesses caused by *Listeria*, *Shigella*, and many *Salmonella* serotypes (CDC 2004b). An estimated 73,000 cases of *E. coli* O157:H7 infection and 61 deaths occur in the US each year. Infection often leads to bloody diarrhea, and occasionally to kidney failure. Most illnesses have been associated with eating undercooked or contaminated ground beef. Person-to-person contact in families and child-care centers are also an important means of transmission. Infection can occur

after drinking raw milk and after swimming in or drinking sewage-contaminated water (CDC 2004a). The USDA Economic Research Service (ERS) has estimated that each year in the US, the cost of acute illnesses from *Listeria* is \$2.3 billion. For *L. monocytogenes*, an estimated 2,500 persons become seriously ill with listeriosis each year. Of these, approximately 500 die. A series of outbreaks associated with the consumption of coleslaw, pasteurized milk, and fresh soft cheese in the early 1980s led to the recognition of *L. monocytogenes* as a major foodborne pathogen (CDC 2004a).

2.2 *Listeria monocytogenes*

2.2.1 General Characteristics

Listeria monocytogenes has been recognized as a human pathogen for more than 60 years and can cause serious invasive illnesses in humans (Mastronicolis et al., et al., 1998). The microorganisms is a small (0.5-2.0 μm), Gram-positive coccobacillus. It is facultatively anaerobic, non-sporeforming and can grow at a temperature range of $> 1\text{ }^{\circ}\text{C}$ to $< 50\text{ }^{\circ}\text{C}$, with the optimal temperature being 30–37°C. The organism survives freezing and can grow in a pH range of 4.0 to 9.5 in the presence of up to 10% NaCl. The pathogen is motile at temperatures ranging from 20 to 25°C but becomes non-motile at higher temperatures, e.g., 37°C (Robinson et al., 2000). A complete list of the metabolic characteristics of *L. monocytogenes* can be found in Table 2.1.

The envelope structure of *L. monocytogenes* is shown in Figure 2.1. The cell wall consists of a thick layer of peptidoglycan that accounts for about 35% of the dry weight of the cell wall (Robinson et al., 2000).

Table 2.1 *Listeria monocytogenes* metabolic characteristics (Mac Faddin 2000).

Test	Result
Catalase	+
Hemolytic in blood agar 5%	β
O-F glucose	F/O
Nitrate reduction	-
Motility at 22°C	+
Glucose	Acidic
Lactose	Variable
Sucrose	Variable
Maltose	Acidic
Manitol	-
Xylose	-
Voges-Proskauer	+
Liquefaction of gelatin at 22°C	-
Urease	-
Arginine Dehydrolase	+

F= fermentative, O= oxidative, (-) = negative, and (+) = positive

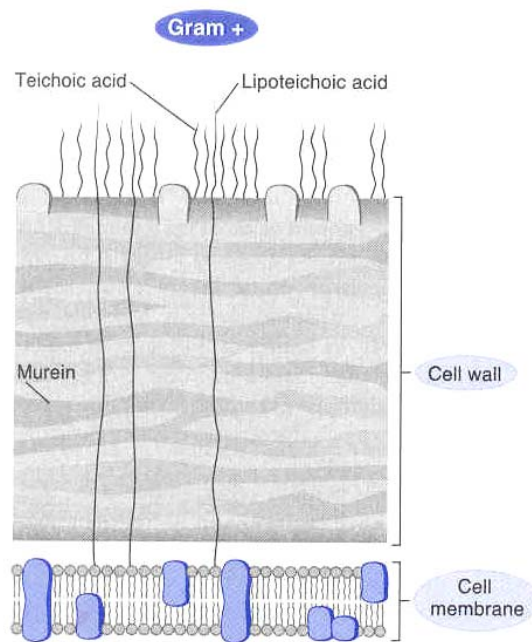


Figure 2.1. Diagram of a Gram-positive bacteria cell wall (Schaechter et al., 1998).

This peptidoglycan layer varies among Gram-positive microorganisms. Additionally, the cell wall of Gram-positive bacteria may contain multiple layers of murein. Murein is composed of glycan sugar chains that are cross-linked by peptides and is a critical component in maintaining the shape and rigidity of Gram-positive bacteria (Figure 2.2).

One strategy is to maintain active transport at low temperature; another is the maintenance of membrane fluidity (Mastronicolis et al., 1998; Li et al., 2002). The process by which a cell adjusts its membrane fluidity is referred to as “homeoviscous adaptation” and it is critical to ensure cell growth at non-optimal temperatures. Bacteria may regulate their membrane fluidity at different temperatures by adjusting the fatty acid composition of the bacterial membrane. A variety of extrinsic factors also affect

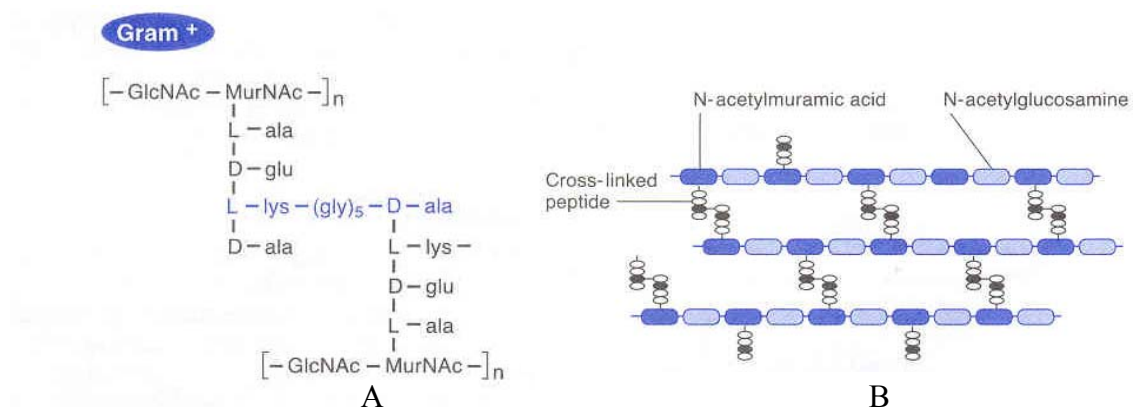


Figure 2.2 (A) Murein structure (B) Composition of murein in Gram-positive bacteria (Schaechter et al., 1998)

membrane fluidity. These include water activity, pH, and the presence of chemicals or antimicrobials. Therefore, the ability of a bacterium to adapt or to alter its membrane fluidity ultimately determines how well the bacterium tolerates changes in environmental conditions that are detrimental to growth (Li et al., 2002).

2.2.2 Sources

Listeria monocytogenes has been isolated from a variety of sources, the primary one being soil and vegetation decay. It has been found to be associated with numerous animals including birds, dogs, sheep, swine, cattle, fish and shellfish, among others (FDA 2001).

L. monocytogenes has been isolated from raw and pasteurized milk, naturally contaminated cheese, vegetables, seafood, liquid whole egg, hard salami, ground meat, raw meat, poultry and ready-to-eat foods, such as hot dogs (ERS 2002). Furthermore, this microorganism has been isolated from a variety of food processing plants. In dairies, it

has been found in raw milk, on cooler floors, in processing rooms, and other areas (Robinson et al., 2000). Finally, some studies found that 1 - 10 % of humans may be intestinal carriers of *L. monocytogenes*.

2.2.4. Illnesses and Outbreaks

The first clinical descriptions of both animal and human diseases caused by *L. monocytogenes* were published in the 1920s (Donnelly 2001). Listeriosis is an acute illness caused by *Listeria monocytogenes*. After ingestion of the contaminated food, the incubation period for infection is in the range of 1 to 8 weeks and averages about 31 days. The manifestation of listeriosis includes septicemia, meningitis, fever, muscle aches, and gastrointestinal symptoms such as nausea and diarrhea. If the infection spreads to the nervous system, symptoms such as headache, stiff neck, loss of balance, confusion, and convulsions can occur. If brain functions are affected, listeriosis may mimic symptoms typical of a stroke (About-Listeria; FDA 2001).

The infective dose of *L. monocytogenes* is unknown and varies depending on the susceptibility of the victim and the strain (FDA 2001). Human cases of *Listeria* are, for the most part, sporadic and treatable. Nonetheless, *Listeria* remains an important threat to public health, especially among those most susceptible to this disease (Anonymous, 2004a). The main target populations are pregnant women and their fetuses, immunocompromised people and the elderly, but healthy people are also considered a target population. When listeric meningitis occurs, the overall mortality may be as high as 70%. Mortality with septicemia is 50% and natal/neonatal infections may result in greater than

80% mortality (FDA, 2001). Listeriosis is a disease that is easily transmitted from mother to fetus through the placenta. It is worrisome to an expectant mother, especially because pregnant women themselves rarely show outward signs of such a devastating infection (Anonymous, 2004a). Listeriosis can only be positively diagnosed by culturing the organism from blood, cerebrospinal fluid, or stool (FDA 2001). Because it is a bacterium, there are several antibiotics that may be used to treat listeriosis. The antibiotics that are most effective are ampicillin, gentamicin, and trimethoprim/sulfamethoxazole. Even with prompt treatment, some infections may result in death. This is particularly likely in those that involve the central nervous system, in the elderly and in persons with other serious medical problems (Anonymous, 2004a).

Numerous studies have shown a clear pattern proving that the primary source of food product contamination by *L. monocytogenes*, prior to release to consumers, is the processing plant environment. The incidence of *L. monocytogenes* in milk in processing plants (33.3%) was higher than in samples tested at dairy farms (5.3%). Before slaughter, *L. monocytogenes* was not commonly detected in poultry fecal samples, but poultry became contaminated after slaughter. An extensive survey of levels of meat contamination indicated that chilling and cutting significantly increased the contamination of pork by *L. monocytogenes*. This was in agreement with a high environmental contamination by the pathogen ranging 71 - 100% in the chilling-cutting area of the processing plant (Kathariou 2002).

In recent years more than 27.4 million pounds of chicken and turkey products, which might have been contaminated with *L. monocytogenes*, were recalled (Teratanavat and Hooker 2004). The Food Safety and Inspection Service (FSIS) of USDA is the main regulatory agencies responsible for the safety of meat and poultry products and provides recall information to the public. From January to April of 2004, the FSIS reported six recalls due to possible *L. monocytogenes* contamination. Some of the products involved included meat bologna, frankfurters, cheese and others. Although outbreaks of listeriosis do not occur as frequently as other foodborne illnesses, the 20 - 30% mortality rate is one of the highest of all foodborne diseases. This high mortality rate is a concern for both the food industry and regulators. Control of this pathogen is therefore of interest to many processors, and new control methods are required and desired for its prevention.

2.3 *Escherichia coli* O157:H7

2.3.1 General Characteristics

Escherichia coli bacteria were discovered in the human colon in 1885 by a German bacteriologist, Theodor Escherich. This scientist also showed that certain strains of these bacteria were responsible for infant diarrhea and gastroenteritis. Initially, this bacterium was called *Bacterium coli* but the name was later changed to *Escherichia coli* to honor the scientist who discovered it (Anonymous, 2004b). The first confirmed isolation of *E. coli* O157:H7 in the United States occurred in 1975 and the bacterium was first identified as a human pathogen in 1982, when it was associated with two foodborne outbreaks of hemorrhagic colitis (Doyle et al., 1997).

Phylogenetic trees based on sequencing of 16S rRNA and 5S rRNA indicates that *Escherichia coli* and *Salmonella* diverged from a common ancestor about 120 to 160 million years ago (Park et al., 2001). Currently, there are four classes of enterovirulent classes of *E. coli*. The most common is enteropathogenic *E. coli* (EPEC) associated with infant diarrhea, Other virulence groups include enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) that cause gastroenteritis in humans. Among the EHEC is the enterohemorrhagic strain, *E. coli* O157:H7. Most recently, two other groups have been described including enteroaggregative (EaggEC) and diffusely adherent *E. coli* (DAEC) (Robinson et al., 2000).

Escherichia coli is a Gram-negative rod that is a member of the Enterobacteriaceae family and represents the majority of the natural microflora in the gut. It is classified as a coliform, which means that it is an asporogenous rod that ferments lactose with gas in less than 48 hours and whose colonies are dark and exhibit a green sheen on Eosin Methylene Blue agar. It is oxidase negative and grows using single carbon sources. Other metabolic characteristics are reported in Table 2.2. *E. coli* O157:H7 differs metabolically from other strains of *E. coli* in that they are slow or non-fermenters of sorbitol and lack the enzyme β -glucuronidase. This serotype cannot grow at high temperatures (44 – 45 °C) but can tolerate very acidic media (pH = 2.5).

E. coli O157:H7 is the causative agent of a foodborne illness that has potentially deadly consequences (Robinson et al., 2000). The virulence of *E. coli* O157:H7 is

Table 2.2 *Escherichia coli* metabolic characteristics (Mac Faddin 2000)

Test	Result
Catalase	+
Oxidase	—
Indol	+
Methyl red	+
Voges-Proskauer	-
Nitrate reduction	+
Fermentation	Acidic
Acid of arabinose, lactose, maltose, mannitol, and sorbitol	+
Citrate utilization	-

(-) = negative, and (+) = positive

attributed to several factors. These factors are one or more Shiga toxins, hemolysin, the adhesin, intimin, Esp E, secreted proteins encoded Type III secretion system and O157 lipopolysaccharide O-antigen (Park et al., 2001). The bacterial cell envelope plays an important role in the adaptation and growth of microorganism in different environments. The cell wall acts as a functional and physical barrier between the inside of the cell and its environment, allowing bacterial resistance to acids, antimicrobials, and other environmental factors (Lugtemberg et al., 1983; Schaechter et al., 1998).

The cell envelope of Gram-negative bacteria is more complex than that of Gram-positive bacteria. The Gram-negative cell envelope is shown in Figure 2.3. It consists of two membranes separated by a layer of peptidoglycan and a cellular compartment known as periplasm. The concentration of peptidoglycan found in Gram-negative bacteria is 20-

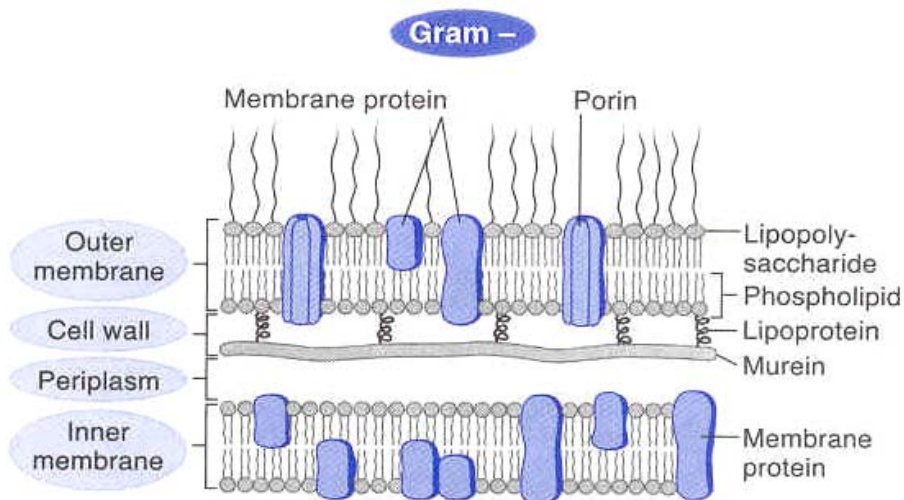


Figure 2.3 Diagram of a Gram-negative bacteria cell wall (Scaechter et al., 1998)

fold less than that present in Gram-positive bacteria (Schaechter et al., 1998). In addition, a bi-layered cell membrane is present on the outside of the peptidoglycan layer preventing the layer from interacting with the extracellular environment (Hancock 1984; Wilson et al., 2001). Another important function of the outer membrane is to endow the bacterial surface with strong hydrophobicity, which is important in evading phagocytosis, some complement resistance, and the capacity to avoid specific immune attack by altering its surface antigen composition.

The bi-layered structure also contains an outer leaflet that is known as the lipopolysaccharide (LPS) layer (Nikaido and Vaara 1985). In Figure 2.4 the structure of the LPS is shown. The presence of the LPS layer in Gram-negative bacteria makes the

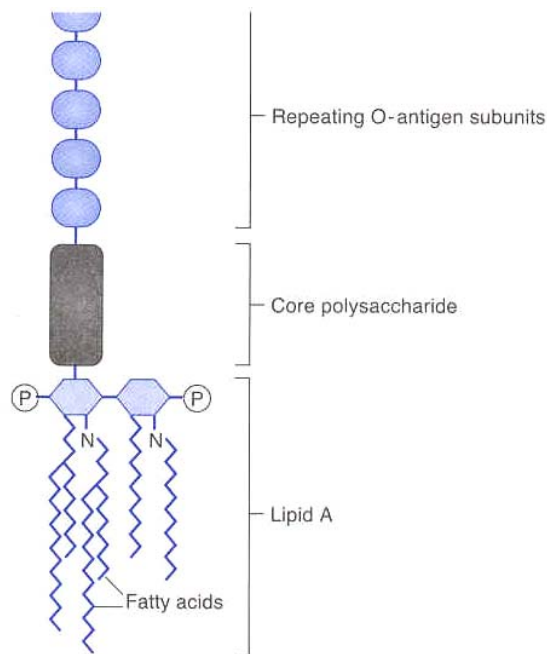


Figure 2.4 The lipopolysaccharide structure (Scaechter et al., 1998).

membrane so chemically distinctive that it is not found elsewhere in nature (Schaechter et al., 1998). Unlike phospholipids that have only two fatty acid chains connected to the backbone structure, lipopolysaccharides have six or seven fatty acids linked to their glucosamine disaccharide backbone (Nikaido and Vaara 1985). Another feature of the LPS is that they bind divalent ions quite strongly. The LPS layer is comprised of three components: Lipid A, the core, and the O or somatic antigen (Park et al., 2001). Lipid A is a glycolipid composed of disaccharides that are attached to short chain fatty acids and phosphate groups (Figure 2.4). Lipid A is responsible for anchoring the LPS to the outer leaflet of the membrane. Attached to the lipid A region is a rough oligosaccharide core containing keto-deoxyoctanoic acid, heptose and hexose residues. These sugar units help to connect the Lipid A and O-antigen components (Schaechter et al., 1998).

The final component of the LPS layer is the O-antigen, which is a long carbohydrate chain comprised of repeating units of D-glucose, L-fructose, 2-acetoamido-2-deoxy-D-galactose, and 4-acetamido-4,6-dideoxy-D-mannose residues (1:1:1:1) with up to 40 sugars in length (Schaechter et al., 1998; Park et al., 2001). The O-polysaccharide is an antigen determinant of the serotype and also appears to play an important role in adherence of the serotype of the host epithelial cells (Park et al., 2001). The O-antigen chains cover the bacterial surface and exclude hydrophobic compounds. The LPS is anchored to the outer membrane by outer membrane proteins through hydrophobic interactions with Lipid A (Schaechter et al., 1998).

Other components of the cell envelope are porins. Porins produce pores or channels that allow the passage of small hydrophobic molecules across the membrane. Many negatively charged groups exist on the backbone as well as on proximal sugar residues such as 2-keto-3-deoxycyonic acid (Nikaido and Vaara 1985). Outer membranes of Gram-negative bacteria allow the influx of nutrients and efflux of waste products. Indeed it was found that the outer membrane of enteric bacteria was permeable to hydrophilic solutes with a molecular weight less than 6000 Daltons. In addition, solutes that carry negative charges diffused through the porins or channels more slowly than their uncharged counterparts. Overall, the bacterial outer membrane shows unusual functional properties such as low permeability toward lipophilic solutes and high permeability to hydrophilic solutes. These characteristics may be attributed to the presence of structural components such as the LPS and porins that in conjunction with other components build a very effective permeation barrier (Nikaido and Vaara 1985).

2.3.2 Sources

E. coli O157:H7 have been detected in dogs, birds, sheep, and deer as well as in humans. *E. coli* O157:H7 outbreaks have been associated with the consumption of undercooked ground beef, and unpasteurized milk (Doyle et al., 1997). Thus, cattle are the most important reservoir of *E. coli* O157:H7. Foodborne outbreaks have been linked not just to beef but also to other food sources such as water, contaminated fruits and vegetables (Park et al., 2001). Many other foods have been linked to *E. coli* O157:H7 infections. For example, newly recognized sources of O157 infections are drinking and recreational

water. A recent study of the fate of *E. coli* O157:H7 in water indicates that the pathogen can survive for long periods of time at 8 °C in water (FDA 2001).

2.3.3 Illnesses and Outbreaks

The CDC estimates that more than 73,000 cases of *E. coli* O157:H7 or *E. coli* occur every year in the US. 2,100 people are hospitalized, and 61 people die as a direct result of *E. coli* infection and complications that can result from the infection (CDC, 2004b). *E. coli* O157:H7 has three principal illness manifestations: hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and , thrombotic thrombocytopenic purpura (TTP) (Doyle et al., 1997, Park et al., 2001). Hemorrhagic colitis is a distinct clinical syndrome that it is characterized by sudden abdominal pain, followed within 24 hr by the onset of non- bloody diarrhea that leads to subsequent bloody diarrhea within several days. The duration of the illness ranges from 2 to 9 days, and the incubation period ranges from 3 to 9 days (Park et al., 2001). Outbreak investigations revealed that more than 90% of cases of diarrhea caused by *E. coli* O157:H7 were bloody (Doyle et al., 1997). Hemolytic uremic syndrome is the most common cause of acute renal failure in children. HUS is a triad of acquired hemolytic anemia, thrombocytopenia and renal failure; hemolytic anemia with fragmented erythrocytes appears to occur as a result of HUS. In contrast to HUS, TTP occurs mainly in adults. This disease is characterized by hemolysis, thrombocytopenia, renal failure, neurological problems, and fluctuating fever. TTP is distinguished from HUS by its association with the central nervous system and the lack of diarrhea (Park et al., 2001). TTP can have a mortality rate in the elderly as high as 50%.

E. coli O57:H7 and its link to food became well known to the public as a result of a 1993 *E. coli* O157:H7 disease outbreak caused by contaminated ground beef. Over 700 people became ill from this outbreak and four children died. In recent years, more outbreaks have been reported and this pathogen is becoming of increasing concern to public health (ERS 2002). Recently, more than 19 million ground beef possibly contaminated with *E. coli* O157:H7, were recalled (Teratanavat and Hooker 2004) and a number of outbreaks reported. In 2003, outbreaks were associated with consumption of water, spinach, pico de gallo, sprouts, leaf lettuce, steaks, ground beef and others (Anonymous 2004a). Other several outbreaks have been linked with person-to-person contact (Doyle et al., 1997).

2.4. Antimicrobial Agents

2.4.1. Introduction

Antimicrobial agents are 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”(Davidson, 2004).

Foodborne illnesses have been a vital concern of public health for years. These diseases are a direct result of the ingestion of contaminated food with pathogenic bacteria and/or their toxins. Controlling foodborne bacteria can reduce the number of outbreaks and assure consumers of a safe food supply (Kim et al., 1995). Therefore, there is increased interest in the development of new methods of reducing or eliminating foodborne pathogens (Burt 2004). Since ancient times, man has searched for ways to

preserve their foods for later use. Traditionally, foods were preserved by using cold, heat, drying and fermentation (Davidson and Branen 1993). Antimicrobials, antioxidants and antibrowning agents are the basic three types of preservatives used in foods (Branen et al., 2002). Antimicrobials agents may be either synthetic compounds or naturally occurring substances and play an important role in extending shelf-life of foods. They have become more important and are used more regularly in recent years to reduce the risk of microbial contamination (Brul and Coote 1999; López-Malo et al., 2000; Branen et al., 2002). Antimicrobials can be obtained from natural sources or chemically synthesized. The increasing demand of reduced additive in foods and the demand for greater convenience have provoked the food industry to search for alternative single antimicrobial agents or new combinations of antimicrobials (López-Malo et al., 2000). Because of the negative consumer perception of synthetic preservatives, attention is shifting towards natural alternatives (Menon and Garg 2001).

Natural antimicrobials can be obtained from animal, plant, microbial and mineral sources. Natural antimicrobials are compounds that exist, for example, in spices, herbs or essential oils. These compounds have for example been used as flavoring agents for thousands of years ago (Kim et al., 1995). Plants, herbs, and spices contain substances that are known to inhibit bacteria, yeast, and molds (Kim et al., 1995; López-Malo et al., 2000). Antimicrobial agents in plants are thought to as a defense mechanism against bacterial attacks. Phenolic compounds and their subclasses such as coumarins, flavonoids and essential oils have antimicrobial functions (Kim et al., 1995).

Antimicrobial activity may depend on the type, genus, species and strain of microorganism treated with the antimicrobial (Davidson and Parish 1989). Most antimicrobial compounds are less effective in foods than in microbiological media because they can interact with other food components (Oka 1964). Antimicrobial action of a chemical compound is typically due to some type of reaction between the microbial cell and the compound. This reaction can be specific or non-specific, and of chemical or physical nature. For any antimicrobial compound it is important how it is transferred from the media to the cell. This is often the first and critical step required for inhibition of the microorganism (Oka 1964).

2.4.2 Spices

A spice can be defined as the dried aromatic parts of natural plants, whose characteristics such as color and constitution may vary depending on year of harvest and place of harvest, among other factors (Hirasa and Takemasa 1998). The word “spice” is derived from the Latin word “species”, meaning specific kind (Uhl 2000). Since ancient times, spices and herbs have been used to add desirable sensory characteristics to food products. They have also been used to season many kinds of foods either alone or in combination with other condiments such as salt, sugar and other ingredients. Spices have various effects in food. Not only do they impart color, flavor and pungency to food but they also act as antioxidants, antimicrobials and enhance the nutritional properties of many foods. Spices are available dried, fresh, whole, ground, crushed, as pureed, as paste, and as extracts (Uhl 2000). Spice aromas and flavors differ according to the amount of essential

oils they contain and the proportion of each essential oil compound (Hirasa and Takemasa 1998).

Studies in the 1980's confirmed the growth inhibition of Gram-negative and Gram-positive bacteria, yeast and molds by some spices such as garlic, onion, cinnamon, cloves, thyme, savory, sage, and others (Shelef 1983; Conner and Beuchat 1984; Deans and Ritchie 1987). The antimicrobial compounds in plant materials are commonly contained in the essential fraction of leaves (rosemary and sage), flowers and flower buds (clove), bulb (garlic, onion), rhizomes (asafetida), fruit (pepper, cardamom) or other parts of the plant (López-Malo et al., 2000). Phytophenols are compounds commonly present in spices and herbs that exhibit antimicrobial activity (Oka 1964; Brul 1999). Antimicrobial agents obtained from spices are hydrophobic and to achieve cell death are generally needed in high concentration above tolerable sensory thresholds (Shelef et al., 1984; Juven et al., 1994 Brul and Coote 1999).

Friedman et al., (2002) reported that the concentration needed to impart bactericidal activity of some spices such as clove, oregano, cinnamon and others, against *E. coli* range from 0.046 to 0.14%. The concentration needed to exhibit bactericidal activity with oregano, thyme, clove bud, and others range from 0.057 to 0.092%. 0.5 % and 1% clove oil restricted the growth of *Listeria monocytogenes* in meat and cheese (Menon and Garg 2001). Many of the major components found in plants, herbs and spices that exhibit antimicrobial activity are phenolic compounds such as terpenes, aliphatic alcohols, aldehydes, ketones, acids, and isoflavonoids (López-Malo et al., 2000).

2.4.3 Essential Oils

Essential oils (EO) are aromatic oily liquids obtained from plant material (Burt 2004). These fractions are comprised of a group of heterogenic organic substances such as aldehydes, alcohols, and esters. EO are the components responsible for the aroma of the plant and are important in the cosmetic (fragrances), pharmaceutical (flavoring agents), and food (spices or condiments and flavoring agents) industries. Typically, they are commercially produced through expression or cold press, fermentation, extraction, or steam distillation (López-Malo et al., 2000; Burt 2004). Depending on the method of extraction, the nature of the volatile can differ within the same type of spice. Usually, essential oils are about 75 to 100 times more concentrated than in the fresh herb and therefore are used at very low levels ranging from 0.01-0.05% in the finished product (Ulh 2000).

Essential oils are generally mixtures of up to 100 different compounds. They can contain a mixture of phenylpropanes, terpenes and aliphatic compounds of low molecular weight such as alkanes, alcohols, aldehydes, ketones, esters and acids. The majority of chemical components of essential oils of spices are terpene compounds consisting of a carbon, hydrogen, and oxygen. Compounds with 10, 15 or 20 carbons are named monoterpenes, sesquiterpenes, or diterpenes (Hirasa and Takemasa 1998). EOs are soluble in ethanol or ether but only sparingly soluble in water (less than 0.01%).

Essential oils can be classified depending on their consistency (fluid essence, balsamic, and oleroresins), origin (naturals, artificial and synthetic), and chemical nature

or major compounds (monoterpenes, sesquiterpenes and phenylpropanes). An estimated 300 EOs are known and all of them are commercially important (Burt 2004). EOs and their components are widely used as food flavors, and are classified by the US FDA as “Generally Recognized as Safe” (GRAS). The antimicrobial activity of essential oils has been recognized and depends on the chemical structure of their components and their concentration (Shelef 1983; Moleyar and Narasimham 1992).

Certain antimicrobial components are hydrophobic, and their activity as antimicrobial agents is therefore limited (Davidson and Naidu 2000). The mechanism of EO has not yet been clearly elucidated. It has been proposed that the antibacterial activity of EO may not be the result of a single mechanism but instead may include several target sites in the cell simultaneously (Figure 2.5). Generally, EOs possessing the strongest antibacterial properties are those that contain phenolic compounds such as carvacrol, eugenol and thymol. It has been proposed that their mechanisms are similar to that of other phenolic compounds, i.e, a disturbance of the cytoplasmic membrane that disrupts the proton motive force and may lead to coagulation of cell contents (Juven et al., 1994; Davidson 1997; Ultee et al., 2002; Burt 2004).

Moleyar and Narismham (1992) showed that cinnamic aldehyde, citral, geraniol, eugenol and menthol showed the strongest antimicrobial effect against *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp. and *Enterobacter* sp. Karapinar and Autuğ (1987) demonstrated that eugenol was the most active compound against *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*.

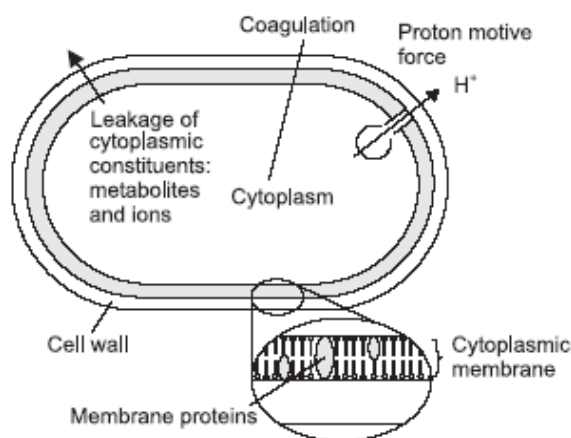


Figure 2.5 Sites of action for EO components (Burt 2004)

2.4.4 Phytophenols

Phenolic compounds in spices and herbs are the most recognized and studied. Phenolic compounds in foods originate from one of the main classes of secondary metabolites in plants (Shahidi and Naczki 1995). These compounds have a molecular weight around 150 to 160 Da and contain a hydroxyl group in the molecule (Shelef 1985). Some of the most potent phenolics include thymol extracted from thyme, carvacrol extracted from oregano, cinnamic aldehyde extracted from cinnamon, and eugenol extracted from cloves.

Several authors have demonstrated that the presence of a hydroxyl group enhances the antimicrobial activity of antimicrobial compounds (Brul and Coote 1999; Cowan 1999; López-Malo et al., 2000; Ultee et al., 2002). Co-existence of phenolic compounds with other compounds such as surfactants, minerals, and the interaction with other food components such as proteins and lipids are factors that control the biological activity of phytophenols. Time, pH, and temperature are other critical factors that affect the function

of phenolic compounds. Microbial targets such as type of organism and strain, stage of growth, and previous exposure to stress and injury are critical factors that contribute to the antimicrobial activity of these compounds (Davidson and Naidu 2000).

Carvacrol. Oregano is a native plant that originated in the Mediterranean and is a common spice in Greece, Italy, and Spain (Farrell 1990). Oregano contains different amounts of essential oils depending on the origin. For example, fresh Greek oregano has about 0.9% of essential oil, and contains 60-70% phenols mainly thymol and carvacrol while Mexican oregano contain 3-4% of essential oils. The antimicrobial activity of oregano and thyme has attributed to their essential oils that contain the terpenes carvacrol and thymol (Davidson and Naidu 2000; Uhl 2000). Carvacrol (5-isopropyl-2-methylphenol, $C_{10}H_{14}O$, Figure 2.6) has a molecular weight of 150.2, a yellowish color, a density of 0.9751 g/ml at 25°C, and a boiling point of 237 to 238°C.

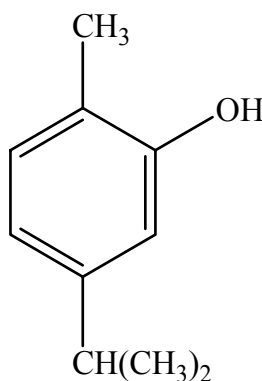


Figure 2.6 Chemical structure of carvacrol

It is incompatible with strong oxidative agents and virtually insoluble in water but soluble in alcohol/ether (Merck index 2001).

Eugenol. Eugenol is a phenolic compound obtained from cloves. Cloves are native to the Moluccus. Arab traders were the first to introduce cloves to Romans and Greeks. Later Europeans fought to monopolize the clove trade due to its high profitability. The name “clove” is a derivative of the French word *clou* and the Spanish word *clavo*, both meaning nail, because of its resemblance to that shape (Uhl 2000).

Clove is one of the most fragrant of all aromatic spices (Farrell 1990). Clove essential oil is typically extracted from clove buds but can also be obtained from leaves and stems. Clove buds have an average essential oil content of 5 to 20% and consist of 93 to 95% eugenol (Davidson and Naidu 2000; Uhl 2000). Eugenol contributes to the pleasant odor of cloves as well as its burning flavor (Farrell 1990).

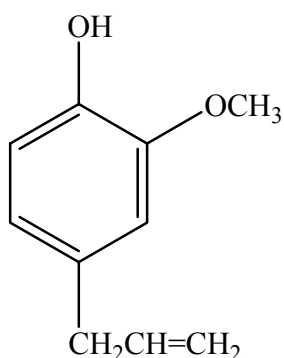


Figure 2.7 Chemical structure of eugenol

Eugenol (2-methoxy-4-(2-propenyl)-phenol, $C_{10}H_{12}O_2$, Figure 2.7) has a molecular weight of 164.20 Da. It is a colorless or pale yellow liquid with a boiling point of 255°C and a density of 1.06 g/ml at 25°C. It is incompatible with strong oxidative agents and is practically insoluble in water but miscible with alcohol, chloroform, and ether (Merck index 2001).

2.5 Surfactant Characteristics

2.5.1 Introduction

Surfactants are one of the most common and most important class of chemicals. Consumers come in contact with surface active agents or *surfactants* on a daily basis. In food products, surfactants are primarily used as emulsifiers that help to stabilize disperse food systems such as foams and emulsions against breakdown (Stauffer 1999). Surfactants are molecules that adsorb readily at solid/liquid, liquid/liquid, or liquid/gas interfaces (Clint, 1992). This is because surfactants are amphiphilic molecules that self-assemble in water or other solvents to form complex three-dimensional structures such as micelles or bilayers (Texter 1999; Pletnev 2001). For example, biological cell membranes are composed mainly of amphiphilic lipids (polar lipids) that self-assemble into a bilayer structure where the hydrophobic groups are oriented such that contact with the water phase is avoided while the hydrophilic parts are oriented toward the outside of the membrane structure towards the solvent. In mammalian cells, the primary component of membranes is phospholipids, but membranes may contain proteins, carbohydrates, and cholesterol as well. Phospholipids are just one type of compounds that belong to the class of surfactants and that play a vital role in living organisms (Clint 1992). Understanding

the behavior of surfactants is important if they are to be used in a wide range of applications such as detergents, emulsifiers, adhesives, and lubricants (Stauffer 1999; Pletnev 2001). Successful production of a stable product with predetermined characteristics often relies on the presence of surfactants. It should be noted that while there are a large number of biologically-derived surfactants available in nature, the majority of surfactants used in industry are synthetically derived (Clint 1992).

2.5.2. Surface Tension

Surface tension can be defined as the internal force that acts perpendicularly to the surface to keep molecules within the interface. Surface tension arises due to a balance of interactions between molecules of the two adjacent phases and of the molecules within each phase (Pletnev 2001). The surface tension of a solution that contains a surfactant is lower than that of a pure solvent. This can be attributed to the fact that surfactant molecules accumulate in the interface and reduce unfavorable interactions between the molecules of the two phases thereby decreasing the overall free energy (Holmberg 2002).

The surface tension is typically measured in dynes/cm which is equivalent to the force necessary to break a film of 1 cm length. Water has a surface tension of 72.8 dynes/cm at 20°C at the air-water interface; ethanol has a surface tension of 22.3 dynes/cm while mercury has a surface tension of 465 dynes/cm (Holmberg 2003).

2.5.3 Surfactant Classification

Surfactant is a word derived from **surface-active-agent** (Clint 1992; Stauffer 1999, Holmberg 2003). Surfactants are amphoteric molecules or amphiphiles which means that

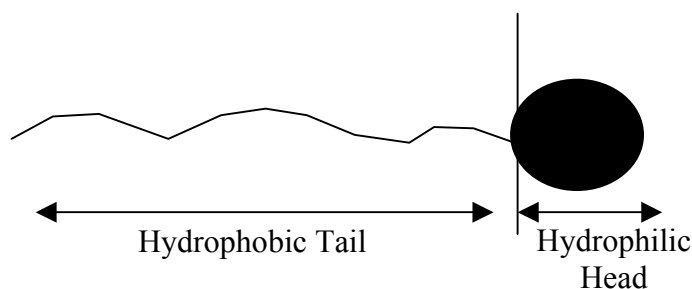


Figure 2.8 Structure of surfactant monomer (Weiss 1999)

they contain both, hydrophilic and hydrophobic parts (Texter 1999). The typical structure is shown in Figure 2.8 and consists of a hydrophobic tail and a hydrophilic head (Weiss 1999). Surfactants may be classified based on their physical properties or functionality (Texter 1999). One of the most important classifications is based on the nature of the polar group, e.g. anionic, cationic, nonionic, and amphoteric (Pletnev 2001).

Anionic Surfactants. Anionic surfactants are amphiphilic compounds that include an anionic group attached or linked to long hydrocarbon chains (Pletnev 2001). Classical examples of anionic surfactants include soaps ($-\text{CO}_2^-$) which are the earliest synthetic detergents, sulfonate ($-\text{SO}_3^-$) and sulfates ($-\text{OSO}_3^-$).

The hydrocarbon part of the surfactant can be a straight-chain aliphatic radical as well as a branched chain, unsaturated radical, and can contain benzene or phenolic residues (Pletnev 2001). A typical structure of an anionic surfactant is shown in Figure 2.9. All of these are widely used in cleaning formulations. The major advantage of sulfonates and sulfates over the carboxylates is their greater tolerance of divalent metal ions in hard water (Clint 1992). The main uses of these surfactants are in soaps, synthetic

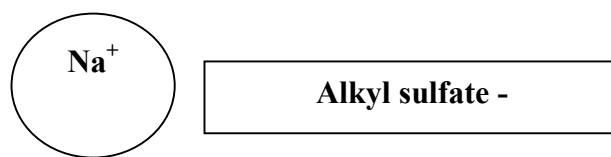


Figure 2.9. Structure of anionic surfactants

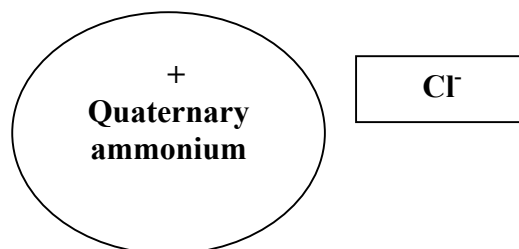


Figure 2.10. Structure of cationic surfactants

detergents, personal care products, corrosion inhibitors, and emulsifiers (Clint 1992). The most important starting materials for the production of anionic surfactants are alkyl benzenes, linear paraffins, linear olefins (terminal or internal double bonds), long chain alcohols and fatty acids (Pletnev 2001).

Cationic Surfactants. Cationic surfactants in solution can be dissociated to form a surface active positively charged cation and a normal anion as shown in Figure 2.10 (Pletnev 2001). The positive charge of cationic surfactants results in strong deposition on negatively charged fibers such as cotton due to electrostatic interactions (Clint 1992). One advantage of cationic surfactants is that they are compatible with nonionic and zwitterionic surfactants. The surface active moiety has a positive charge, and thus adsorbs strongly onto most interfaces (Rosen 1989). For example, an emulsion may break when it

encounters a negatively charged substrate thus allowing deposition of the active phase onto the substrate. A big disadvantage is that most cationic surfactants are not compatible with anionic surfactants (Rosen 1989).

It should also be noted that cationic surfactants are not effective in cleaning applications and therefore are not used as detergents (Pletnev 2001). The main applications of these surfactants is as sanitizers/disinfectants and antistatic agents (Clint 1992; Pletnev 2001). These are usually quaternary ammonium, imidazolinium or alkyl pyridinium compounds. Quaternary ammonium in particular accounts for approximately 90% of the total cationic production (Clint 1992; Pletnev 2001). The total world production of cationic surfactants has recently reached two million tons per year and they comprise a quarter of the total world surfactant production (Pletnev 2001).

Amphoteric Surfactants. Amphoteric surfactants are surfactants that may carry both anionic and cationic charges (Pletnev 2001). An example of an amphoteric structure of such a surfactant is shown in Figure 2.11. The term amphoteric or zwitterionic surfactants refers to compounds that have pH-dependent amphoteric properties (Pletnev 2001).

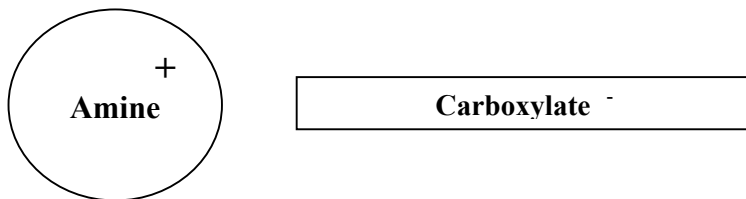


Figure 2.11. Structure of amphoteric or zwitterionic surfactants

Examples of amphoteric surfactants are betaines ($-N^+ (CH_3)_2CH_2CO^-$) and sulfobetaines ($-N^+ (CH_3)_2CH_2SO^-$).

These compounds are milder to the skin than the anionic surfactants and are used in toiletries and baby shampoos. Among the important naturally occurring surfactants in this class are the lecithins (Clint 1992).

Nonionic Surfactants. Nonionic surfactants are amphiphilic compounds with a hydrophilic and a hydrophobic part. They do not dissociate into ions and therefore, have no charge (Pletnev 2001). An example of nonionic surfactants is shown in Figure 2.12. Nonionic surfactants include primarily ethoxylated alcohols ($((OCH_2CH_2)_nOH)$), and alkylphenols that have polyoxyethylene chain as their hydrophilic group (Clint 1992; Pletnev 2001). A major advantage of these surfactants is that they are compatible with most other types of surfactants (Rosen 1989). Nonionic surfactants are primarily used as

detergents and emulsifiers. This group also includes semi-polar compounds such as amine oxides, sulfoxides and phosphine oxides, but the only commercially important ones are the amine oxides. Interestingly, the amine oxide surfactants are able to acquire some charge depending on the pH value. Even polyethers, such as

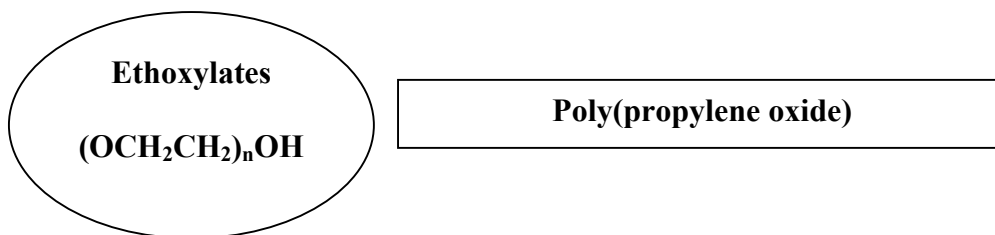
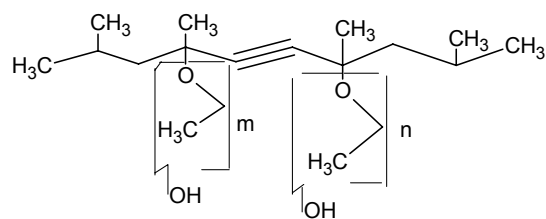


Figure 2.12. Structure of nonionic surfactants

polyethylene oxides, are protonated under acidic conditions and exist in their cationic form. Carboxylic acids are nonionic under neutral and acidic pH but under basic conditions are negatively charged (Pletnev 2001).

Recently, there has been an increase in the variety of nonionic head groups used in nonionic surfactants including the pyrrolidones, alkanolamides and their ethoxylated derivates, and even sugars (Clint 1992). Normally, nonionic surfactants in the food industry are used as mixtures of several different compounds. In food applications the most used surfactants are the nonionic because of their characteristics. Ethoxylated nonionic surfactants show the lowest interfacial tension as well as high solubilization and detergency around their clouding point.

Surfynol[®] 465. *Surfynol*[®] 465 is a nonionic surfactant with two ethoxylated groups. Its chemical name is ethoxylated 2, 4, 7, 9-tetramethyl-5-decyn-4, 7 diol and its structure is shown in Figure 2.13. It is a mild surfactant that is widely utilized as a humectant. It has an HLB of 13 and a CMC of 0.67 %. It has 10 moles of ethylene oxide, a pH ranging



$m + n = \text{number of moles of ethylene oxide}$

Figure 2.13. *Surfynol*[®] 465 and 485W structure

from 6 to 8, a viscosity of less than 2000 cps at 20°C and a specific gravity of 1.038 at 25°C. This surfactant is soluble in water, xylene, carbon tetrachloride, mineral oil, and ethylene glycol. The surfactant is regulated under 21CFR 175.105, 175.300, 176.180 and 176.200.

Surfynol[®] 485W. Surfynol[®] 485W is a nonionic surfactant with two ethoxylated groups. It is a yellowish liquid with a weak odor. Its chemical name is ethoxylated 2, 4, 7, 9-tetramethyl-5-decyn-4,7 diol (Figure 2.13). It is widely utilized as a humectant and has emulsifier properties. It has an HLB of 17 and a CMC of 1.65 %. It has 30 moles of ethylene oxide, a pH of 7, a viscosity of less than 3000 cps at 20°C and a specific gravity of 1.07 at 25°C. This surfactant is soluble in water, xylene, carbon tetrachloride, mineral oil, and ethylene glycol. It can be dispersed in soy oil, and is insoluble in kerosene. The surfactant is regulated under 21CFR 175.105, 175.300, 176.180 and 176.200.

2.5.4 Hydrophilic Lipophilic Balance (HLB)

Surfactants consist of a hydrophobic and a hydrophilic portion. The balance between these two groups governs the functionality and how they behave at an oil-water interface. This balance is referred to as the Hydrophilic Lipophilic Balance (HLB) and indicates the proportion of the hydrophilic to hydrophobic parts in a surface active agent. The HLB may range from 0 to 20. The greater the hydrophilic tendency, the higher the HLB). Emulsifiers with a low HLB value tend to form oil-in-water (O/W) emulsions while those with a high HLB value tend to form water-in-oil (W/O) emulsions. Surfactants with very

high HLB numbers are considered good solubilization agents. Initially, the HLB was calculated HLB as (Stauffer 1999):

$$HLB = \frac{L}{T} \times 20 \quad (1)$$

where L is the molecular weight of the hydrophilic portion of the molecule and T is the total molecular weight. However, in 1954 Griffin developed another mathematical description of the HLB that quickly became more accepted in the surfactant community. This equation is used to determine the HLB of different types of nonionic surfactants, particularly ethoxylated alcohols and esters of polyhydric fatty acids:

$$HLB = 20 \left(1 - \frac{S}{A} \right) \quad (2)$$

where S is the saponification and A is the acid number. For the determination of an HLB of a surfactant such as polysorbate the following formula may be used:

$$HLB = \frac{E + P}{5} \quad (3)$$

where E is the weight percent of the oleophilic chains and P is the weight percent of the polyhydroxylated alcohols. For the determination of the HLB of a mixture of surfactants the following formula should be used:

$$X = 100 \times \frac{HLB_{AB} - HLB_B}{HLB_A - HLB_B} \quad (4)$$

Where X is the surfactant ratio of component A to B.

Determination of the HLB has the advantage that classification of surfactant mixtures is possible. Some of the disadvantages of the HLB number are that the HLB of ionic surfactants cannot be calculated due to the fact that their charge varies depending on pH. Another disadvantage is that the HLB calculation is based on the molecular structure of the emulsifiers and does not take into account the combination of the oil/water phase of the emulsification system (Becher 1965). Different applications and functions of surfactants according to their HLB number are shown in Table 2.3. Nevertheless, it is thought that there is an analogy between HLB and the structure of the molecules (Figure 2.14)

2.5.5 Micelles

At low surfactant concentrations in water surfactants exist solely as monomers in the aqueous phase. At a certain concentration, the so called “critical micelle concentration” (CMC), surfactants begin to self-assemble and form microstructures such as micelles and bilayers. Above the CMC, the colligative properties of the solution (freezing point, boiling point, electric conductivity) change (Noskov and Grigoriev 2001).

Micelles are aggregates of monomers of surfactant molecules where the hydrophobic tails point to the inside of the micelle, i.e., away from the polar phase, while the hydrophilic heads are oriented towards the water or polar phase (Stauffer 1999). The number of surfactant molecules required to form a micelle is called the aggregation number and it is one of the most important characteristics of a micelle (Noskov and Grigoriev 2001)

Table 2.3 Characteristics of surfactants depending on the HLB (Weiss 1999)

HLB	Application
<3	Surface films
3-6	Water-in-Oil emulsifiers
7-9	Wetting agents
8-15	Oil- in-Water emulsifier
13-15	Detergents
15-18	Solubilizers

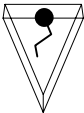
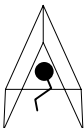
<u>Aggregate</u>	<u>Shape</u>	<u>Characteristic in solution</u>	<u>HLB</u>
 Micelles		Clear solution	>13
 Lamellar Phase		Milky dispersion	7-10
 Inverted Aggregates		Lumps of emulsifiers in equilibrium with surplus of water	<7

Figure 2.14. Surfactant shapes depending on the HLB (modified from Bergensthal and Cleasson 1990)

Micelles have a spherical shape with a diameter around 4 to 10 nm (Holmberg 2003). At high surfactant concentrations, micelles become increasingly larger and can no longer maintain their spherical shape. They adopt elliptic, cylindrical or laminar structures as shown in Figure 2.15 (Holmberg 2002). These systems are highly dynamic, with surfactant monomers adsorbing and desorbing from the interface on an extremely short timescale (Clint 1992).

2.5.6 Critical Micelle Concentration (CMC)

The narrow concentration range where aggregates start to form and where the physicochemical properties of the solution suddenly change is the CMC (Pletnev 2001).

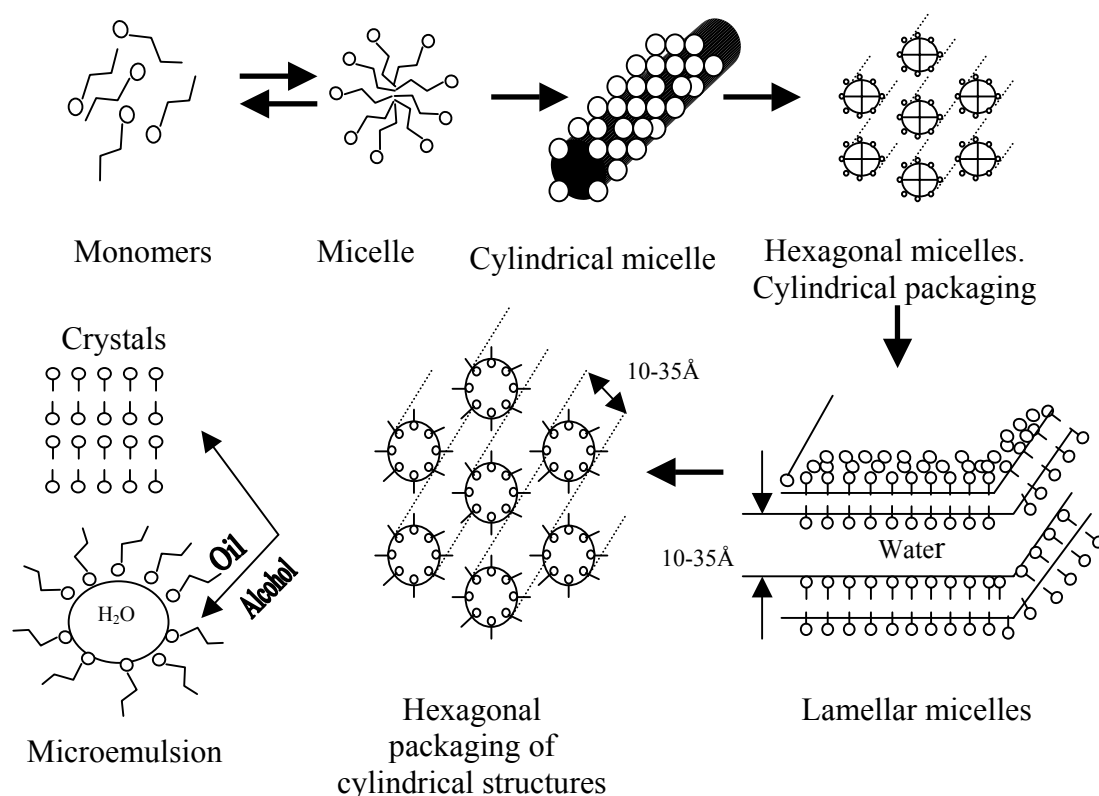


Figure 2.15 Structures formed by surfactant monomers at different concentrations (Holmberg 2002, Vol. 1)

In Figure 2.16, the changes in key physical properties as a function of surfactant concentration are shown. For example, the conductivity of aqueous solutions of ionic surfactants decreases drastically just above the CMC (Noskov and Grigoriev 2001).

The CMC is one of the key characteristics of a surfactant and is almost always required to ensure the adequate functionality of surfactants in industrial applications (Holmberg 2003). For example, detergency and ability to foam is typically low below the CMC and increases strongly after the surfactant concentration exceeds the CMC. For the

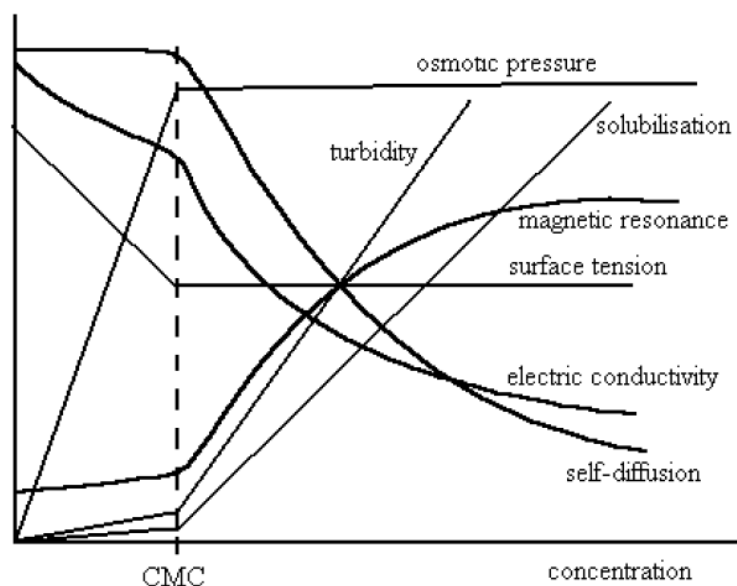


Figure 2.16. Schematic representation of physical changes above CMC (Pletnev 2001)

majority of surfactants, the CMC is around 1 %. A low CMC indicates a greater interaction between the surfactant and the solvent phase. The shorter the polar chain the lower the CMC. In addition, if the temperature decreases, the CMC also decreases (Clint 1992). The CMC depends on nature of the hydrophobic group, the system pH and presence of electrolytes. (Clint 1992). There are several methods available to determine the CMC experimentally. These include determining surface tension, conductivity, light scattering, solubilization and autodiffusion as a function of surfactant concentration.

The surface tension of a surfactant solution may be measured by classical methods such as the du Nuoy ring method (Clint 1992; Holmeberg 2002). Conductivity measurements are based on the fact that the surfactant has electrolyte properties. The

electrical conductivity of a surfactant in an aqueous system consequently changes when reaching the CMC because of altered electrostatic interactions of the micelles with the solvent phase compared to the surfactant monomers (Clint 1992). Generally, at concentrations larger than the CMC, there is a large decrease in conductivity since micelles are less efficient carriers of charge than the dissociated monomers (Clint 1992). Light scattering is one of the most precise methods to determine the formation of micelles. The aggregation of surfactant molecules causes a strong increase in light scattering (Clint 1992). Dynamic light scattering may be used to assess the size of micelles. This method is based on the principle of Brownian motion, i.e., the diffusive motion of the particles are determined by their size. At a constant temperature, larger particles move slower than smaller particles (Holmberg 2002).

Finally, solubilization can be used to measure the CMC due to the fact that surfactant micelles provide an environment in which hydrophobic substances can be incorporated or solubilized. However the compound to be solubilized may affect the CMC because they influence the formation of the micelles.

2.5.7 Cloud Point

Pletnev (2001) defined the cloud point as the temperature at which the solution becomes turbid and is a consequence of the dehydration of the polyethylene chains. In other words, aqueous solutions of polyoxyethylene nonionic surfactants become turbid and strong light scatterers upon being heated to a temperature that is known as the cloud point. This is then followed by a phase separation (Holmberg 2003) that may be reversed by cooling

the solution below the cloud point temperature. The separation of the phases is believed to be due to the increase in aggregation number of micelles and the decrease in the interaction within the micelles. This results from dehydration of the oxyethylene oxide in the polyoxyethylene hydrophilic group with increasing temperature (Rosen 1989). The temperature at which the phase separation occurs depends on the structure and chain length of the polyoxyethylene surfactant. The higher the number of oxyethylene chains, the higher the cloud point (Rosen 1989). The cloud point depends strongly on the presence of co-solutes and in general electrolytes can either increase or decrease the cloud point (Holmberg 2003).

2.6 Solubilization

The first to give a definition of solubilization were McBain and Hutchinson in 1955. They defined solubilization as the “increase in the solubility of an insoluble or slightly soluble substance in a given medium” (Holmberg 2003). This phenomenon is a property of regular micelles in that they promote solubility of compounds that would otherwise be insoluble in aqueous solutions (Texter, 1999). Solubilization is different from emulsification in that in solubilization the non-polar compound, or the compound to be solubilized, is in the same phase as the solubilizing solution and the system is thermodynamically stable. The emulsification process is defined as the dispersion of one liquid phase into another (Rosen 1989).

Solubilization of an insoluble molecule in a surfactant solution is primarily governed by fundamental thermodynamic properties and structure (Holmberg 2003). The location

of the solubilized compound depends on the nature of the material solubilized and also reflects the type of interaction that occurs between the surfactant and the compound solubilized (Rosen 1989). The incorporation of a compound into micelles can change the shape of the micelle considerably. With the incorporation of larger quantities of non polar material, micelles become more asymmetric and form an inverted lamellar micelle that eventually may assume the shape of a spherical inverted micelle in the non polar medium (Figure 2.15; Rosen 1989).

One of the most important parameters in solubilization is the maximum amount of material that can be incorporated or the “maximum additive concentration” (MAC). This concentration is also referred to as the saturation concentration or the solubilization capacity (Weiss 1999; Holmberg 2002). The solubilization capacity is defined as the number of moles of compound to be solubilized per mole of surfactant in the micelles. It often remains constant for a particular surfactant over a wide range of concentrations above the CMC. However, some surfactants at high concentration show an increase in solubilization capacity (Rosen 1999).

The inclusion of hydrophobic molecules in the interior of the micelles causes a reduction in the free energy of the system, which ultimately drives solubilization. This is because hydrophobic interactions between solvent molecules and the hydrophobic compound are reduced when the hydrophobic material is removed from the aqueous phase. Secondly, the micelles may assume a more optimal curvature that may not be

possible to be assumed in the absence of an included compound that stabilizes the micelle (Holmberg 2003).

3. Materials and Methods

3.1 Materials

All solutions were prepared with distilled and deionized water. Eugenol (4-allyl-2-methoxyphenol) and carvacrol (5-isopropyl-2-methyl-phenol) were purchased from Sigma Chemical Co. (St. Louis, MO). Nonionic surfactants Surfynol[®] 465 and 485W were provided by Air Products and Chemical, Inc. (Allentown, PA). The critical micellar concentration was 0.65 % (w/w) for Surfynol[®] 465 and 1.65 % (w/w) for Surfynol[®] 485W. pH was adjusted with 0.1N HCl or NaOH.

3.2 Preparation of carvacrol/eugenol containing surfactant micelles

Aqueous surfactant solutions were prepared by dispersing Surfynol[®] 465 and 485W in water at room temperature to obtain surfactant solutions with concentrations of 1, 2, 3.5, 5, 7.5 and 10 % (w/w). Eugenol and carvacrol were added to surfactant solutions at concentrations ranging from 0.025 % to 3 % (w/w). Solutions were stirred until the absorbance remained constant indicating that solubilization was complete (typically less than 10 minutes). Solutions were filter sterilized using a 0.22 μ m cellulose acetate membrane (Corning, Corning, NY) and stored at $25 \pm 2^{\circ}\text{C}$ until used but not longer than 2 weeks.

3.3 Maximum additive concentration (MAC)

The MAC was defined as the highest concentration of a lipophilic compound that could be incorporated into a micellar surfactant solution at a given surfactant concentration

(Weiss and McClements, 2000). It was determined by measuring the absorbance of surfactant solutions ranging in concentrations between 1 and 10 % at 632 nm using a uv-visible, spectrophotometer (Hewlett Packard 8452A Diode Array Spectrophotometer, Palo Alto, CA) after addition of essential oil components (0.025% to 3 %). The wavelength of 632 nm was chosen to avoid interference with intrinsic absorbance of eugenol and carvacrol.

3.4 Stability Measurements

Surfactant solutions were prepared as above. Solutions were adjusted to pHs ranging from 3 to 9 with NaOH or HCl. Surfactant solutions were heated from 25 to 85°C for Surfynol[®] 485W and from 25 to 70°C for Surfynol[®] 465 at 5 degree intervals. Solution turbidity for each temperature treatment was measured at 632 nm using a ultraviolet-visible, spectrophotometer (HP 8452A).

3.5 Particle Size Determination

Surfactant solutions were prepared as above. For these experiments, surfactant solutions without essential oil components and surfactant solutions that contained specific concentrations of EO components were selected and the particle size of empty and swollen micelles measured using a dynamic light scattering particle sizer ZetaPALS, with particle size software (Brookhaven Instruments, Co., Holtsville, NY) at a 90° scattering angle and at a wavelength of 632nm.

3.6 Nuclear Magnetic Resonance Spectroscopy

The 1D proton nuclear magnetic resonance experiments were performed on a Varian Mercury 300 Spectrometer (Palo Alto, CA) with proton resonance at 300 MHz. The spectra were acquired in regular one pulse sequences with a 30 degree proton pulse and a 3s relaxation delay at room temperature. The samples were prepared by dispersing 5 % (w/w) Surfydol[®] 465 and 485W in deuterium oxide at room temperature. Eugenol and carvacrol were added to surfactant solutions at concentrations of 0.5 % (w/w) for Surfydol[®] 465 and 0.6 % (w/w) for Surfydol[®] 485W.

3.7 Bacteria and growth conditions

Growth inhibition against four different *Listeria monocytogenes* strains (Scott A, 101, 108, 310) and four *Escherichia coli* O157:H7 (H1730, F4546, 932, E0019) strains was investigated. The source of the cultures was the University of Tennessee Department of Food Science and Technology culture collection. Bacterial cultures were maintained on tryptic soy agar (Difco, Sparks, MD) slants at 4°C. A loopful of the culture was transferred to tryptic soy broth (TSB) (Difco), and incubated at 32°C for 24 hr. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 18 hr.

For the first experiment a microbroth dilution assay (Parish and Davidson, 1993) was used to determine the minimum inhibitory concentration of combinations of essential oil and surfactants. Microtiter plate wells were filled with 120 µl of micellar solutions and 120 µl of inoculated double strength TSB ($\sim 5 \times 10^7$ CFU/ml). Plates were incubated at 32°C and optical density (OD) at 630 nm was monitored periodically over 24 hr using

an EL_x800 Universal Microplate Reader (Bio-Tex Instruments, Inc., Winooski, VT). Minimum inhibitory concentrations (MIC) were defined as the lowest concentration at which growth was completely inhibited after 24 hr. All samples were run in duplicate.

For the second experiment the same bacteria and growth condition were used with the exception that TSB was adjusted to a pH of 5, 6, and 7 using either NaOH or HCl (0.1N). A microbroth dilution assay (Parish and Davidson, 1983) was used to determine the growth inhibition of combination of 0.2, 0.5, and 0.9 % eugenol encapsulated in Surfynol[®] 485W at 5 %. All solutions were adjusted to a pH of 5, 6, and 7. Microtiter plate wells were filled with 120 µl of micellar solutions and 120 µl of inoculated double strength TSB ($\sim 5 \times 10^7$ CFU/ml). Plates were incubated at 32°C and 22°C and optical density (OD) at 630 nm was monitored periodically over 24 hr as above. Plates were also incubated at 10°C and optical density (OD) at 630nm was monitored every 24 hr for 168 hours as above. For this experiment TSB and solutions containing the encapsulated EO components were adjusted to a pH of 7 and tested. All samples were run in duplicate.

4 Growth Inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by Carvacrol and Eugenol Encapsulated in Surfactant Micelles

4.1 Abstract

Growth inhibition of four strains of *Escherichia coli* O157:H7 (H1730, F4546, 932, and E0019) and *Listeria monocytogenes* (Scott A, 101, 108, and 310) by essential oil (EO) components (carvacrol and eugenol) solubilized in nonionic surfactant micelles (Surfynol[®] 465 and 485W) was determined. Concentrations of EO components encapsulated varied between 0.02 and 1.25 % depending on compound, surfactant type and surfactant concentration (0.5 to 5 %). Eugenol encapsulated in Surfynol[®] 485W micelles was most efficient in inhibiting growth of the pathogens. 1 % of Surfynol[®] 485W and 0.15 % eugenol was sufficient to inhibit growth of all strains of *E. coli* O157:H7 and three of four strains of *L. monocytogenes* (Scott A, 310, and 108). The fourth strain of *L. monocytogenes*, 101, was inhibited by 2.5 % Surfynol[®] and 0.225 % eugenol. 1 % Surfynol[®] 485W in combination with 0.025 % carvacrol was effective in inhibiting three of four strains of *E. coli* O157:H7. Strain H1730 was the most resistant strain requiring 0.3 % of carvacrol and 5 % of surfactant for complete inhibition. Growth inhibition of *L. monocytogenes* by combinations of carvacrol and Surfynol[®] 465 varied between 0.15 and 0.35 % and 1 and 3.75 %, respectively. Generally, the antimicrobial activity of Surfynol[®] 465 in combination with eugenol was higher than the combination with carvacrol. The potent activity was attributed to increased solubility of essential oil

components in the aqueous phase due to the presence of surfactants and improved interactions of antimicrobials with microorganisms.

4.2 Introduction

Escherichia coli O157:H7 and *Listeria monocytogenes* are two of the major pathogens present in food systems. *Escherichia coli* O157:H7 is recognized as a major cause of hemorrhagic colitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Zottola and Smith, 1991; Kim *et al.*, 1995; Kim *et al.*, 2001). Foodborne illness outbreaks caused by *E. coli* O157:H7 have been associated with undercooked ground beef, unpasteurized milk, fruit juices and certain raw vegetables (Doyle. 1997, Zottola and Smith. 1991; Kim *et al.*,2001). In the case of *L. monocytogenes*, outbreaks have been linked to consumption of milk, soft cheeses, ready-to-eat meat products, fish, and vegetables (Aureli. 1990; Doyle 1997; Menon 2001).

Because of the need to find more effective and broad spectrum food antimicrobials, naturally occurring food antimicrobials, such as spice extracts, have gained increased attention as antimicrobial agents in food products. Spice extracts are natural compounds that have been classified as “generally recognized as safe” (GRAS) (Moleyar *et al.*, 1992; Smith-Palmer. 1998; Menon. *et al.*, 2001, Lambert, 2001). Spices and herbs have been in use for centuries to flavor and extend the shelf life of a wide variety of foods (Achinewhu *et al.*, 1995).

The antimicrobial activity of spices has been attributed to the presence of essential oil components (Shelef. 1983; Davidson and Naidu 2000). Essential oils are very potent

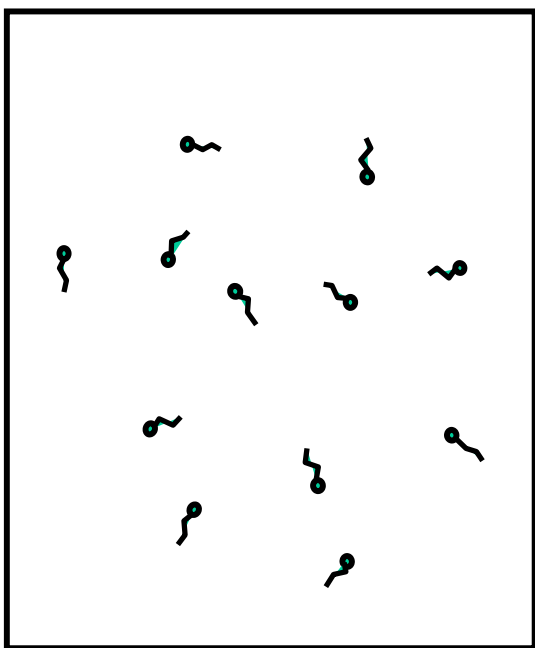
antimicrobial agents that have been recognized by many researchers to be effective inhibitors of various bacteria (Kim *et al.*, 1995; Baganboula, 2004; Ultee, Paster, Balzyk, Shelef). EO components also are effective inhibitors of fungal growth (Conner and Beuchat 1984; Inouye *et al.*, 2001; Sridhal 2003).

Essential oils are composed of a complex mixture of volatile, mostly non-polar components that may be obtained by distillation or solvent extraction from plants, herbs and spices. Approximately 5% of the mass of spices, herbs, and leafy vegetables consists of essential oils. Essential oils contain components such as alcohols, aldehydes, esters, ketones and phenolics. However, the majority of antimicrobial activity has been attributed to the presence of phenolics.

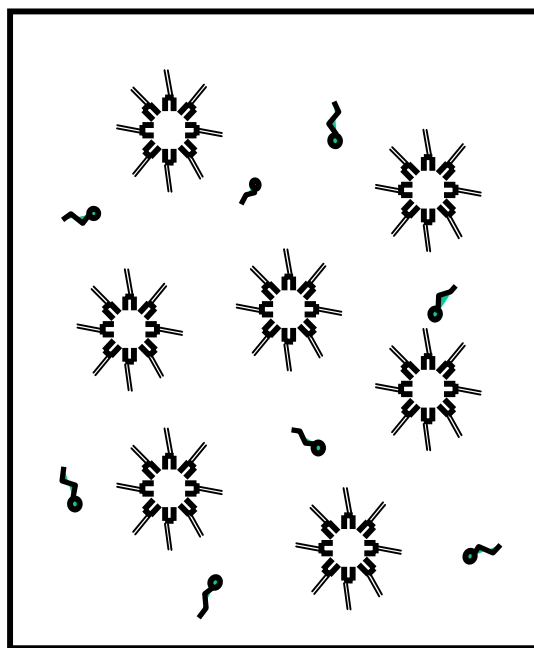
Many authors have reported on the efficacy of phytochemicals as antimicrobial agents, especially carvacrol from oregano and thyme and eugenol from clove, respectively (Aktuğ and Karapinar 1986; Deans and Ritchie 1987). Balzyk *et al.*, (1998) found that eugenol completely inhibited *L. monocytogenes* and *E. coli* O157:H7 growth in model microbial systems at greater than 1000 µg/ml. Significantly higher concentrations of the spice essential oils were required to inhibit the same pathogens in food systems (Bagamboula *et al.*, 2004). The addition of high concentrations of spice essential oils required to inhibit foodborne pathogens generally reduces the sensory acceptability of foods (Smith-Palmer *et al.*, 1998). This can be an important limitation for their use as antimicrobial agents. Therefore, the development of new methodologies that lead to a reduction in the use concentration but maintain antimicrobial activity of these compounds

is an important task for improving the balance between sensory properties and antimicrobial efficacy (Lambert *et al.*, 2001). Regardless of the nature of the target microorganisms, essential oils are always less effective when applied in food systems. This is because essential oils are mostly hydrophobic compounds and their use as preservatives is limited to food products that contain lipid or fat fractions in which the antimicrobials may be dispersed (Gill *et al.*, 2002, Bagamboula *et al.*, 2004). Since microorganisms reside primarily in the aqueous phase of the food system, the rate of mass transport of active compounds to the microorganisms is small and thought to be the reason for their low activity in foods. One possible solution to that limitation is to encapsulate these compounds in surfactant micelles. Surfactants are amphiphilic molecules that, above a specific concentration, or the so-called “critical micellar concentration” (CMC), self assemble to form complex structures with sizes in the tens to hundreds of nanometers (Figure 4.1). The surface of these micelles is hydrophilic while the interior of micelles is hydrophobic. As a consequence, micelles may be dispersed in water and contain in their interior lipophilic compounds such as essential oils.

The objective of this study was to determine the capacity of two nonionic micellar surfactant systems to encapsulate eugenol and carvacrol and allow solubilization in the aqueous phase and to determine the antimicrobial activity of the combined surfactant-essential oil component systems against two pathogenic bacteria, *Listeria monocytogenes* and *Escherichia coli* O157:H7.



A



B

Figure 4.1. Comparison of the arrangement of surfactant monomers below (A) and above (B) the critical micelle concentration.

4.3 Materials and Methods

4.3.1 Materials

All solutions were prepared with distilled and deionized water. Eugenol (4-allyl-2-methoxyphenol) and carvacrol (5-isopropyl-2-methyl-phenol) were purchased from Sigma Chemical Co. (St. Louis, MO). Nonionic surfactants Surfynol[®] 465 and 485W were provided by Air Products and Chemical, Inc. (Allentown, PA). The critical micellar concentration was 0.65 % (w/w) for Surfynol[®] 465 and 1.65 % (w/w) for Surfynol[®] 485W. pH was adjusted with 0.1N HCl or NaOH.

4.3.2 Preparation of carvacrol/eugenol containing surfactant micelles

Aqueous surfactant solutions were prepared by dispersing Surfynol[®] 465 and 485W in water at room temperature to obtain surfactant solutions with concentrations of 1, 2, 3.5, 5, 7.5 and 10 % (w/w). Eugenol and carvacrol were added to surfactant solutions at concentrations ranging from 0.025 % to 3 % (w/w). Solutions were stirred until the absorbance remained constant indicating that solubilization was complete (typically less than 10 minutes). Solutions were filter sterilized using a 0.22 μ m cellulose acetate membrane (Corning, Corning, NY) and stored at $25 \pm 2^{\circ}\text{C}$ until used but not longer than 2 weeks.

4.3.3 Maximum additive concentration (MAC)

The MAC was defined as the highest concentration of a lipophilic compound that could be incorporated into a micellar surfactant solution at a given surfactant concentration (Weiss and McClements, 2000). It was determined by measuring the absorbance of

surfactant solutions ranging in concentrations between 1 and 10 % at 632 nm using a uv-visible, spectrophotometer (Hewlett Packard 8452A Diode Array Spectrophotometer, Palo Alto, CA) after addition of essential oil components (0.025% to 3 %). The wavelength of 632 nm was chosen to avoid interference with intrinsic absorbance of eugenol and carvacrol.

4.3.4 Bacteria and growth conditions

Growth inhibition against four different *Listeria monocytogenes* strains (Scott A, 101, 108, 310) and four *Escherichia coli* O157:H7 (H1730, F4546, 932, E0019) strains was investigated. The source of the cultures was the University of Tennessee Department of Food Science and Technology culture collection. Bacterial cultures were maintained on tryptic soy agar (Difco, Sparks, MD) slants at 4°C. A loopful of the culture was transferred to tryptic soy broth (TSB) (Difco), and incubated at 32°C for 24 hr. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 18 hr. A microbroth dilution assay (Parish and Davidson, 1993) was used to determine the minimum inhibitory concentration of combinations of essential oil and surfactants. Microtiter plate wells were filled with 120 µl of micellar solutions and 120 µl of inoculated double strength TSB ($\sim 5 \times 10^7$ CFU/ml). Plates were incubated at 32°C and optical density (OD) at 630 nm was monitored periodically over 24 hr using an Elx800 Universal Microplate Reader (Bio-Tex Instruments, Inc., Winooski, VT). Minimum inhibitory concentrations (MIC) were defined as the lowest concentration at which growth was completely inhibited after 24 hr. All samples were run in duplicate.

4.3.5 Statistical Analysis

Tukey's multiple range test was used to determine significant differences ($P < 0.05$) between treatments. Least squares means were analyzed using the general linear model of the Statistical Analysis System (SAS Institute, Cary, NC).

4.4 Results

4.4.1 Maximum additive concentration (MAC) of eugenol and carvacrol in Surfynol[®] 465 and 485W.

Figure 4.2-A shows typical results obtained from the spectrophotometric analysis of the titration of surfactant solutions with essential oil components. Increasing concentrations of carvacrol were titrated into Surfynol[®] 465 solutions at surfactant concentrations ranging from 1 to 10 %. Absorbance remained zero as carvacrol was titrated into the solution until a critical concentration was reached. Absorbance then increased significantly, indicating that micelles were fully saturated and were no longer able to take up more carvacrol. The concentration at which the turbidity increases is referred to as the "maximum additive concentration" (MAC). For example, at 1% Surfynol[®] 465, the absorbance increased from zero to above 4 at 0.04 % of carvacrol. Therefore, the MAC for Surfynol[®] 465 at 1 % surfactant titrated with carvacrol was 0.04 %. In contrast, solutions of Surfynol[®] 465 at 10 % were able to incorporate as much as 3 % carvacrol, more than 70 times the amount at 1 %.

Figure 4.2-B shows a plot of the MAC as a function of the essential oil component concentrations for the four combinations used in this study. For all surfactant-essential oil component combinations, the MAC increased with increasing emulsifier concentration.

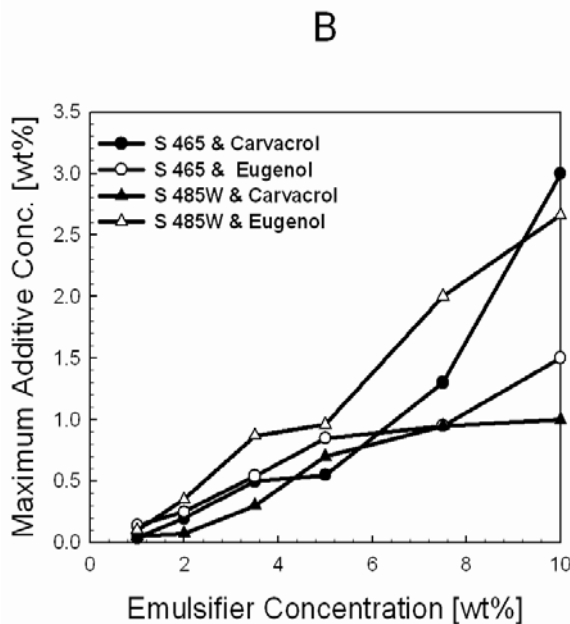
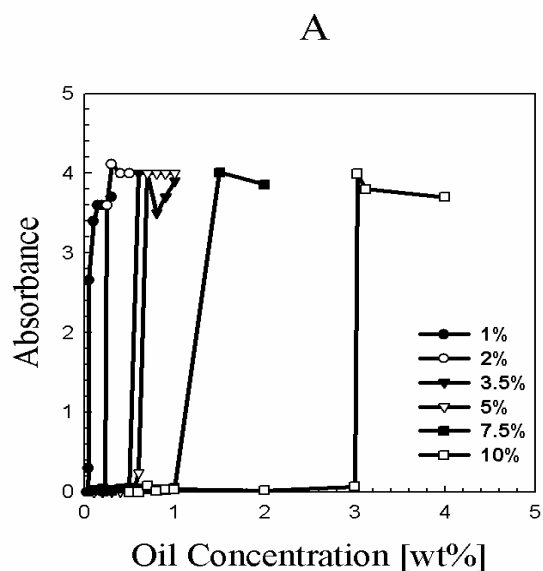


Figure 4.2 Maximum amount of essential oil that could be incorporated into the surfactant micelles. (A) Different carvacrol concentrations solubilized in Surfydol[®] 465 as measured by the increase in absorbance at 632nm (B) Comparison of the maximum amount that could be incorporated into the surfactant-base micelles of the four systems used (Surfydol[®] 465 with the eugenol or carvacrol and Surfydol[®] 485W with eugenol or carvacrol).

As shown for Surfynol[®] 465 and carvacrol (Figure 4.2-A), as long as the concentration of the carvacrol or eugenol added to the surfactant solutions was smaller than the MAC, the turbidity of the solution remained zero indicating that all of the compound was incorporated by the micelles and none remained in the surrounding aqueous phase. Conversely, if the essential oil components were added at concentrations above the MAC, solutions became turbid indicating that micelles were saturated and fractions of the compounds remained dispersed in the aqueous phase in the form of droplets that eventually phase separated. Figure 4.2 indicates that the maximum uptake of essential oil depended on the type of the surfactant. The largest concentration of uptake was observed with Surfynol[®] 485W and eugenol, while the lowest was with Surfynol[®] 485W and carvacrol. All subsequent antimicrobial experiments were conducted with carvacrol or eugenol below the MAC

4.4.2 Minimum inhibitory concentration (MIC) of surfactant-essential oil component combinations.

Table 4.1 shows the MICs (defined as the lowest concentration where growth was completely inhibited ($OD \leq 0.07$ for > 24 hr)) for the different surfactant-essential oil component combinations against *L. monocytogenes* and *E. coli* O157:H7 strains. In presence of Surfynol[®] 485W, *Listeria monocytogenes* generally required higher concentrations of essential oil component for inhibition than *E. coli* O157:H7, while in the presence of Surfynol[®] 465, higher concentrations were required to inhibit *E. coli* O157:H7. Generally, Surfynol[®] 485W and eugenol were the most efficient system against both pathogens tested. Inhibition of the microorganisms was strain dependent. For

Table 4.1 Minimum inhibitory concentrations of each surfactant-phytoperol system against strains of *Listeria monocytogenes* or *Escherichia coli* O157:H7 after 24 hr at 32°C.

Surfynol [®] 485W			Surfynol [®] 465		Surfynol [®] 485W		Surfynol [®] 465	
+ Carvacrol			+ Carvacrol		+ Eugenol		+ Eugenol	
S485W	Carv		S465	Carv	S485W	Eug	S465	Eug
(% w/w)	(% w/w)		(% w/w)	(% w/w)	(% w/w)	(% w/w)	(% w/w)	(% w/w)
<i>L. monocytogenes</i>								
Scott A	1	0.025	3.75	0.275	1	0.15	1.75	0.1
310	5*	0.35*	3.75	0.275	1	0.15	2.5*	0.3*
101	3.75	0.15	2.5	0.15	2.5*	0.225*	1.75	0.1
108	1	0.35	5*	0.275*	1	0.15	2.5	0.1
<i>E. coli</i> O157:H7								
H1730	5*	0.3*	5	0.3	1	0.15	5*	0.5*
E0019	1	0.025	5	0.3	1	0.15	1.75	0.15
F4546	1	0.025	5	0.3	1	0.15	3.75	0.2
932	1	0.025	5	0.3	1.75*	0.15*	1	0.1

*Concentrations at which the most resistant strains show inhibition, SF = Surfactant Concentration; Carv = Carvacrol; Eug = Eugenol.

example, 2.5 % Surfynol[®] 485W and 0.225 % eugenol were required to inhibit *L. monocytogenes* strain 101 whereas strains 310, Scott A, and 108 were inhibited by 0.1 % and 0.15 %, respectively (Table 4.1). With Surfynol[®] 485W and 465, the maximum concentration of eugenol required for inhibition of all strains of both microorganisms was 0.225 and 0.5 %, respectively. For the same surfactants with carvacrol, the highest concentration for complete inhibition of all strains was 0.35 and 0.3 %, respectively.

4.4.3 Growth kinetics of pathogens in the presence of essential oil component–surfactant combinations.

The growth kinetics of *E. coli* O157:H7 and *Listeria monocytogenes* in the presence of the surfactants alone is shown in Fig. 4.3. The growth kinetics of the most sensitive strain of *E. coli* O157:H7, strain H1730 (Fig. 4.3A and 4.3C), and *L. monocytogenes*, strain Scott A (Fig. 4.3B and 4.3D), indicated that Surfynol[®] 465 (Fig. 4.3A and 4.3B) had greater antimicrobial activity than Surfynol[®] 485W (Fig. 4.3C and 4.3D). In all cases, the surfactant did allow growth and increases in concentration above 1% did not completely inhibit either microorganism.

The growth of *Escherichia coli* O157:H7 strains in the presence of surfactant encapsulated essential oil components are shown in Fig. 4.4 – 4.5. The most resistant of the strains are shown in Fig. 4.4 and the least resistant strains in Fig. 4.5. 5 % surfactant was evaluated with a range of concentrations of carvacrol or eugenol (0.1 to 0.8 %). Surfynol[®] 485W with eugenol was most effective against the most resistant strain, H1730 (Fig. 4.4D), causing total inhibition at approximately 0.3% eugenol. The least effective

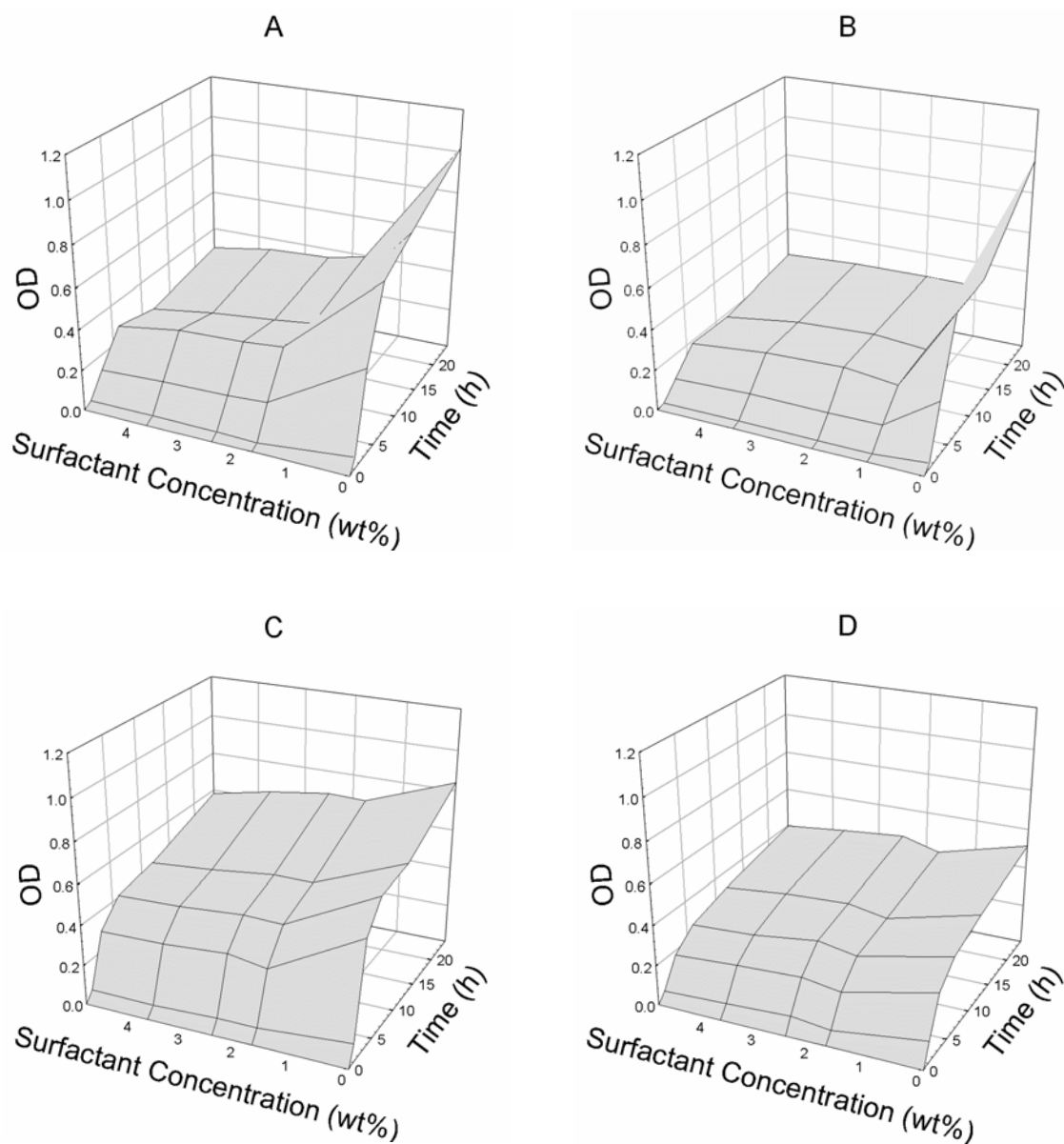


Figure 4.3. Growth of *E. coli* O157:H7 strain H1730 and *L. monocytogenes* strain Scott A in the presence of surfactant without essential oil at pH of 7 for 24 hours. (A) *E. coli* O157:H7 H1730 in the presence of Surfyinol[®] 465 (B) *L. monocytogenes* Scott A in the presence of Surfyinol[®] 465 (C) *E. coli* O157:H7 H1730 in the presence of Surfyinol[®] 485W (D) *L. monocytogenes* Scott A in the presence of Surfyinol[®] 485W.

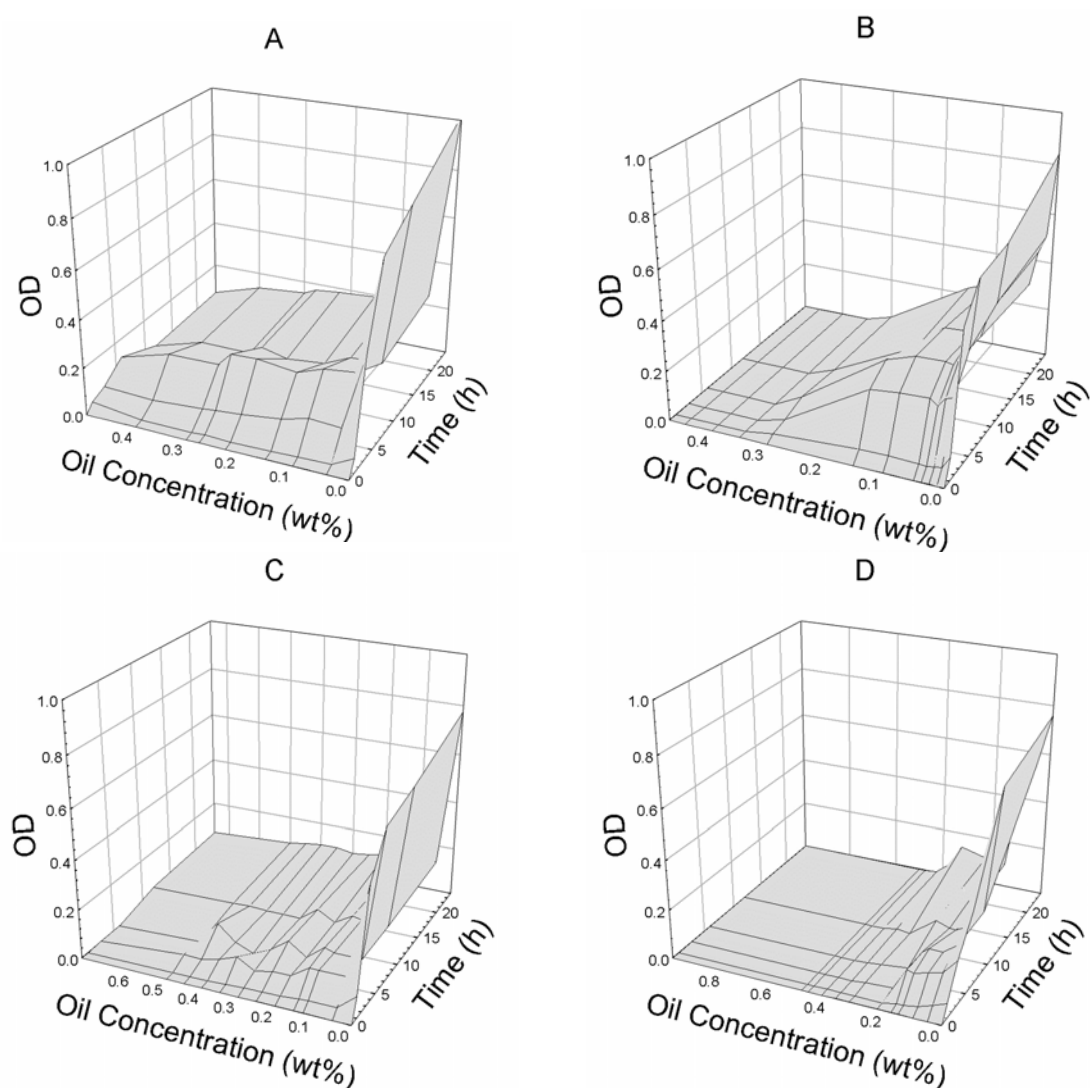


Figure 4.4. Growth of the most resistant strains of *E. coli* O157:H7 as measured by OD₆₃₀ to surfactant (5%) with eugenol or carvacrol at pH of 7 for 24 hr. (A) *E. coli* O157:H7 H1730 in the presence of Surfynol[®] 465 with carvacrol (B) *E. coli* O157:H7 H1730 in the presence of Surfynol[®] 485W with carvacrol (C) *E. coli* O157:H7 H1730 in the presence of Surfynol[®] 465 with eugenol (D) *E. coli* O157:H7 932 in the presence of Surfynol[®] 485W with eugenol.

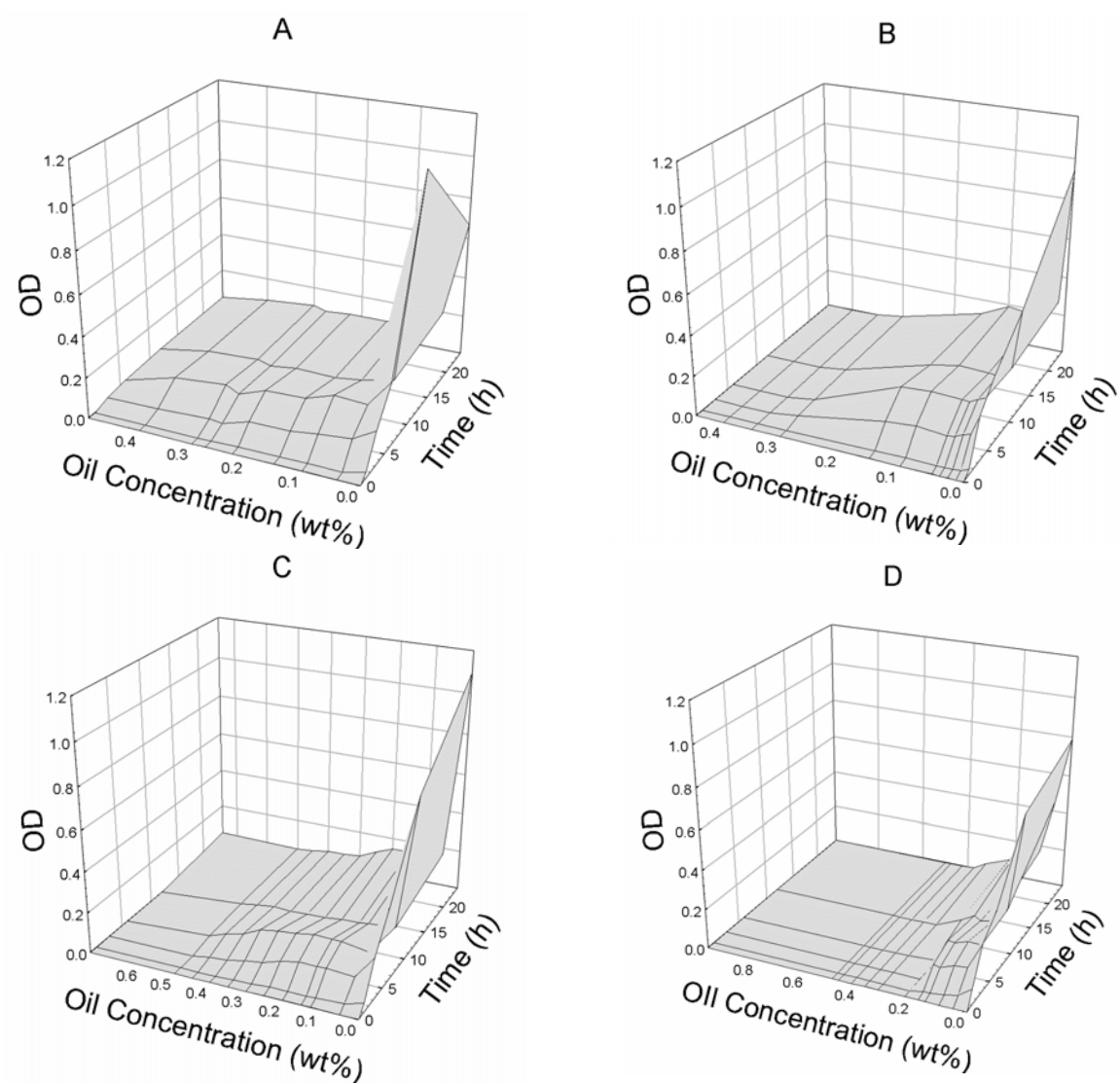


Figure 4.5 Growth of the least resistant strains of *E. coli* O157:H7 as measured by OD₆₃₀ to surfactant (5%) with eugenol or carvacrol at pH of 7 for 24 hr.. (A) *E. coli* O157:H7 E0019 in the presence of Surfynol[®] 465 with carvacrol (B) *E. coli* O157:H7 F4546 in the presence of Surfynol[®] 485W with carvacrol (C) *E. coli* O157:H7 932 in the presence of Surfynol[®] 465 with eugenol (D) *E. coli* O157:H7 F4546 in the presence of Surfynol[®] 485W with eugenol.

combinations were Surfynol[®] 465 and 485W with carvacrol (Fig. 4.4A, 4.4B, 4.5A, and 4.5B). However, all emulsifier-essential oil component combination was inhibitory to the four strains of *E. coli* O157:H7.

The growth of *Listeria monocytogenes* strains in the presence of surfactant encapsulated essential oil components is shown in Fig. 4.6 - 4.7. The most resistant strains to the combinations are shown in Fig. 4.5 and the least resistant strains in Fig. 4.7. Surfactant (5 %) was evaluated with a range of concentrations of carvacrol or eugenol (0.1 to 0.8 %). The most effective system against all strains was Surfynol[®] 485W with eugenol (Fig. 4.6D, 4.7D), Even against the most resistant strains, total inhibition was demonstrated at 0.3-0.4% eugenol. Surfynol[®] 465 with eugenol also demonstrated inhibition against the most resistant strain of *L. monocytogenes*, 310, at > 0.5%. Surfynol[®] 465 and 485W with carvacrol were much less effective against the most resistant strains, 108 and 310, respectively. The least resistant strain overall was Scott A.

4.5 Discussion

Because of their high solubilization capacity, Surfynol[®] 465 and 485W were used to produce micelles. Surfynol[®] 485W could incorporate up to 2.66 % of essential oil component at 10 % and at 5 % up to 0.96 % (Fig. 4.2B). Other surfactants have different MACs depending on the structure of the surfactant, size and shapes of the micelles, and the type of oil used. Weiss (1999) investigated the different MACs with Tween 20, 40, 60, 80 and Triton X-100, Triton SP-190 and Triton SP-175 incorporating

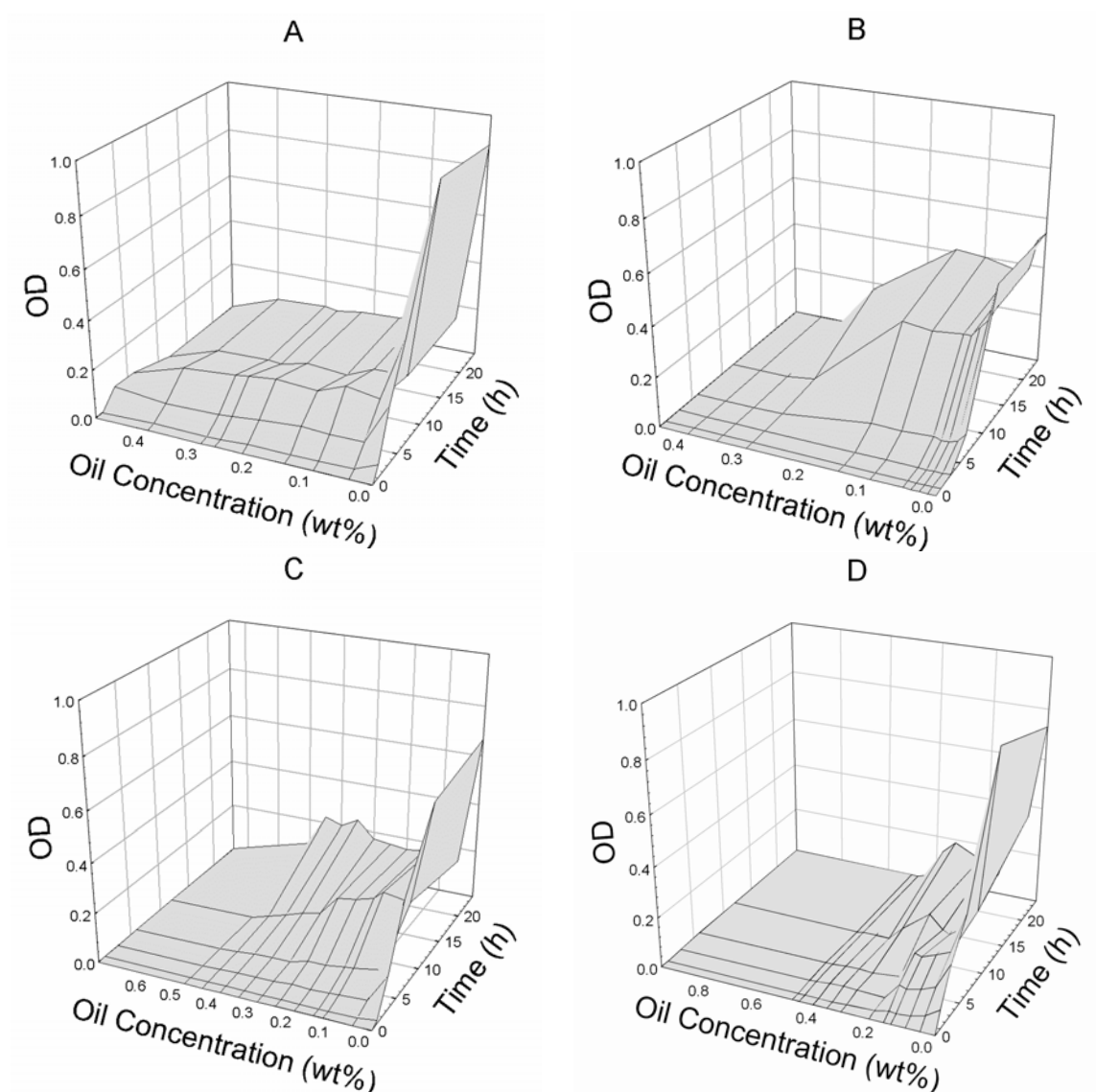


Figure 4.6 Growth of the most resistant strains of *L. monocytogenes* as measured by OD₆₃₀ in presence surfactant (5%) and carvacrol or eugenol at pH of 7 for 24 hr. (A) *L. monocytogenes* 108 in presence of Surfynol[®] 465 with carvacrol (B) *L. monocytogenes* 310 in presence of Surfynol[®] 485W with carvacrol (C) *L. monocytogenes* 310 in presence of Surfynol[®] 465 with eugenol (D) *L. monocytogenes* 101 in presence of Surfynol[®] 485W with eugenol.

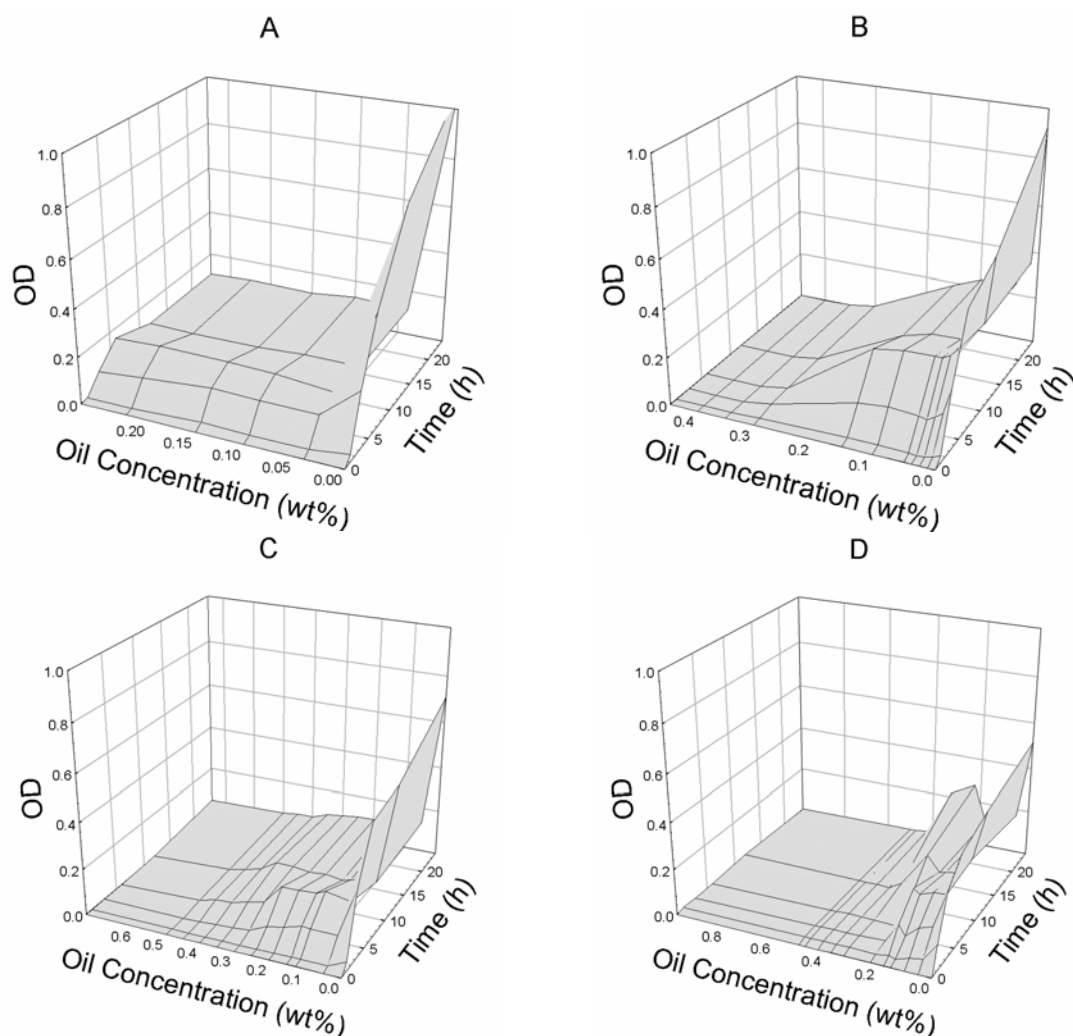


Figure 4.7 Growth of the least resistant strains of *L. monocytogenes* as measured by OD₆₃₀ in presence surfactant (5%) and carvacrol or eugenol at pH of 7 for 24 hr.. (A) *L. monocytogenes* 101 in presence of Surfynol[®] 465 with carvacrol (B) *L. monocytogenes* Scott A in presence of Surfynol[®] 485W with carvacrol (C) *L. monocytogenes* Scott A in presence of Surfynol[®] 465 with eugenol (D) *L. monocytogenes* Scott A in presence of Surfynol[®] 485W with eugenol.

n-tetradecane. Tween 20 solubilized only 1/10 of the amount of the oil compared to Triton SP-175. The maximum amount of essential oil that can be incorporated into the micelles depends on the surfactant structure, hydrophilic-lipophilic balance (HLB), chemical nature of the essential oil, and the interaction between them. In this study, turbidity decreased to zero within 10 minutes upon addition of carvacrol and eugenol to surfactant solutions indicating that nanoparticles rapidly incorporated the phytophenols compared to the slow solubilization kinetics of Tween 20, 40 and 60 that sometimes require days.

Carvacrol and eugenol are hydrophobic compounds that typically associate with lipid fractions in foods. Nevertheless, they possess a free hydroxyl group in their structure that imparts some polar characteristics. Because of this, eugenol and carvacrol have some solubility (< 0.01 mol/L in water (ACS, 2002)) in the aqueous phase, even in the absence of surfactants. However, concentrations of these compounds required to achieve significant inhibitory activity are substantially larger than the intrinsic solubility of eugenol or carvacrol in water. For use as an antimicrobial solution, food processors could choose to solubilize these phenolic compounds in ethanol or propylene glycol, but increasingly, the presence of even small concentrations of these compounds may pose regulatory hurdles. A second option would be to produce emulsions that contain carvacrol and eugenol. However, emulsions produced with carvacrol and eugenol rapidly break down due to Ostwald ripening, i.e., growth of larger oil droplets at the expense of smaller ones (Weiss, 1999). One of the best solutions to enhance the solubility of these compounds is, therefore, to solubilize them in surfactants micelles.

For an antimicrobial to be efficient it has to be available in the aqueous phase, in which the antimicrobial activity takes place. It has been shown that non-ionic surfactants such as Tween 80 may cause an increase in hydrophilicity of thymol and cellular membrane proteins causing an increased interaction between compounds in the aqueous phase and phytophenols (Juven *et al.*, 1993). It is hypothesized that solubilization of carvacrol and eugenol into micellar systems may improve the contact between the cell membrane and the phytophenols. In this case, the Surfynol[®] 465 and 485W may act as vehicles that transport the essential oils through the aqueous phase to the bacterial cell surfaces in addition to providing thermodynamic stability (Rosen, 1989).

The difference in solubilization capacity and antimicrobial activity can be attributed to the size and structure of the micellar capsules and their interaction with the essential oils components, carvacrol and eugenol. Essential oil components may be localized either in the interior of the micelle or near the surface depending on the interaction of the solubilized component with the surfactant head and tail groups. Ultee *et al.*, (2002) demonstrated that the free hydroxyl group is key to the antimicrobial properties of phenolic compounds such as carvacrol. Therefore availability of this group at the surface of the micellar nanocapsules would be beneficial. The size of micelles may also vary depending on the properties of the surfactant monomers. If the hydrophilic-lipophilic balance (HLB) of the amphiphilic molecules approaches to 7, the structure of the surfactant aggregates approaches that of a bilayer, i.e., the curvature is reduced and the size of the aggregates is increased. Consequently, more lipophilic material may be encapsulated in their interior.

Low solubility of essential oil appears to play an important role in their efficiency as antimicrobials in food systems. Some researchers have used nonionic surfactants (Tween 20, Tween 80) to produce emulsions or disperse the oil into the aqueous phase to study the inhibitory properties of essential oil components. For example, Juven *et al* (1994), prepared aqueous emulsions of thymol with Tween 80 at 125 – 1000 µg/ml (0.1%). In their study, Tween 80 up to 1000 µl did not cause any changes in viable counts of *Salmonella typhimurium* in absence of thymol under anaerobic conditions. However, in presence of the thymol, Tween 80 decreased the antimicrobial effect of the compound with increasing concentration of the Tween. It should be noted however, that emulsion droplets were incorporated in a solidified agar and therefore did not undergo rapid breakdown or phase separation. Similar results were found by Inouye *et al.*, (2001) who used 0.05% Tween 80 to prepare an emulsion to test the antimicrobial efficiency of essential oil components against molds. Again, addition of Tween 80 resulted in an increase in the MIC against *A. fumigatus* using broth dilution assays but no significant change in the MIC using agar dilution assays were found. Remmal *et al.*, (1993) also found that the MIC varied depending on the emulsifier used. Balzyk *et al.*, (1997) showed that at 1000 µg/ml of eugenol, growth of *E. coli* O157:H7 and *L. monocytogenes* was not observed, demonstrating the antimicrobial potency of this essential oil against these two pathogenic bacteria. Tween 20 was used at $\geq 0.25\%$ to disperse the compound in the aqueous phase. Some eugenol may have been incorporated into the surfactant micelles that were spontaneously formed by this surfactant but with the MIC of Tween 20 being very low, the eugenol concentrations were most likely $< 0.05\%$ (Weiss, 1999).

Clearly, there is a distinct difference between adding antimicrobials in the form of emulsion droplets and adding antimicrobials in the form of swollen micelles. It should also be noted that in a food system, emulsion droplets would inevitably cream and phase separate.

4.6 Conclusion

In summary, the system that showed most inhibition against *L. monocytogenes* and *E. coli* O157:H7 was Surfynol[®] 485W with eugenol. Inhibition was highly strain dependent. The activity of the encapsulated essential oil was found to be dependent on the type of surfactant used as a carrier. Nanocapsules made from Surfynol[®] 485W were generally more inhibitory than micelles formed with Surfynol[®] 465. The surfactant may play an important role in the interaction between the essential oil and the bacterial cell membrane, possibly allowing the essential oil to have better access the membrane. Eugenol and carvacrol, being phenolic compounds, interfere with the essential functions of the cytoplasmic membrane, such as proton motive force and active transport (Davidson, 1993). In addition, while it is well known that phenolic compounds are generally more effective against Gram-positive than Gram-negative bacteria (Shelef 1983, Narasimharm, 1992, Blazyk et al, 1998), our study shows that nanoencapsulated micellar phenolic compounds are nearly equally effective against both Gram-negative and Gram-positive bacteria. Overall, the encapsulation of essential oil components in surfactant micelles is a promising new method to potentially overcoming the limited antimicrobial activity of these compounds in aqueous systems. Obviously, these systems

must be evaluated in food systems prior to concluding that they would have success in this milieu.

5. Stability and Antimicrobial Efficiency of Eugenol Encapsulated in Surfactant Micelles as Affected by Temperature and pH

5.1 Abstract

Growth inhibition of four strains of *Escherichia coli* O157:H7 (H1730, F4546, 932, and E0019) and *Listeria monocytogenes* (Scott A, 101, 108, and 310) by eugenol solubilized in micellar nonionic surfactant solutions (Surfynol[®] 485W) adjusted to pH 5, 6 and 7 and incubated at 10, 22 and 32°C was determined. Concentrations of eugenol ranged from 0.2 to 0.9 % at a surfactant concentration of 5 %. Antimicrobial activity was assessed using a microbroth dilution assay. Eugenol encapsulated in surfactant micelles inhibited both microorganisms at pH 5, 6, and 7. At pH 5, some inhibition occurred in the absence of eugenol, i.e., by the surfactant itself (OD_{24hr} , *L. monocytogenes*=0.07 and OD_{24hr} , *E. coli* O157:H7=0.09), but addition of > 0.2 % eugenol led to complete inhibition of both microorganisms. Inhibition decreased with increasing pH, that is the MIC was 0.2, 0.5 and 0.5 % of micellar encapsulated eugenol solutions at pH 5, 6 and 7, respectively. The encapsulated essential oil component in surfactant micelles was effective at all three temperatures tested (10, 22, and 32°C) indicating that the activity of encapsulated eugenol was not affected by a shift to lower (refrigeration) or higher (incubation) temperatures. Overall, strains of *Escherichia coli* O157:H7 were more sensitive than strains of *Listeria monocytogenes*. Improved activity was attributed to increased solubility of eugenol in the

aqueous phase due to the presence of surfactants and improved interactions of antimicrobials with microorganisms.

5.3 Introduction

Listeria monocytogenes and *Escherichia coli* O157:H7 are an important cause of foodborne illnesses throughout the World. The US Center for Disease Control and Prevention reported that approximately 76 million illnesses occur each year in the United States attribute to foodborne pathogens (CDC 2004). A reduction of foodborne illness outbreaks through controlling the growth of foodborne pathogens is of key interest to the food industry to assure consumers a safe food supply. Interest in the development of new methods to reduce or eliminate foodborne pathogens is consequently increasing (Kim et al., 1995, Burt 2004). Because of the need to find more effective food antimicrobials that have a broad-spectrum activity and difficulties with gaining regulatory approval for synthesized antimicrobials, naturally occurring food antimicrobials, such as spice extracts, have gained increased attention for application in food products (Brul and Coote 1999; López-Malo et al., 2000).

The antimicrobial activity of spices and herbs has been attributed to the presence of active components in the essential oil extracts of these plants (Shelef. 1983; Davidson and Naidu 2). Several researchers have shown that EO components inhibit the growth of pathogenic bacteria (Shelef 1983; Karapinar and Aktuğ 1987; Kim et al., 1995; Baganboula et al., 2004; Ultee 1998; Paster et al., 1990; Balzyk and Holley 1998; Kim et al., 2004). Many of these essential oil components are phenolic compounds such as carvacrol and eugenol (López-Malo 2000).

A general mode of action for phenolic compounds has been proposed that involves insertion of the antimicrobial in the cytoplasmic membrane followed by a disruption of membrane integrity and proton motive force and possibly eventual coagulation of cell contents (Juven et al., 1994; Davidson 1997; Ultee et al., 2002; Burt 2004). Differences in susceptibility to antimicrobial agents have been found between Gram-positive and Gram-negative bacteria. This has been attributed to the fact that a rigid wall of peptidoglycan surrounds Gram-positive bacteria while Gram-negative bacteria have the outer membrane in addition to the cell wall (Nikaido and Vaara 1985; Pagán et al., 1997). Because of the outer membrane, Gram-negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs (Nikaido and Vaara 1992; Nikaido 1996; Helander et al., 1998). However, the outer membrane is not completely hydrophobic and some compounds may pass through the membrane through porins (Plésiat and Vaara 1985; Nikaido 1995; Helander 1998).

Growth inhibition of pathogens by antimicrobials depends on environmental conditions such as temperature, pH and ionic strength, factors that generally influence the growth of microorganisms. These factors are interdependent. For example microbial thermotolerance, i.e. the ability to grow at or survive elevated temperatures, can increase after microorganisms have been stressed by other factors such as acid shock, or presence of salts or other compounds, such as antimicrobials (Pagán et al., 1997). This is because bacteria have the ability to adapt to these conditions by producing stress compounds and modifying their lipid composition which allows them to maintain vital biological membrane activity (Pagán et al., 1997; Li et al., 2002). Ideally, effectiveness of new

antimicrobials should therefore be evaluated for key target organisms under different growth conditions such as varying pH and temperatures. This is particularly important for *L. monocytogenes*, a pathogen that is able to grow under a wide range of temperature conditions, -0.4 to 50°C (Walker and Stringer 1987; Junttila et al., 1988; Farber and Peterkin 1991).

We previously demonstrated that encapsulation of essential oil components (eugenol and carvacrol) in surfactant micelles (Surfynol[®] 465 and 485W) increased their inhibitory effect against *E. coli* O157:H7 and *L. monocytogenes* at pH 7 and 22°C. The objective of this study was to determine the stability and antimicrobial activity of the most efficient system, eugenol encapsulated in Surfynol[®] 485W, at different pHs and incubation temperatures against two pathogenic bacteria; *Listeria monocytogenes* and *Escherichia coli* O157:H7.

5.3 Materials and Methods

5.3.1 Materials

All solutions were prepared with distilled and deionized water. Eugenol (4-allyl-2-methoxyphenol) and carvacrol (5-isopropyl-2-methyl-phenol) were purchased from Sigma Chemical Co. (St. Louis, MO). Nonionic surfactants Surfynol[®] 465 and 485W were provided by Air Products and Chemical, Inc. (Allentown, PA). The critical micellar concentration was 0.65 % (w/w) for Surfynol[®] 465 and 1.65 % (w/w) for Surfynol[®] 485W. pH was adjusted with 0.1N HCl or NaOH.

5.3.2 Preparation of eugenol containing Surfynol[®] 485W micelles

Aqueous surfactant solutions were prepared by dispersing Surfynol[®] 465 and 485W in water at room temperature to obtain surfactant solutions with concentrations of 1, 2, 3.5, 5, 7.5 and 10 % (w/w). Eugenol and carvacrol were added to surfactant solutions at concentrations ranging from 0.025 % to 3 % (w/w). Solutions were stirred until the absorbance remained constant indicating that solubilization was complete (typically less than 10 minutes). Solutions were filter sterilized using a 0.22 μ m cellulose acetate membrane (Corning, Corning, NY) and stored at $25 \pm 2^{\circ}\text{C}$ until used but not longer than 2 weeks.

5.3.3 Temperature and pH stability of eugenol containing micelles

The stability of micellar encapsulated eugenol as a function of pH and temperature was determined by measuring the absorbance of a variety of surfactant-antimicrobial combination (5 % Surfynol[®] 485W and 0.1-0.9 % eugenol) that were adjusted to pH 3 to 9 using HCl and NaOH as a function of temperature (25–90 $^{\circ}\text{C}$) at a wavelength of 632 nm using a UV-visible spectrophotometer (Hewlett Packard 8452A Diode Array Spectrophotometer) The wavelength of 632 nm was chosen to avoid interference with the intrinsic absorbance of eugenol.

5.3.4 Bacteria and growth conditions

Growth inhibition against four different *L. monocytogenes* strains (Scott A, 101, 108, 310) and four strains of *E. coli* O157:H7 (H1730, F4546, 932, E0019) was investigated. Bacterial cultures were maintained on slants stored at 4 $^{\circ}\text{C}$. A loopful of the culture was transferred to tryptic soy broth (TSB) (Difco Laboratories, Sparks, MD), and incubated at

32°C for 24 hours. Prior to exposure to antimicrobials, each strain was sub-cultured in TSB for 18 hours. A microbroth dilution assay (Parish and Davidson, 1993) was used to determine the minimum inhibitory concentration of eugenol and Surfynol[®] 485W at pH 5, 6 and 7. Microtiter plate wells were filled with 240 µl of a 1:1 mixture of micellar solutions and inoculated double strength TSB ($\sim 5 \times 10^7$ CFU/ml) adjusted to pH 5, 6 and 7 using HCl. Plates were incubated at 10, 22 and 32°C. For temperatures of 22 and 32°C, the optical density (OD) at 630 nm was monitored periodically over 24 hr using an Elx800 Universal Microplate Reader (Bio-Tex Instruments, Inc., Winooski, VT). At 10 °C, optical density (OD) at 630 nm was read every 24 hours for 168 hours to account for the slower growth kinetics at the reduced temperature.

5.3.5 Statistical Analysis

Tukey's multiple range test was used to determine significant differences ($P < 0.05$) between treatments. Least squares means were analyzed using the general linear model of the Statistical Analysis System (SAS Institute, Cary, NC).

5.4 Results

5.4.1 Maximum additive concentration (MAC) of eugenol in Surfynol[®] 485W

The maximum additive concentration (MAC) is the largest amount of solute that can be incorporated into a micellar surfactant solution at a given surfactant concentration and is a key parameter for systems that contain swollen micelles because it governs the applicable concentration range. The MAC increased from 0.35 to 2.66 % as the surfactant concentration increased from 2 to 10 %. Absorbance remained zero as eugenol was titrated into the solution until the MAC was reached (data not shown). Absorbance

increased significantly above the MAC indicating that micelles were fully saturated and were no longer able to take up eugenol. Table 5.1 shows the MAC for eugenol in a solution containing Surfynol[®] 485W. Above the MAC, eugenol remained dispersed in the aqueous phase in the form of emulsion droplets that eventually phase separated. The MAC for Surfynol[®] 485W at 5 % surfactant, a typical surfactant concentration used in many emulsion applications, for eugenol was 0.96 %. Consequently, all subsequent antimicrobial experiments were conducted at a Surfynol[®] 485W concentration of 5% and eugenol concentrations below the MAC (0.96 %) to prevent phase separation and ensure testing of partially and fully filled micelles.

5.4.2 Temperature and pH stability of encapsulated surfactant micelles

Stability of micelles prepared with 5 % Surfynol[®] 485W and eugenol concentrations ranging from 0 to 0.9 % was evaluated. Thermal and pH stability was determined by measuring the absorbance of surfactant Surfynol[®] 485W solutions in the absence (0 %) and presence of eugenol (0.1 – 0.9 %) at temperature ranging from 25 to 90°C. Figure 5.1A shows the results of such experiments at pH 7. The absorbance of solutions remained zero until a critical temperature was reached, at which the absorbance rapidly increased. The critical temperature indicated the so-called “cloud point,” i.e, the point where micelles phase invert and release the encapsulated compound because of increased dehydration of the polar head group of the surfactant (Rosen1989; Toerne 2001). The cloud point decreased with increasing eugenol concentration. For example, at a eugenol concentration of 0.1 %, micelles remained stable even if solutions were heated to 90 °C

Table 5.1 Maximum amount of essential oil component that could be incorporated into the surfactant micelles prepared with Surfynol[®] 485W with eugenol

<i>Surfactant Concentration</i> [%]	<i>Surfynol[®] 485W with Eugenol</i> [%]
2	0.35
3.5	0.87
5	0.96
7.5	2
10	2.66

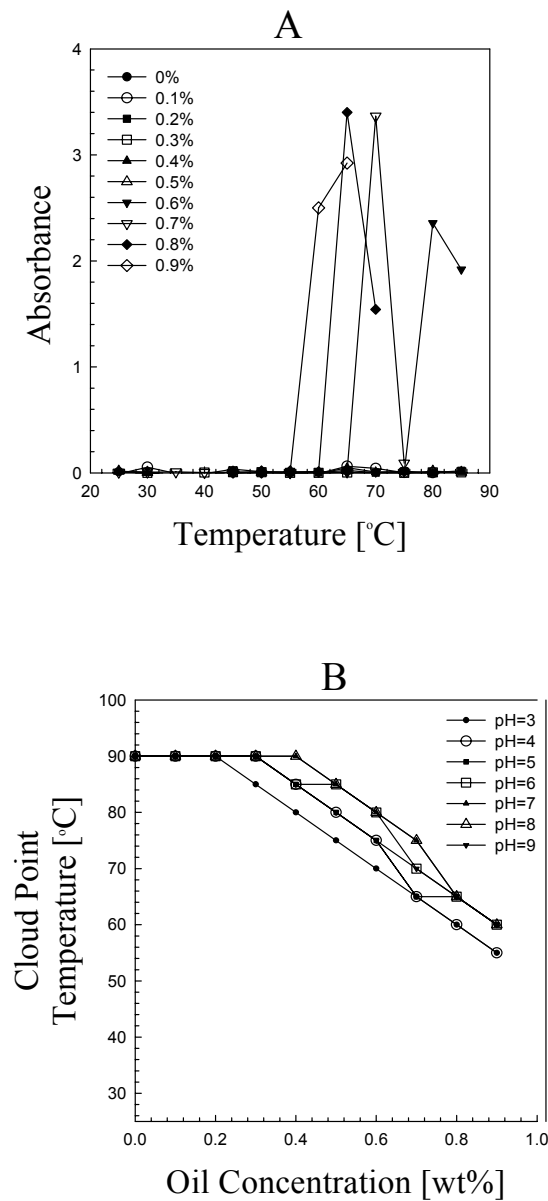


Figure 5.1 Stability Surfylnol[®] 485W and eugenol nanoparticles at pH ranging from 3 to 9 and temperatures ranging from 25 to 90°C at eugenol concentrations of 0 to 0.9 %. to the (A) Absorbance of a 5 % Surfylnol[®] solution titrated with 0 to 0.9 % eugenol as a function of temperature at pH 7. (B) Cloud point (temperature where solution turbidity increases above zero) as a function of pH and temperature for Surfylnol[®] solutions containing 0 to 0.9 % eugenol.

In contrast at a eugenol concentration of 0.9 %, the cloud point decreased to 55°C (Figure 5.1B). Results indicate that the thermal stability of micelles decreased with concentration of antimicrobial.

Similarly, the thermal stability of eugenol-containing Surfynol® 485W micelles at different pH was evaluated (Figure 5.1B). Variation in pH did not affect the thermal stability of micelles. At pH 3, a small (5°C) decrease in the overall thermal stability of the surfactants was observed, but compared to the decline of thermal stability with increasing essential oil component concentration, the decrease was not significant.

5.4.3 Influence of pH on growth kinetics of *Listeria monocytogenes* in the presence and absence of eugenol at 22 and 32°C

The growth of the most resistant strains of *L. monocytogenes* (Scott A) at pH 5, 6 and 7 at incubation temperatures of 22 and 32 °C in the presence and absence of eugenol-Surfynol® combinations is shown in Figure 5.2. The presence of the surfactant in the absence of eugenol (Surfynol®) did not affect the growth of *Listeria*. The optical density of both the control and the Surfynol® 485W solution increased from 0.02 (base optical density of media and antimicrobial preparation) to maximally 0.4 after 24 hr. Slightly reduced growth in the presence of surfactant was observed in the case of *L. monocytogenes* at pH 5 at 22 and 32 °C (Figure 5.2A and 5.2D). As expected growth in the absence of eugenol was both temperature and pH dependent. The optical density after

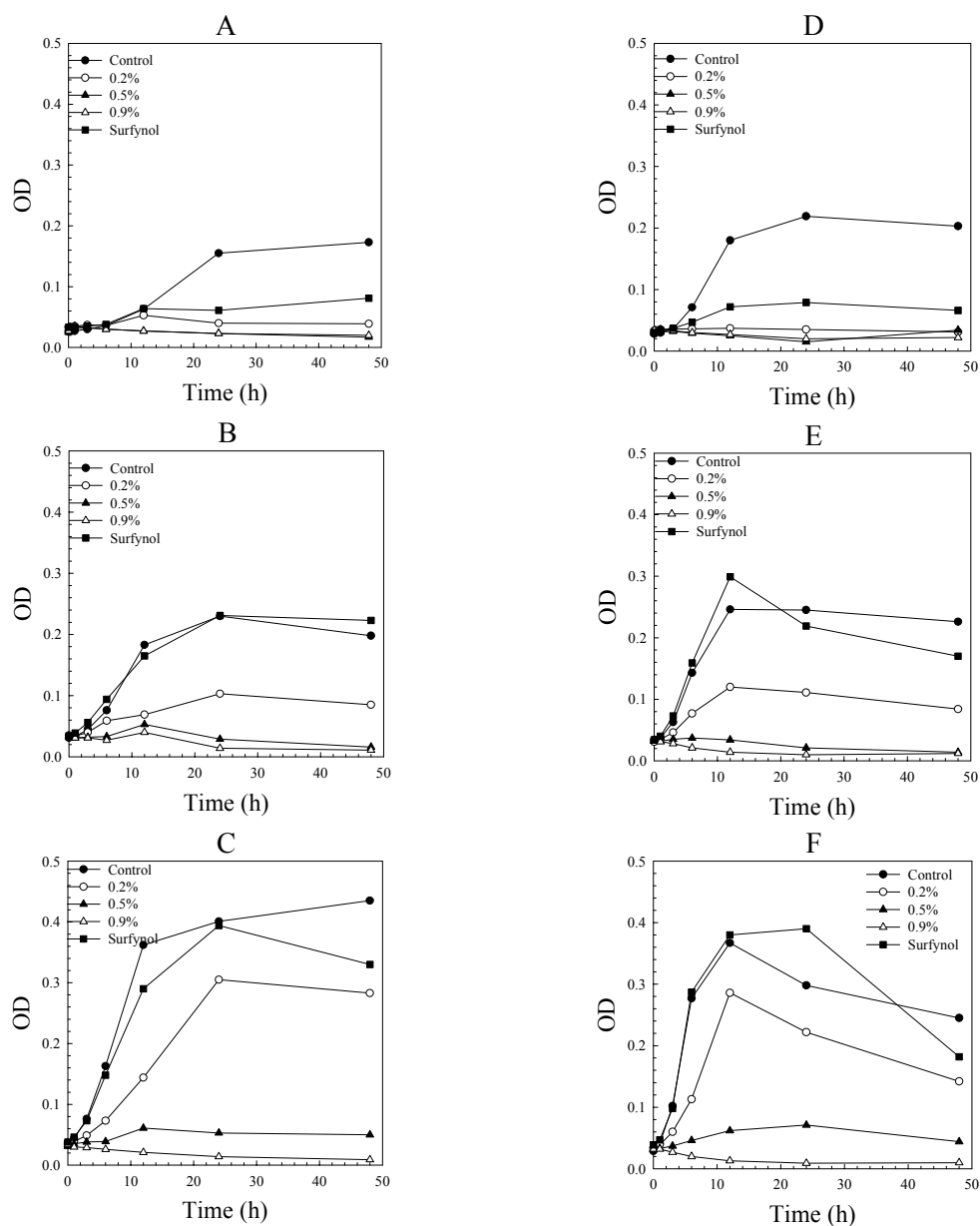


Figure 5.2 Growth of *Listeria monocytogenes* Scott A as measured by OD at 632 nm in the presence of 5 % Surfylnol® 485W and 0, 0.2, 0.5 and 0.9 % eugenol as a function of incubation time at (A) pH=5, 22°C, (B) pH=6, 22°C, (C) pH=7, 22°C, (D) pH=5, 32°C, (E) pH=6, 32°C, (F) pH=7, 32°C.

24 hr 22°C increased to 0.19, 0.24 and 0.4 at pH 5, 6 and 7, respectively, indicating that growth was slightly inhibited at lower pH (Figure 5.2 A,B,C). Growth at 32°C at pH 7 was more rapid than at 22 °C (Figure 5.2F). No significant difference in growth at pH 5 and 6 between 22 and 32°C was observed (Figures 5.2A, D and 5.2B, E). Similar results were observed for all other strains (data not shown).

Incorporation of eugenol in surfactant micelles strongly inhibited growth of *L. monocytogenes* (Figure 5.2) confirming the potent antimicrobial activity of the eugenol-Surfynol® 485W combination. Above a specific concentration (0.5 % eugenol), growth of *L. monocytogenes* was completely inhibited. Growth inhibition at 22°C with 0.2 % eugenol was pH dependent, i.e., the optical density after 24 hr increased from 0.02 to 0.03, 0.11 and 0.32 at pH 5, 6 and 7, respectively (Figure 5.2 A,B,C) and similar results were observed at 32°C (Figure 5.2 D,E,F). Using an MIC definition based on a minimum of optical density increase of 0.05 between 0 and 24 hr, this suggests that the MIC decreased from 0.5 to 0.2 upon a reduction of pH to 5 regardless of incubation temperature (Figure 5.2A and 5.2D). Less resistant strains were inhibited by 0.2 % of eugenol at pH 6 and 7 as well (data not shown), that is no growth was observed in the presence of any of the tested eugenol-Surfynol® combinations.

5.4.4 Influence of pH on growth kinetics of *E. coli* O157:H7 in the presence and absence of eugenol at 22 and 32°C

The growth of the most resistant strains of *E. coli* O157:H7 (F4546) at pH 5, 6 and 7 at incubation temperatures of 22 and 32 °C in the presence and absence of eugenol-

Surfynol[®] combinations is shown in Figure 5.3. Again, presence of Surfynol[®] micelles without eugenol did not inhibit growth of *E. coli* compared to the control. Growth of the controls and with Surfynol[®] 485W only micelles at 22 °C decreased at lower pHs, i.e., the optical density decreased from 0.6 to 0.52 and 0.21 as the pH was reduced from 7 to 5, respectively (Figure 5.3 A,B,C). Growth of controls was slightly greater at 32 than at 22°C. The optical density increased at pH 7 to a maximum of 1.1 at 32°C compared to 0.6 at 22°C. Addition of eugenol containing Surfynol[®] micelles completely suppressed growth of *E. coli* O157:H7 at all tested concentrations (Figure 5.3). There was no increase in optical density of culture suspensions containing 0.2 % eugenol and 5 % Surfynol[®] over the 48 hr incubation period.

5.4.5 Growth kinetics of four strains of *L. monocytogenes* in the presence and absence of eugenol at 10°C

L. monocytogenes is a psychrotrophic microorganism and can thus grow at low temperatures typical for refrigeration. The change in optical density as a function of time of all four tested strains of *L. monocytogenes* incubated at 10°C at pH 7 is shown in Figure 5.4. Results indicated that maximal growth of the controls was reached after 60 hr. Addition of ≥ 0.2 % eugenol inhibited growth of all strains for the entire incubation

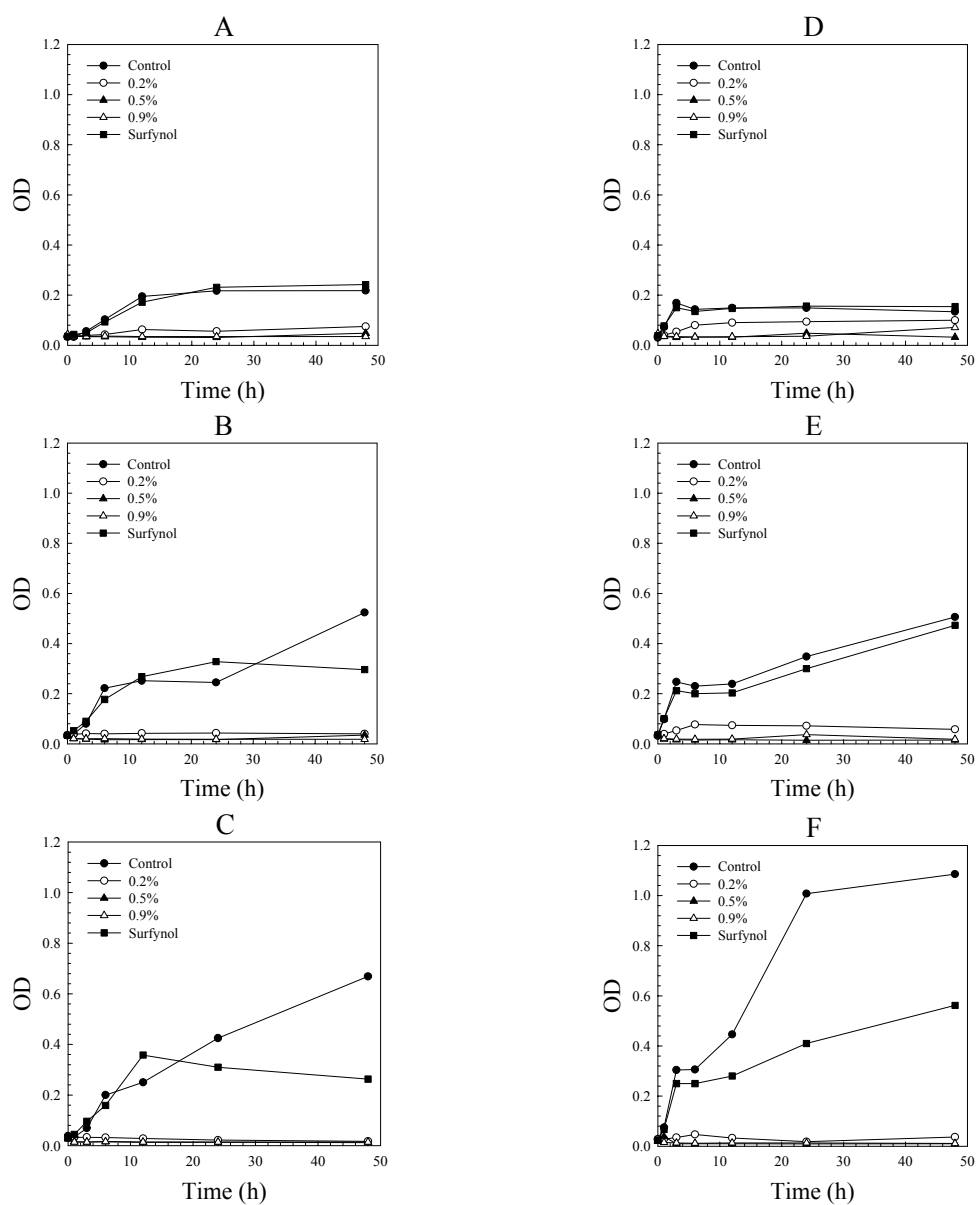


Figure 5.3 Growth of *E. coli* O157:H7 F4546 as measured by OD at 632 nm in the presence of 5 % Surfylnol[®] 485W and 0, 0.2, 0.5 and 0.9 % eugenol as a function of incubation time at (A) pH=5, 22°C, (B) pH=6, 22°C, (C) pH=7, 22°C, (D) pH=5, 32°C, (E) pH=6, 32°C, (F) pH=7, 32°C.

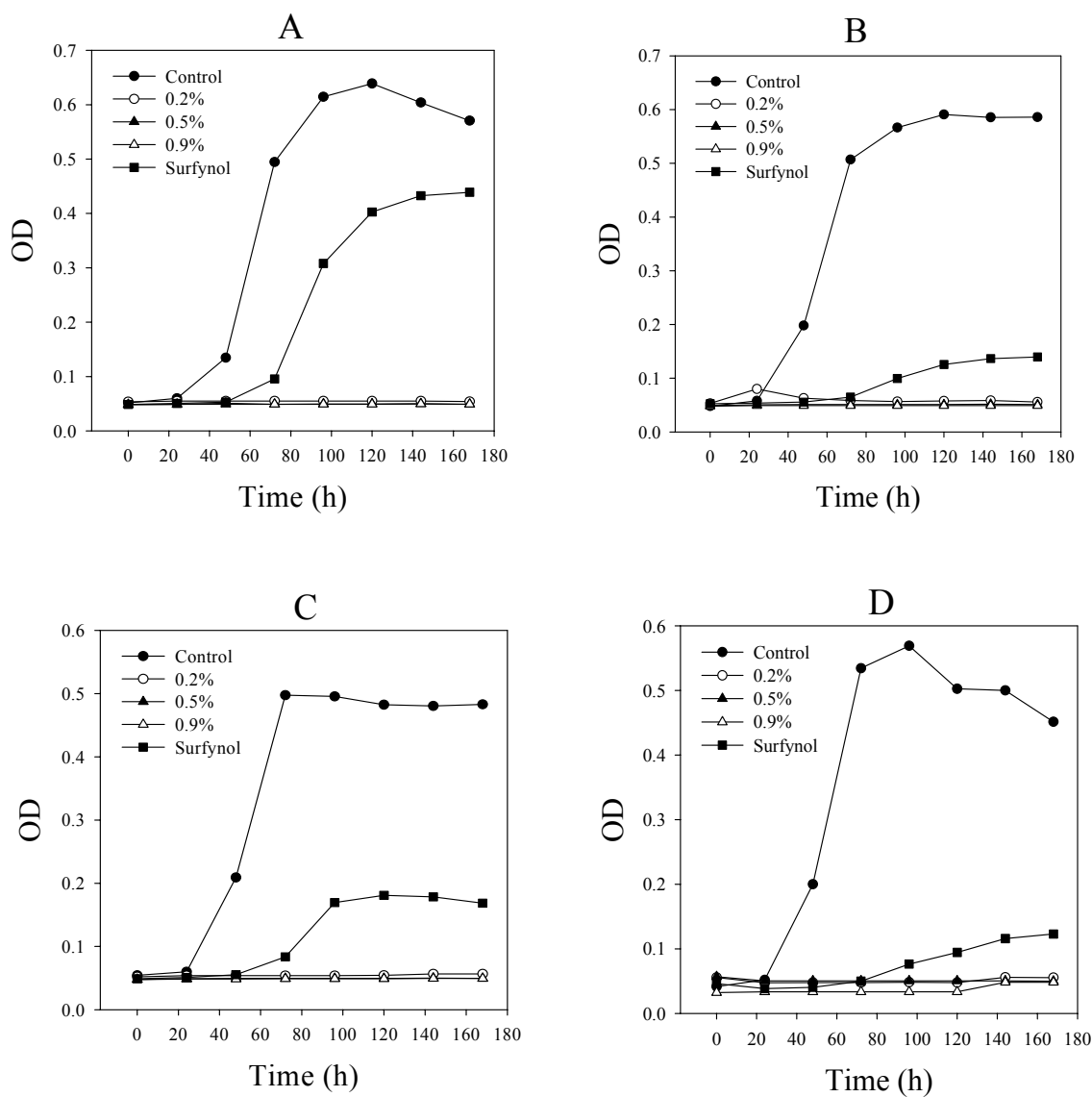


Figure 5.4 Growth of four strains of *L. monocytogenes* at 10°C as represented by increase in OD at 632 nm in the presence of 5 % Surfylnol[®] 485W and 0, 0.2, 0.5 and 0.9 % eugenol as a function of incubation time at pH=7: (A) 108, (B) Scott A, (C) 310, (D) 101

period (168 hr) indicating that the micellar encapsulated eugenol was effective even at 10°C. Similar results were found at pH 5 and 6 (data not shown).

5.5 Discussion

Surfactant micelles incorporate relatively large amounts of non-polar compounds in their interior. The formation of micelles in an aqueous phase and the inclusion of hydrophobic compounds is thermodynamically driven. Surfactant micelles are nanometer-sized aggregates of surfactants; amphiphilic molecules that are surface active. The self-assembly process enables the hydrophobic tails of surfactant monomers to minimize undesirable interactions with water molecules by forming spherical particles in which the hydrophobic tails are located in the interior of the aggregate and the hydrophobic head groups are located at the exterior of the aggregate where they can interact with water. Consequently, the interior of micelles is hydrophobic in nature and is said to have properties similar to that of an organic solvent. Micelles are therefore capable of encapsulating lipophilic antimicrobials, a class to which phenolics such as eugenol and carvacrol belong. The solubilization capacity is a function of the nature of the surfactant monomer which determines the size of the micelles through surfactant-surfactant interactions and the chemical nature of the antimicrobial, which determines the surfactant-antimicrobial interactions.

Surfynol[®] 485W, a surfactant used in the food industry, has been demonstrated to have a very high solubilization capacity. MAC values are generally very large. Because of this, the surfactant was chosen as a model system for our experiments. Surfynol[®]

485W can incorporate up to 2.66 % of essential oil component at 10 % and 0.96 % at 5% (Table 5.1). Other surfactants have typical MACs that are significantly lower than that of the Surfynol[®]. In a solubilization study with a series of short chain alkanes and alkenes, Weiss (1999) demonstrated that the MAC of Tween 20 for hexadecane at a surfactant concentration of 5 % was less than 0.045 % at 5 which is approximately 5% (1/20th) of that of Surfynol[®] 485W and eugenol. It has been suggested that this extraordinary capacity of Surfynol[®] to include phenolics such as eugenol and carvacrol is due to the formation of a mixed micelle, a micelle that consists of a shell composed of both the solute and the surfactant monomers. This is, for example, the case for compounds that possess polar groups that may interact with water molecules. Eugenol, a 2-methoxy-4-(2-propenyl)-phenol, has a methoxyl and phenol group which may well show some interaction with water despite the fact that the overall solubility of the molecule in water is virtually zero. The mixed micellar structure may contribute to the enhanced antimicrobial activity of the micelle since eugenol molecules can directly interact with cell membranes upon collision of the micelle with bacterial surfaces. As such, mixed micelles can be thought of as transport vehicles that allow the antimicrobial to better interact with foodborne pathogens. They act as virtual “solubilizers” by increasing the amount of antimicrobial that can be dissolved in the aqueous phase. Since mass transport process is driven by the concentration difference between the aqueous phase and the interior of the bacterial cell and cell membrane, an increase in the rate of antimicrobial uptake followed by accelerated destabilization and loss of biological activity of bacterial membranes is observed. Clearly, this differentiates the high antimicrobial activity

achieved using the nanostructured systems with traditional applications of eugenol, including ethanol or emulsion-based systems, they have significantly reduced and selective antimicrobial activity. .

Smith –Palmet et al., (1998) showed that at concentration of less than 1% plant essential oils exhibited bactericidal activity. At lower concentrations, i.e., 0.075%, essential oils of cinnamon, clove, and thyme showed bacteriostatic activity.

Because of EO components low solubility, they are incorporated into agar media forming an emulsion adding emulsifiers or solvents like ethanol to disperse the EO component (Remmal et al., 1993; Juven et al., 1994; Inouye et al., 2001). It is hypothesized that the use of emulsifiers and other solvents may actually interfere with the activity of essential oil components. Remmal et al., (1993) showed when 10% of the EO was added into 2.5 % of emulsifying agent or 2 w/w% into ethanol, the antimicrobial activity varied. Ethanol had higher interference than those systems prepared with Tween 20, 80 and Triton X100. For example, oregano essential oil was found to be more efficient than thyme essential oil against *Bacillus megaterium* when ethanol or Tween 80 were used, while it was less active when the emulsion was prepared with Tween 20. This data showed that the type of emulsifying agent or solubilizing agent used may interfere with the activity of essential oil or essential oil components.

Nonionic surfactant micelles have been shown to be thermodynamically stable systems these are able to withstand elevated temperatures. Nevertheless, temperature does have a significant effect on the organization and stability of surfactant micelles. The

cloud point of a nonionic surfactant is the temperature at which a surfactant solution becomes turbid. This point varies depending on the structure of the nonionic surfactant (i.e. length of the polyoxyethylene groups), and the non polar compound solubilized. The development of a cloud point indicates a phase separation that is due to the increase in aggregation number of the micelles and the low intermicellar repulsion that is a direct consequence of the dehydration of the oxyethylene groups in the polyoxyethylene chain with increasing temperature (Toerne 2001). Addition of co-surfactants has shown to generally lower the temperature stability of micelles. Similar results were found in this study, where the temperature stability of Surfynol® 485W micelles upon inclusion of eugenol decreased.

A change in systems pH had little or no effect on stability of the encapsulated eugenol system. This may be attributed to the fact that the surfactant used is a non-ionic and carries a zero net charge regardless of system pH. Unlike anionic or cationic surfactants, Surfynols® do not dissociate into a positively charged cations or negatively charged anions. The results are therefore not unexpected and confirm that nonionic surfactants are not only highly functional emulsifiers for food systems where pH may vary depending on application but are also best suited to serve as carrier systems for lipophilic antimicrobials. It should be noted that results may be significantly different if ionic surfactants are used, an investigation that is currently underway in our laboratories.

A comparison of *E. coli* O157:H7 and *L. monocytogenes* susceptibility to these systems indicate that *E. coli* O157:H7, a Gram-negative bacterium, is more strongly

inhibited by the Surfynol[®]-eugenol combinations. These results are interesting because other studies have shown that Gram-negative bacteria are, in general, more resistant to hydrophobic compounds and other toxic substances due to the presence of the outer membrane (Helander et al., 1998). The precise reason behind this observation is not clear but other authors have suggested that biological membranes (such as the outer membrane) may be solubilized by surfactants above 1 % (Galembeck et al., 1998; Helander et al., 1998). For example, it has been reported that Tween 20 improved the diffusion and enhanced the antimicrobial activity of nisin (Helander et al., 1998). The inclusion of the phenolic antimicrobial in a surfactant micelle results in a 10 nm-sized particle that has a hydrophilic surface. The change in the molecular nature of the antimicrobial may allow passage through the previously impermeable membrane; however more detailed studies on the interaction of a mixed micellar system with model membranes will be required to verify this hypothesis.

5.6 Conclusions

Our study suggests encapsulation of phenolic antimicrobials such as eugenol is an excellent mean to improve their antimicrobial efficacy against both Gram-positive and Gram-negative pathogens. The capsules were stable under a wide range of pH and temperature conditions. Small concentrations of eugenol (0.2 or 0.5 %) were sufficient to achieve complete inhibition of all tested organisms and strains. The inhibitory efficacy was not significantly affected by the incubation temperature. The capsules prevented growth of *E. coli* O157:H7 and *L. monocytogenes* at 10, 22 and 32°C suggesting that systems could efficiently be used in the food industry for foods that are stored at room

temperature or under refrigeration without experiencing a loss of activity. Inhibition slightly improved at lower pH. Finally, our results have important implications for the food industry as it may offer a new means to produce shelf stable food products that offer added protection against spoilage organisms and pathogens.

6 Characterization and Stability of Phytophenols

Encapsulated in Surfactant Micelles

6.1 Abstract

Phytophenols such as eugenol and carvacrol are antimicrobials that can inhibit growth of foodborne pathogens. Encapsulation in nanoparticles may offer a new method to increase solubility in the aqueous phase. The objective of this study was to determine properties, and efficiency of encapsulation of phytophenols in surfactant micelles and the stability at different temperatures and pHs. Carvacrol and Eugenol containing micelles were prepared by dispersing Surfynol® 485W and Surfynol® 465 in water at room temperature. Stability was determined by UV-visible spectroscopy at different pH and temperatures to obtain stability phase diagrams; particle size was determined by dynamic light scattering, and structural information about mixed micellar systems was obtained by nuclear magnetic resonance spectroscopy (NMR).

Incorporation of carvacrol and eugenol to surfactant-based nanoparticles was very rapid (approximately 10min). Depending on the surfactant-antimicrobial combination, the particles produced varied in size between 5 to 20 nm. All encapsulated micelles were stable over a wide range of pHs. However, temperature and essential oil component concentration influenced the stability of the mixed micelles, i.e. micelles destabilized at lower temperatures with increasing encapsulated essential oil component concentration. For example, 0.9 % of eugenol encapsulated in Surfynol® 485W exhibited turbidity (cloud point) at 55 °C, while at 0.5 %, 70°C was needed to reach the cloud point.

Encapsulation of phytophenols in surfactant micelles offers an excellent means to incorporate large amounts of these compounds in aqueous systems by dramatically increasing the solubility. This is one potential solution to the application problem of phytophenols in many food systems.

6.2 Introduction

Food antimicrobials such as phytophenols are compounds that are able to reduce the growth of pathogens in food systems thereby improving microbial safety of the product (Shelef, 1983; Branen and Davidson 1993). The major problem with antimicrobials is their low solubility in aqueous systems and resulting low antimicrobial activity in food systems (Oka 1964). Therefore, high concentrations of these compounds are required to be effective antimicrobials applied to food systems. Solubilization of these hydrophobic compounds in surfactant micelles may overcome that limitation.

Surfactants are amphiphilic molecules containing a hydrophobic and hydrophilic group. Above a specific concentration called the “critical micelle concentration” (CMC), the surfactant monomers spontaneously self-assemble to form spherical aggregates (Kumar et al., 1994; Alargova et al., 1995). The simplest aggregate form is the micelle (Hereida and Bukovac 1992; Kumar et al., 1994; Texter 1999). At the molecular level, interfacial forces control the curvature of the surfactant determining the shape of the surfactant aggregates (Kumar et al., 1994). Self-assembly micellar surfactant aggregates under the appropriate conditions are able to dissolve relatively large amounts of oil in comparison with water (Texter 1999; Weiss and McClemments 2000; Christov et al.,

2002; Dungan et al., 2003). The process by which the oil is incorporated into the surfactant aggregates is called solubilization. Micelles can solubilize the oil or the solute by direct or indirect solubilization (Sailaja et al., 2003). Direct solubilization is a diffusion process while indirect solubilization is an uptake of the solute from the continue phase present around the micelles (Sailaja et al., 2003). The material that can be solubilized in the micelles is relatively small in size (Texter 1999). The ability of surfactant aggregates to incorporate hydrophobic material may be of great importance for the pharmaceutical, biological, chemical and food industries (Cerichelli and Mancini 2000).

Environmental factors such as temperature, salinity and pH may have an effect on the solubilization of nonionic surfactant (Zhou and Zhu 2004). Temperature has a significant effect on the stability of nonionic surfactant micelles, especially those containing polyoxyethylene groups (Rosen 1989; Toerne et al., 2001). Nonionic surfactants become turbid on heating and if heating continues a phase separation will follow due to the dehydration of the oxyethylene oxygens located in the polyoxyethylene groups (Rosen 1989).

In recent years, there has been an increased interest in the investigation of surfactant aggregates using NMR spectroscopy (Griffiths et al., 2001; Triba et al., 2004). In general ^{31}P , ^2H , or ^{14}N NMR is used due to the anisotropic magnetic properties of these nuclei (Triba et al., 2004). On the other hand, ^1H is abundant and a sensitive nucleus and its chemical shift allows the differentiation of molecules in heterogeneous mixtures.

Another advantage of this type of NMR technique, besides its sensitivity, is that molecules can be differentiated by their chemical shift, therefore by superposition of the spectrum, structural changes in the compounds can be elucidated (Triba et al., 2004). Aromatic molecules such as benzene have been investigated. Because their ring structure ¹H NMR chemical shift is affected by surfactant molecules and can give evidence in the region of the micelle in which they are located (Cerichelli and Mancini 2000). NMR has been an important tool in the investigation of the dynamics of the self-assembly process, solubilization rate, and to determine shape and size (Cerichelli and Mancini 2000; Doyle and Marangoni 2004; Triba et al., 2004)

The objective of this study was to determine the solubilization capacity, size, structure and location of the hydrophobic compound within the micelles as well as the stability to determine if the micelles had utility as a transport mechanism of essential oil components for use as antimicrobial preservatives in food systems.

6.3 Materials and Methods

6.3.1 Materials

All solutions were prepared with distilled and deionized water. Eugenol (4-allyl-2-methoxyphenol) and carvacrol (5-isopropyl-2-methyl-phenol) were purchased from Sigma Chemical Co. (St. Louis, MO). Nonionic surfactants Surfynol[®] 465 and 485W were provided by Air Products and Chemical, Inc. (Allentown, PA). The critical micellar concentration was 0.65 % (w/w) for Surfynol[®] 465 and 1.65 % (w/w) for Surfynol[®] 485W. pH was adjusted with 0.1N HCl or NaOH.

6.3.2 Preparation of carvacrol/eugenol containing surfactant micelles

Aqueous surfactant solutions were prepared by dispersing Surfyrol[®] 465 and 485W in water at room temperature to obtain surfactant solutions with concentrations of 1, 2, 3.5, 5, 7.5 and 10 % (w/w). Eugenol and carvacrol were added to surfactant solutions at concentrations ranging from 0.025 % to 3 % (w/w). Solutions were stirred until the absorbance remained constant indicating that solubilization was complete (typically less than 10 minutes). Solutions were filter sterilized using a 0.22 μ m cellulose acetate membrane (Corning, Corning, NY) and stored at $25 \pm 2^{\circ}\text{C}$ until used but not longer than 2 weeks.

6.3.3 Maximum additive concentration (MAC)

The MAC was defined as the highest concentration of a lipophilic compound that could be incorporated into a micellar surfactant solution at a given surfactant concentration (Weiss and McClements, 2000). It was determined by measuring the absorbance of surfactant solutions ranging in concentrations between 1 and 10 % at 632 nm using a uv-visible, spectrophotometer (Hewlett Packard 8452A Diode Array Spectrophotometer, Palo Alto, CA) after addition of essential oil components (0.025% to 3 %). The wavelength of 632 nm was chosen to avoid interference with intrinsic absorbance of eugenol and carvacrol.

6.3.4 Particle size determination

Surfactant solutions were prepared as above. For these experiments, surfactant solutions without essential oil components and surfactant solutions that contained specific

concentrations of EO components were selected and the particle size of empty and swollen micelles measured using a dynamic light scattering particle sizer ZetaPALS with particle size software (Brookhaven Instruments, Co., Holtsville, NY) at a 90° scattering angle and at a wavelength of 632nm.

6.3.5 Stability of encapsulated surfactant micelles

Aqueous surfactant solutions were prepared by dispersing Surfydol[®] 465 and 485W in water at room temperature to obtain surfactant solutions with concentrations of 1, 2, 3.5, 5, 7.5 and 10 %. Eugenol and carvacrol were added to surfactant solutions at concentrations ranging from 0.025 % to 3 %. Solutions were stirred until the absorbance remained constant indicating that solubilization was completed (typically less than 10 minutes) and stored at $25 \pm 2^{\circ}\text{C}$ until used. All solutions were adjusted to pH ranging from 3 to 9 with NaOH and HCl (0.1N). Surfactant solutions prepared with two different surfactants and with two essential oil components at different concentrations and adjusted to pH ranging from 3 to 9 were heated from 25 to 85°C for Surfydol[®] 485W and from 25 to 70°C for Surfydol[®] 465 and every 5 degrees solutions were measured at 632 nm using a UV-visible, spectrophotometer (Hewlett Packard 8452A Diode Array Spectrophotometer) after titration with essential oils and adjusting the pH.

6.3.6 Nuclear magnetic resonance (NMR) spectroscopy.

The 1D proton nuclear magnetic resonance experiments were performed on a Varian Mercury 300 Spectrometer (Palo Alto, CA) with proton resonance at 300 MHz. The spectra were acquired in regular one pulse sequences with a 30 degree proton pulse and a 3s relaxation delay at room temperature. The samples were prepared by dispersing 5 %

(w/w) Surfynol[®] 465 and 485W in deuterium oxide at room temperature. Eugenol and carvacrol were added to surfactant solutions at concentrations of 0.5 % (w/w) for Surfynol[®] 465 and 0.6 % (w/w) for Surfynol[®] 485W.

6.4 Results

6.4.1 Maximum Additive Concentration (MAC) of eugenol in Surfynol[®] 485W

Increasing concentrations of carvacrol and eugenol were titrated into Surfynol[®] 465 and 485W solutions at surfactant concentrations ranging from 1 to 10 % (Fig. 6.1). Absorbance remained zero as carvacrol or eugenol was titrated into the solution until a critical concentration was reached. Absorbance then increased significantly, indicating that micelles were fully saturated and were no longer able to take up more carvacrol. The concentration at which the turbidity increases is referred to as the “maximum additive concentration” (MAC). For example, at 1% Surfynol[®] 465, the absorbance increased from zero to above 4 at 0.04 % of carvacrol (Figure 6.1D). Therefore, the MAC for Surfynol[®] 465 at 1 % surfactant titrated with carvacrol was 0.04 %. In contrast, solutions of Surfynol[®] 465 at 10 % were able to incorporate as much as 3 % carvacrol, more than 70 times the amount at 1 %.

Four systems were tested, Surfynol[®] 465 and 485W in combination of eugenol or carvacrol. Surfynol[®] 485W in combination with eugenol was the system that could incorporate the greatest amount of essential oil component; therefore, for further experiments this system was used. Figure 6.2 shows the MAC for this system.

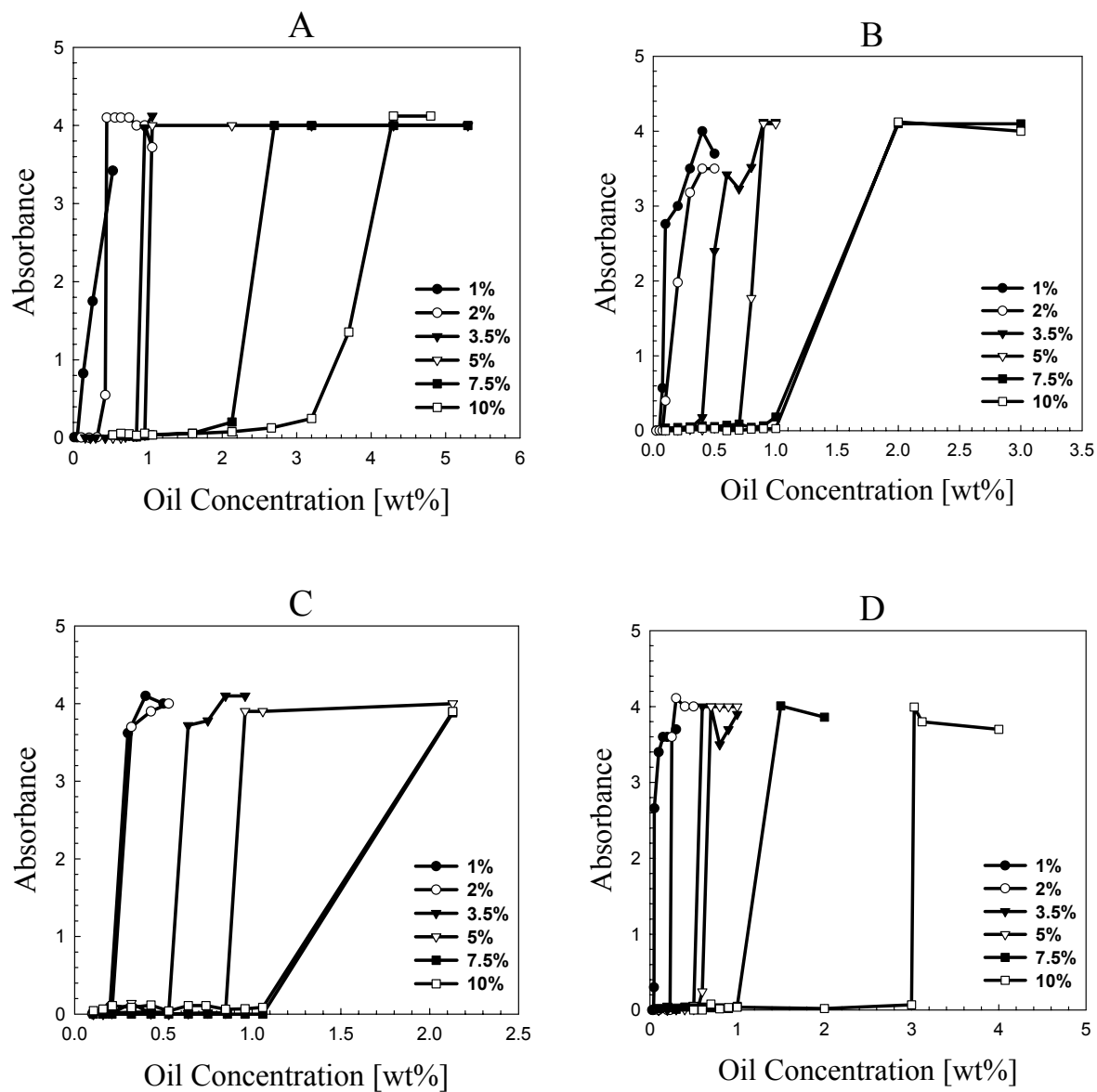


Figure 6.1. Maximum Additive Concentration (MAC). Comparison of the maximum of amount that can be incorporated into the surfactant-base micelles of the four systems used. Surfynol[®] 465 with the combination of eugenol or carvacrol and Surfynol[®] 485W with the combination of eugenol or carvacrol.

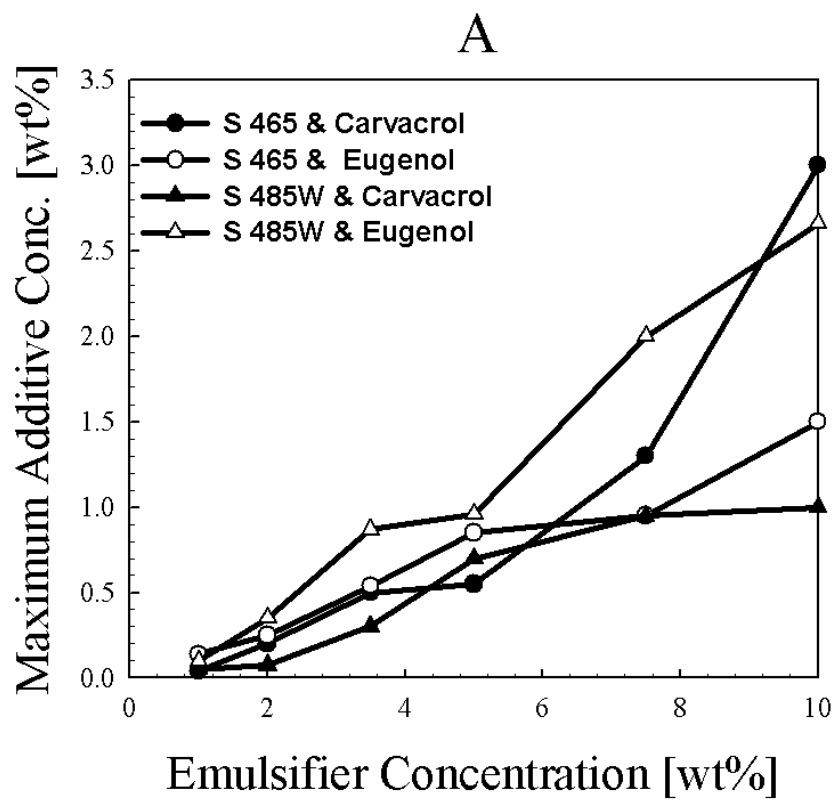


Figure 6.2 Maximum amount of essential oil component that could be incorporated into the surfactant micelles. (A) Eugenol in Surfyrol[®] 485W, (B) Carvacrol in Surfyrol[®] 485W, (C) Eugenol in Surfyrol[®] 465, and (D) Carvacrol in Surfyrol[®] 465. All were measured by the sudden increase in absorbance at 632nm

For all surfactant-essential oil component combinations, the MAC increased with increasing emulsifier concentration. As long as the concentration of eugenol or carvacrol added to the surfactant solutions was smaller than the MAC, the turbidity of the solution remained zero indicating that all of the compound was incorporated by the micelles and none remained in the surrounding aqueous phase. Conversely, if the essential oil components were added at concentrations above the MAC, solutions became turbid indicating that micelles were saturated and fractions of the compounds remained dispersed in the aqueous phase in the form of droplets that eventually phase separated. All subsequent antimicrobial experiments were conducted with Surfynol[®] 485W at 5% with eugenol below the MAC due to its antimicrobial properties (data not shown) and the large amount of essential oil component and to be certain that the CMC was reached.

6.4.2 Particle size determinations

Particle size was determined by dynamic light scattering at a scattering angle of 90° to minimize dust effects. The results obtained by DLS are presented in table 1-4 for each of the system tested at different concentrations. For Surfynol[®] 485W, the size of empty micelles was 7nm and for Surfynol[®] 465 was 5.7nm. Surfynol[®] 485W containing encapsulated carvacrol had a particle size approximately of 10 nm (table 6.1), and containing eugenol varied between 10.5 to 13.1 nm (table 6.2). Surfynol[®] 465 containing encapsulated carvacrol had a size range of 17.4 to 19.7 nm (table 6.3) and for eugenol the size varied between 12.8nm and 15.8nm (table 6.4).

Table 6.1 Particle size of Surfynol® 485W with carvacrol

Surfactant concentration [%]	Essential oil component concentration	Particle Size [nm]
	[%]	
5	0.4	8.3
	0.6	10.5
7.5	0.6	7.4
	0.8	8.6
	1	10.9
10	0.5	7.6
	1	8
	1.25	10.6

Table 6.2 Particle size of Surfydol[®] 485W with eugenol

Surfactant concentration [%]	Essential oil component concentration	Particle Size [nm]
	[%]	
5	0.6	7.9
	0.8	8.4
	0.96	10.9
7.5	0.8	10.3
	1	12.5
10	1.2	7.2
	2	13.1

Table 6.3 Particle size of Surfynol® 465 with carvacrol

Surfactant concentration [%]	Essential oil component concentration	Particle Size [nm]
	[%]	
2	0.1	9.6
	0.2	17.4
3.5	0.2	8.9
	0.3	13.1
	0.4	19.7
5	0.3	9.5
	0.4	13.6
	0.5	18.7
7.5	0.4	8.4
	0.5	10.3
	0.7	15.9
10	0.5	8.8
	1	18.8

Table 6.4 Particle size of Surfydol® 465 with eugenol

Surfactant concentration [%]	Essential oil component concentration [%]	Particle Size [nm]
2	0.2	9.2
	0.3	8.9
3.5	0.4	11.1
	0.5	15.0
	0.4	7.4
5	0.5	9.6
	0.85	13.1
	0.6	9.2
7.5	0.8	11.4
	0.95	12.8
	0.4	9.0
10	0.6	9.4
	1	15.3

6.4.3 Stability of encapsulated surfactant micelles

Stability of micelles prepared with Surfynol[®] 485W and Surfynol[®] 465 with eugenol or carvacrol was measured. Concentration of essential oil component varied depending on the surfactant and type of essential oil component. Thermal and pH stability was determined by measuring the absorbance of surfactant Surfynol[®] 485W and 465 at 5 % solutions in the absence (0 %) of essential oil components and in presence of essential oil components. Concentrations of eugenol ranged from 0.1 to 0.9 % and of carvacrol from 0.1 to 0.5 % in combination with Surfynol[®] 485W. In combination with Surfynol[®] 465, concentration of eugenol ranged from 0.1-0.85 % and for carvacrol ranged from 0.1 to 0.55 %. The four systems were tested at different temperatures ranging from 25 to 90°C at pHs ranging from 3 to 9. Figure 6.3 shows the results of such an experiment at pH ranging from 3 to 9

The critical temperature or the so-called “cloud point” is the point where the micelles phase invert and release the encapsulated compound because of increased dehydration of the polar head group of the surfactant (Rosen, 1989; Toerne 2001). The cloud point decreased with increasing eugenol concentration. Surfynol[®] 485W, which has a cloud point above 90°C, exhibited high thermostability. For Surfynol[®] 485W with eugenol at concentration of 0.1 %, micelles remained stable even if solutions were heated above 85°C. In contrast, at 0.9 % eugenol, the cloud point decreased to 55°C (Figure 6.3A). With carvacrol the same type of result was seen. At 0.1 % eugenol, micelles remained stable above 90°C while at 0.5 %, the cloud point decreased to 55°C at low pH and around 70°C at higher pHs (Figure 6.3B). Surfynol[®] 465 in absence of essential oil

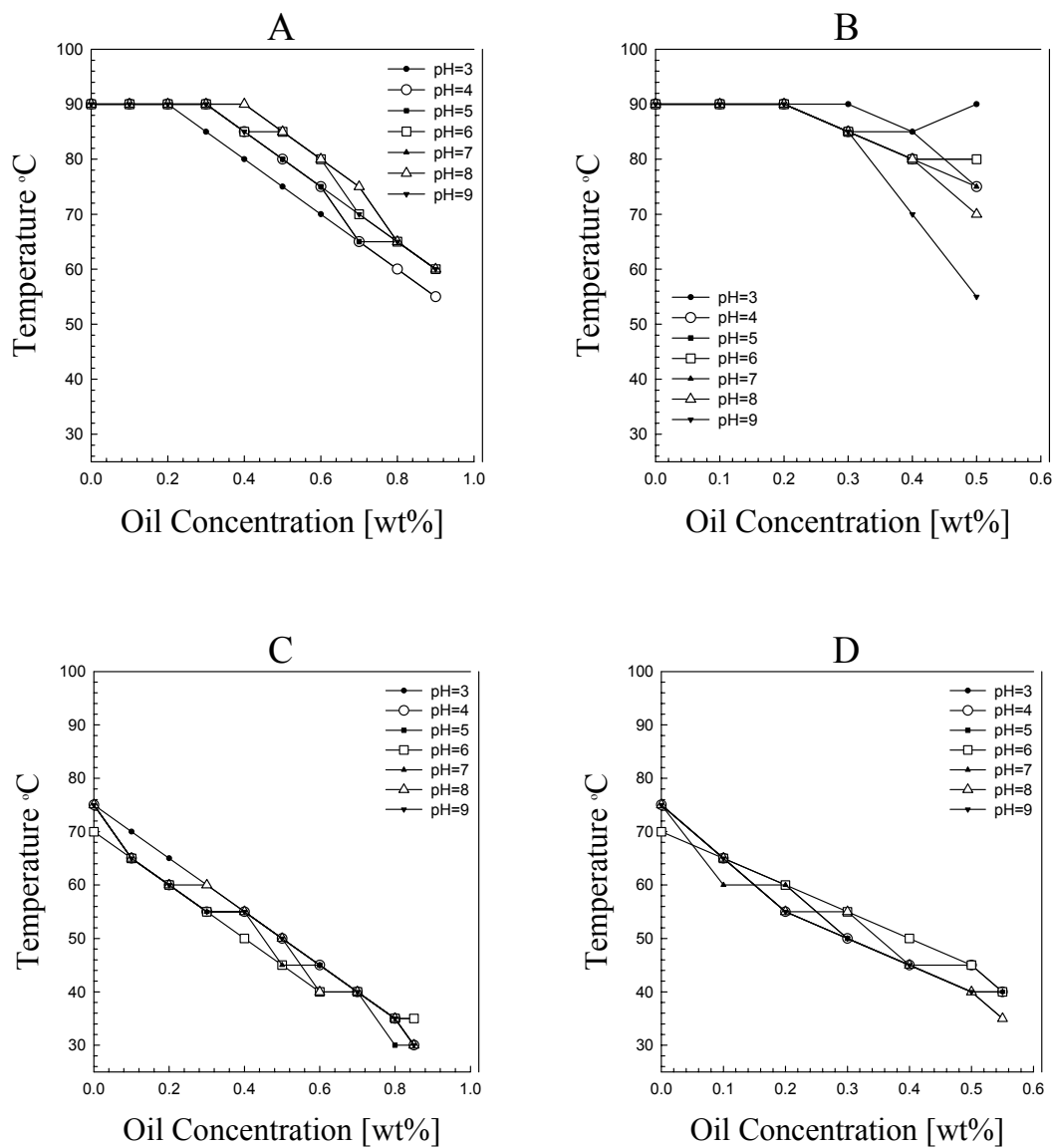


Figure 6.3 Effect of pH, temperature, and oil concentration in the stability of phytophenol encapsulated surfactant micelles. (A) Surfynol[®] 485W with eugenol, (B) Surfynol[®] 485W with carvacrol, (C) Surfynol[®] 465 with

components required lower temperatures (75°C) than Surfynol® 485W to reach the cloud point. In combination with 0.1% eugenol 60°C was needed to reach the cloud point while at 0.85 % only 30°C was needed (Figure 6.3C). In combination with carvacrol the same trend is apparent. At 0.1 % carvacrol the cloud point was at 70 °C while it decreased 0.55% to 35°C (Figure 6.3D). Results indicated that the thermal stability of micelles decreased with concentration of essential oil component. As is shown in Figure 6.4, pH has a little or not effect in the stability of surfactant micelles. The most dramatic changes were shown at pH of 3. In comparison with the other pHs, there was a variation of 5° C to reach the cloud point .With exception of Surfynol® 485W with carvacrol that the cloud point change from around 90°C at pH 3 (Figure 6.4A) to 80°C at pH 5 (Figure 6.4B) and to 75°C at pH of 7 (Figure 6.4C), and to 55°C at pH of 9 (Figure 6.4D). Therefore Surfynol® 485W with carvacrol is the more affected by pH than other systems. Overall, pH does not play a role in the stabilization of surfactant micelles

6.4.4 Structure of mixed micelles

¹H NMR spectra were run in solutions with 5 % of Surfynol® 485W and 465 in presence and absence of added carvacrol or eugenol. In figure 6.5C, the mixed micellar solution is shown Chemical shifts of the ¹H spectra are shown in figure 6.5. Figure 6.5A shows the eugenol spectra and Figure 6.5B shows the Surfynol® 485W at 5% spectra. Comparing the location of the peaks in the eugenol spectra to those in the mixed micellar solution, there is a change in the chemical shift of all peaks with the exception of the water peak that includes the hydroxyl group present in eugenol.

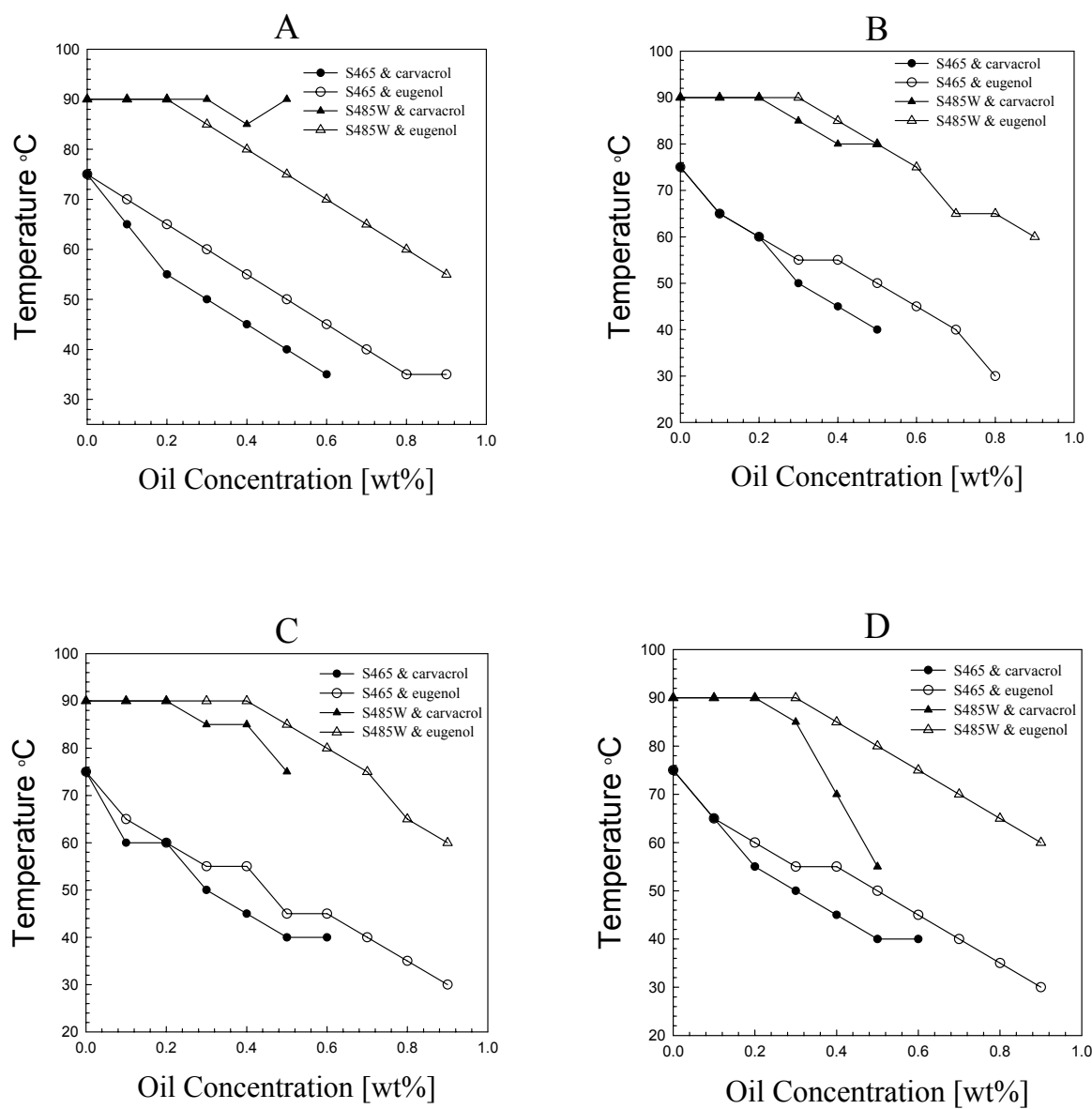


Figure 6. 4 Comparison between the stability varying the pH of four systems prepared. (A) pH=3, (B) pH=5, (C) pH=7, (D) pH=9

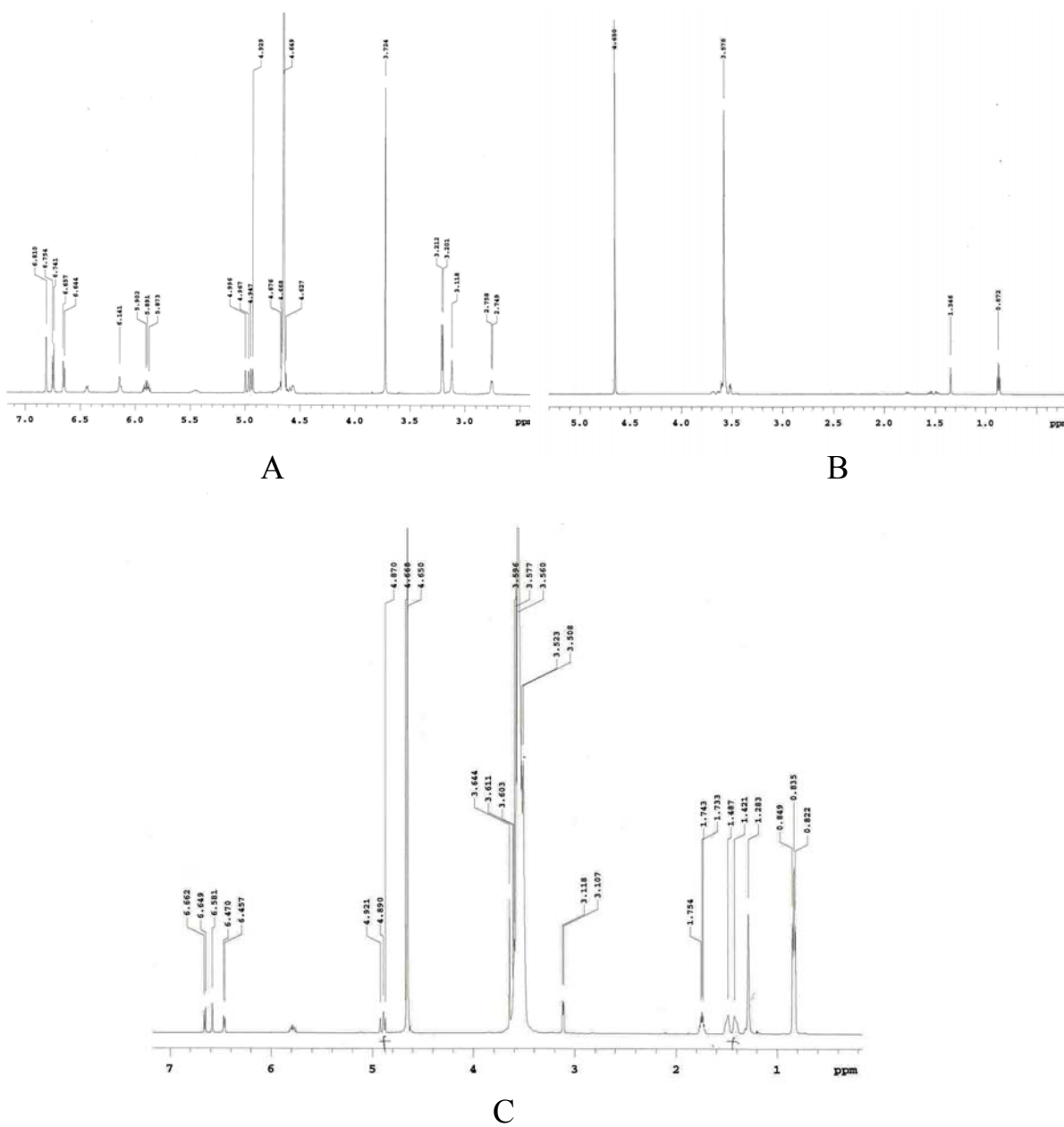


Figure 6.5 Typical 1D or pulse spectra acquired by NMR measurements. (A) Eugenol spectra (B) Surfynol[®] 485W spectra (C) Mixed micelles spectra

Comparing the peaks of change of the peaks involving the $-CH_3$ groups in the backbone of the hydrophobic tail of surfactant structure. The shift of carvacrol or eugenol and Surfynol[®] 465 and 485W followed the same trends. The different chemical shift by solutions prepared in absence of phytophenols showed small variations due to the variation of the hydrophilic head (results not shown). I. In Figure 6.6A, eugenol groups that showed a chemical shift are highlighted in yellow and those groups of Surfynol[®] 485W that showed a chemical shift are also highlighted in yellow. The predicted structure of an empty micelle formed with Surfynol[®] 485W is shown in Figure 6.6C and the predicted structure of the mixed micelles is shown in Figure 6.6D based on the result obtained from the ^1H NMR spectra. of Surfynol[®] 485W with the mixed micellar system, it is showed a chemical eugenol, (D) Surfynol[®] 465 with carvacrol.

6.4 Discussion

The solubilization capacity or the maximum amount of essential oil component (MAC) that can be solubilized is influenced by several factors such as the structure of the hydrophobic molecule, type of surfactant, hydrophile-lipophile balance (HLB), surfactant hydrophobic and hydrophilic chain lengths, surfactant concentration, and the interaction between surfactant and hydrophobic molecule. Because of their high solubilization capacity, Surfynol[®] 465 and 485W were used to produce micelles. It was shown that, as the surfactant concentration increased, the solubilization capacity or MAC also increased. Surfynol[®] 485W could incorporate up to 2.66 % of eugenol at 10 % w/w surfactant and 0.96% at 5 % while the same surfactant can incorporate up to 1 % of carvacrol at 10 % of

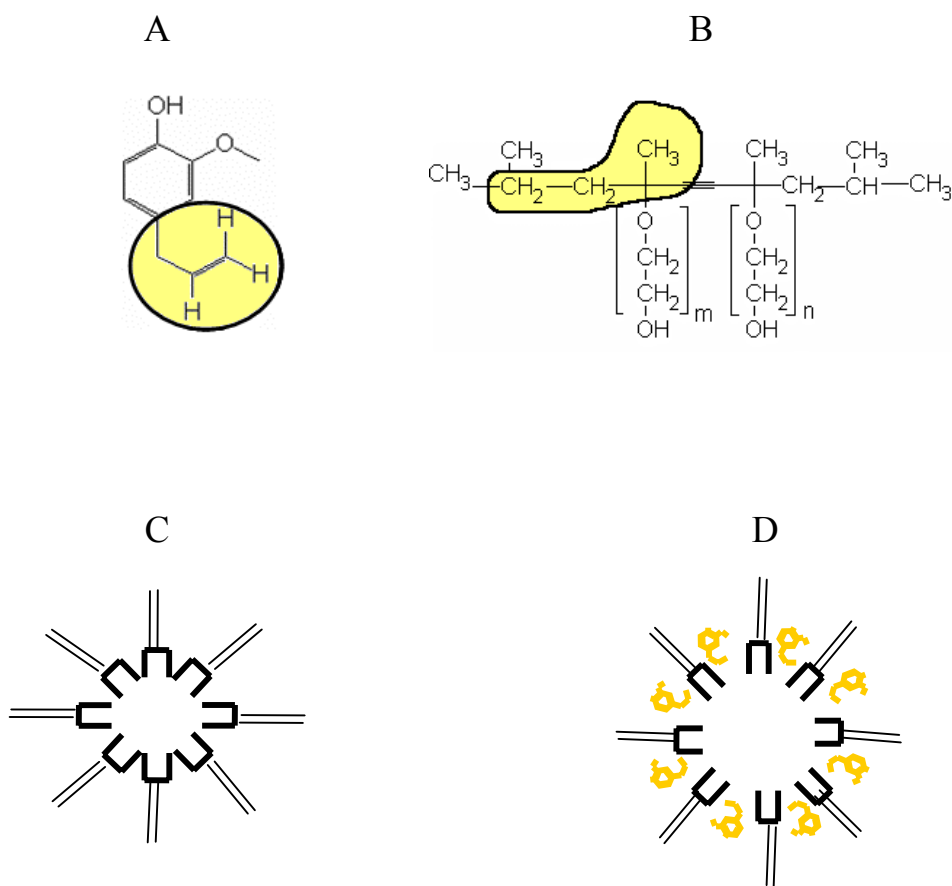


Figure 6.6 Group interaction in the formation of mixed micelles (in yellow) formed with eugenol and Surfynol[®] 485W. (A) Eugenol structure, (B) Surfynol[®] 485W structure, (C) Predicted structure of micelles (7nm in diameter), (D) Predicted structure of mixed micelles (8nm in diameter)

surfactant and at 5 % can incorporate 0.70 %. Therefore, the MAC is affected by the type of hydrophobic molecule encapsulated.

Weiss (1999) investigated the MACs of Tween 20, 40, 60, 80 and Triton X-100, Triton SP-190 and Triton SP-175 incorporating *n*-tetradecane. Tween 20 solubilized only 1/10 of the amount of the oil of Triton SP-175. Weiss and McClements (2000) reported that the solubilization capacity increased as the HLB number decreased from ~17 to 12. In this experiment the nonionic surfactant used, Surfynol[®] 465 and 485W, have HLB values of 13 and 17 respectively. With these surfactants specifically, the solubilization capacity increased with the increase of HLB. However, smaller micelles were formed with Surfynol[®] 485W because the HLB was approximately 17. Therefore a more optimal curvature of the surfactant increases as the HLB number moves away from 7. Overall, micelles could take up more eugenol and carvacrol being the interaction between surfactant-oil one of the most important factors influencing the MAC

In this study, turbidity of the solution decreased to zero within 10 minutes upon addition of carvacrol and eugenol to surfactant solutions indicating that micelles rapidly incorporated the phytochemicals. This is compared to the slow solubilization kinetics of Tween 20, 40 and 60 that sometimes require days (Weiss 1999).

The measured particle size of swollen micelles was a function of both the surfactant type and the essential oil component used. The size of the empty micelles was a function of the surfactant used. An increase of particle size means that the essential oil component has been solubilized in the micelle. Eventually, the size of the combination of

essential oil component and surfactants reaches a maximum once all the micelles have been fully saturated. Addition of more essential oil component leads to unincorporated component remaining in the aqueous phase.

Temperature has a significant effect on the organization and stability of surfactant micelles. Mixed micelles showed stability over the entire range of pH tested. Surfynol[®] 485W with carvacrol was the most affected by the pH. For instance, at pH 9 and high essential oil component concentration, lower temperatures were needed to cause turbidity. While, with lower pHs, at the same essential oil component concentration, higher temperatures were needed to reach the clouding point. Surfynol[®] 465 was less thermoresistance at higher pHs whereas, Surfynol[®] 465 was less thermoresistant at low pHs reaching the cloud point at pH 3. A change in system pH had little or no effect on stability of the encapsulated eugenol and carvacrol systems. This may be attributed to the fact that the surfactant used is a non-ionic surfactant that carries a zero net charge regardless of system pH. Unlike anionic or cationic surfactants, Surfynols[®] do not dissociate into a positively charged cation or a negatively charged anion. The results are therefore not unexpected and confirm that nonionic surfactants are not only highly functional emulsifiers for food systems where pH may vary depending on application but are also best suited to serve as carrier systems for lipophilic antimicrobials. It should be noted that results may be significantly different if ionic surfactants are used, an investigation that is currently underway in our laboratories

As was expected, temperature and essential oil component concentration played a major role in the stability of mixed micelles. The higher the essential oil component concentration, the lower the clouding temperature. For example, 0.9 % of eugenol exhibited turbidity (clouding temperature) at 55°C, while at 0.5 % required 70°C to reach the clouding point. Overall, Surfynol® 485W was more resistant to changes in temperature, being stable above 90°C at low essential oil component concentrations. In contrast, Surfynol® 465 was less stable, reaching the clouding point at 0 % of essential oil component at 75°C. These results were expected due to the differences in the hydrophilic polyoxyethylene chain (Surfynol® 485W contain 30 moles while Surfynol® 465 contain 15 moles). An interesting note is that Surfynol® 485W and 465 containing eugenol become yellow above pH 8. This can be due to the change in curvature due to the competition with the water of the ions formed by the HCl and NaOH.

Nuclear magnetic resonance can give structural information on surfactant aggregates. In this study it is proposed that oil is located in between the surfactant monomers interacting with the hydrophobic tails having the hydroxyl group phasing towards the aqueous phase (Figure 6.6D). Cerichelli and Manici (2000) investigated the structure of the solubilization of benzene in surfactant aggregates formed with cetyltrimethylammonium by ^1H and ^{13}C NMR. They found that the benzene was located in regions of the aggregate formerly occupied by water molecules making their hypothesis that the water content in the clefts can be replaced with benzene without any dramatic change in the cleft volume. Depending on the surfactant structure and the type of oil used, different type of aggregates or mixed micelles are going to be formed.

Because of the structure of the surfactant, it is hypothesized that the micelles formed have a bracket-like structure. The molecules are spherical due to hydrophobic interactions from the $-CH_3$ groups at the end of the hydrophobic chain in the surfactant molecule maintaining the curvature.

Thermodynamic properties of a micellar solution are governed in terms of the Gibbs free energy (Kwon and Kim 2002). In this study it is hypothesized that when the eugenol is in solution they stabilize the micelles due to the reduction of free energy by the increasing in enthalpy and decreasing in entropy.

6.5 Conclusions

Solubilization of phytophenols in surfactant micelles offers an excellent means to incorporate large amounts of essential oil components in aqueous systems, dramatically increasing their solubility. Swollen micelles have particle size in the nanometer size range and are therefore, not visible to the naked eye. The surfactant micelles, especially those prepared with Surfynol[®] 485W showed pH and thermal stability which has important implications for the applications in food systems. As such, solubilization in micellar systems may solve the major limitation that currently prevents phytophenols to be applied in food systems

7. Conclusions

Encapsulation in surfactant-based nanoparticles offers an excellent mean to solubilize hydrophobic compounds such as carvacrol and eugenol increasing their solubility in aqueous media. As a result, increasing their antimicrobial activity due to microbial growth in aqueous environments. These nanoparticles are effective against Gram-positive bacteria and Gram-negative bacteria being more effective against *E. coli* O157:H7, a Gram-negative bacterium. Furthermore, antimicrobial activity of the nanoparticles was stable to changes in pH and temperature thereby maintaining activity under the conditions tested.

Surfactant based nanoparticles are very stable at pHs ranging from 3 to 9. They are also stable under a wide range of temperatures, especially those prepared with Surfynol[®] 485W that can withstand more than 90 °C. The thermostability of surfactant nanoparticles has important implications for their application in food systems.

Encapsulation of hydrophobic antimicrobials offer a new methods to improve the antimicrobial activity and application of phytophenols. This technique may provide another tool to improve food safety and overcome the major limitation that currently prevents application of essential oil components to foods.

References

- Achinewhu, S. C., C. C. Ogbonna, et al. (1995). "Chemical composition of indigenous wild herbs, spices, nuts and leafy vegetables used as foods." Journal of Plant Food and Human Nutrition **32**: 31-36.
- American Chemical Society (ACS) (2002).
- Anonymus (2004). About-E. coli. **2004**.
- Anonymus (2004). About-Listeria. **2004**.
- Aureli, P., A. Constantine, et al. (1992). "Antimicrobial activity of some plant essential oil against *Listeria monocytogenes*." Journal of Food Protection **5**: 344-348.
- Bagamboula, C. F., M. Uyttendeale, et al. (2004). "Inhibitory effect of the thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*." Journal of Food Microbiology **21**(33-42.).
- Balszyk, M. and A. Holley (1998). "Interaction of monolautin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms." International Journal of Food Microbiology **39**: 175-183.
- Branen, A. L. and P. M. Davidson (1993). Antimicrobials in Foods. New York, Marcel Deker.
- Burl, S. and P. Coote (1999). "Preservative agents in foods. Mode of action and microbial resistance mechanism." International Journal of Food Microbiology **50**: 1-17.
- Burst, S. (2004). "Essential oils: their antibacterial properties and potential applications in foods-a review." International Journal of Food Microbiology.

- Centers for Disease Control and Prevention (CDC) (2004). "Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food Selected Sites." **53**(16): 338-343.
- Centers for Disease Control and Prevention (CDC) (2004). Foodborne Infections. From Division of Bacterial and Mycotic Diseases. **2004**.
- Clint, J. H. (1992). Surfactant aggregation. New Delhi, Great Britain., Blackie.
- Conner, D. E. and L. R. Beuchat (1984). "Effects of essential oils from plants on growth of food spoilage yeast." Journal of Food Science **49**: 429-434.
- Cowan, M. M. (1999). "Plant products as antimicrobial agents." Journal of Clinical Microbiology Reviews. **12**: 564-582.
- Davidson, P. M. (1993). Parabens and phenolic compounds. New York, Marcel Dekker.
- Davidson, P. M. (1997). Chemical preservatives and natural antimicrobial compounds. Washington, ASM.
- Davidson, P. M. (2004). Antimicrobial Agents Definition. S. Gaysinsky.
- Davidson, P. M. and A. S. Naidu (2000). Phyto-phenols. Boca Raton, Florida, CRC.
- Davidson, P. M. and M. E. Parish (1989). "Methods for testing the efficacy of food antimicrobials." Journal of Food Technology **43**: 148-155.
- Deans, S. G. and G. Ritchie (1987). "Antibacterial properties of plant essential oils." International Journal of Food Microbiology **5**: 165-180.
- Deans, S. G. and P. Svoboda (1989). "Antibacterial of summer savory (*Satureja hortensis* L) essential oil and its constituents." Journal of Horticulture Science **64**(205-210.).

- Doyle, M. P., L. R. Beuchat, et al. (1997). Food Microbiology: Fundamentals and Frontiers. Washington DC., ASM Press.
- Doyle, M. P., T. Zhao, et al. (1997). Escherichia coli O157:H7. Washington, ASM.
- Farrell, K. T. (1990). Spices, condiments and seasonings. New York, NY., AVI.
- Food and Drugs Administration (FDA) (2001). Foodborne pathogenic microorganism and natural toxins handbook. Bad Bug Book. C. f. F. S. a. A. Nutrition. **2004**.
- Friedman, M. P., P. P. Henika, et al. (2002). "Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*." Journal of Food Protection **65**: 1545-1560.
- Hancock, R. E. W. (1984). "Alterations in outer membrane permeability." Annual Reviews in Microbiology **38**(237-264.).
- Hirasa, K. and M. Takemasa (1998). Spice science and technology. New York, NY., Marcel Deker.
- Holmberg, K. (2002). Handbook of applied surface and colloidal chemistry. New York, NY., Wiley.
- Holmberg, K., B. Jonsson, et al. (2003). Surfactants and polymers in aqueous solutions. New York, NY., Wiley.
- Inouye, S., T. Tsuruoka, et al. (2001). "Effect of sealing and Tween 80 on the antifungal susceptibility testing of essential oils." Journal of Microbiology Immunology **45**: 201-208.

- Juven, B. J., J. Kanner, et al. (1994). "Factors that interact with the antibacterial action of thyme essential oil and its active constituents." Journal of Applied Bacteriology **76**: 626-631.
- Karapinar, M. and S. E. Aktug (1987). "Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole." International Journal of Food Microbiology **4**: 161-166.
- Katharinos, S. (2002). "*Listeria monocytogenes* virulence and pathogenicity, a food safety perspective." Journal of Food Protection **65**: 1811-1829.
- Keweloh, H., G. Weyrauch, et al. (1990). "Phenol-induced membrane changes in free and immobilized *Escherichia coli*." Journal of Applied Microbiology and Biotechnology **33**: 66-71.
- Kim, J., M. R. Marshal, et al. (1995). "Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes." Journal of Food Science. **60**: 1364-1375.
- Kim, J., M. R. Marshal, et al. (1995). "Antibacterial activity of some essential oils against five foodborne pathogens." Journal of Agricultural and Food Chemistry. **43**: 2839-2845.
- Kim, O. H., S. W. Park, et al. (2004). "Inactivation of *Escherichia coli* O157:H7 by cinnamic aldehyde purified from *Cinnamomum cassia* shoot." Journal of Food Microbiology **21**: 105-110.

- Lambert, R. J. W., S. P.N., et al. (2001). "A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol." Journal of Applied Microbiology **18**: 647-650.
- Li, J., M. L. Chikindas, et al. (2002). "Temperature and surfactant induced membrane modifications that alter *Listeria monocytogenes* nisin sensitivity by different mechanisms." Journal of Applied Environmental Microbiology. **68**: 5904-5910.
- Lopez-Malo, A., S. M. Alzamora, et al. (2000). Natural antimicrobials from plants. Gaithersburg, Maryland, Aspen.
- Lugtemberg, B. and L. Van Alphen (1983). "Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria." Biochimica et Biophysica Acta **737**: 51-115.
- MacFaddin, J. F. (2000). Biochemical test for identification of medical bacteria. Philadelphia, PA., Lippincott Williams and Wilkins.
- Mastronicolis, S. K., J. B. German, et al. (1998). "Influence of cold shock on the fatty-acid composition of different lipid classes of the foodborne pathogen *Listeria monocytogenes*." Journal of Food Microbiology **15**(299-306.).
- Menon, K. V. and S. R. Garg (2001). "Inhibitory effect of clove oil on *Listeria monocytogenes* in meat and cheese." Journal of Food Microbiology **18**: 647-650.
- MerkCo. (2001). The merck index. Eahaway, NJ., Merck.
- Moleyar, V. and P. Narasimham (1992). "Antibacterial activity of essential oil components." International Journal of Food Microbiology. **16**: 337-342.

- Nikaido, H. and M. Vaara (1985). "Molecular basis of bacterial outer membrane permeability." Microbiology Reviews **49**: 1-32.
- Noskov, B. A. and D. O. Grigoriev (2001). Adsorption from micellar solutions. Amsterdam, The Netherlands., Elsevier.
- Oka, S. (1964). "Mechanism of antimicrobial effect of various food preservatives." International Symposium of Food Microbiology. **4**: 3-16.
- Pagan, R., S. Condon, et al. (1997). "Effects of several factors on the heat-shock-induced thermotolerance of *Listeria monocytogenes*." Journal of Applied Environmental Microbiology. **63**: 3225-3232.
- Park, S., R. W. Worbo, et al. (2001). "*Escherichia coli* O157:H7 as an emerging foodborne pathogen: a literature review." Critical Reviews in Biotechnology. **21**: 27-48.
- Pletnev, M. Y. (2001). Chemistry of surfactants. Amsterdam, The Netherlands., Elsevier.
- Robinson, R. K., C. A. Batt, et al. (2000). Encyclopedia of Food Microbiology. San Diego, CA, Academic Press. **2**: 1195-1237.
- Rosen, M. J. (1989). Surfactant and interfacial phenomena. New York, NY., Wiley-Interscience.
- Schaechter, M., N. C. Engleberg, et al. (1998). Mechanism of Microbial Diseases. Baltimore, Maryland., Williams and Wilkins.
- Shashidi, F. and M. Naczki (1995). Food phenolics. Sources, chemistry, effects and applications. Lancaster, PA., Technomic publishing.
- Shelef, L. A. (1983). "Antimicrobial effect of spices." Journal of Food Safety **6**: 29-44.

- Shelef, L. A., E. K. Jyothi, et al. (1984). "Growth of enteropathogenic and spoilage bacteria in sage-containing broth foods." Journal of Food science **49**: 737-740.
- Smith-Palmer, A., J. Stewart, et al. (1998). "Antimicrobial properties and plant essential oils and essences against five important food-borne pathogens." Letters in Applied Microbiology **26**: 118-122.
- Sridhal, S. P., R. V. Rajagopal, et al. (2003). "Antifungal activity of some essential oils." Journal of Agricultural and Food Chemistry. **51**: 7596-7599.
- Stauffer, C. E. (1999). Emulsifiers. St. Paul, MN, Egan Press Handbook Series.
- Texter, J. (1999). Characterization of surfactants. Cincinnati, OH., Hansen.
- Ulh, S. R. (2000). Handbook of spices, seasoning and flavoring. Pennsylvania, Technomic.
- Ultee, A., L. G. M. Gorris, et al. (1998). "Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*." Journal of Applied Microbiology. **20**: 211-218.
- Ultee, A., E. P. W. Kets, et al. (1999). "Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*." Journal of Applied and Environmental Microbiology **85**: 221-218.
- Ultee, A. and E. J. Smith (2002). "The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*." Journal of Applied and Environmental Microbiology **63**: 620-624.
- Weiss, J. (1999). Effect of mass transport process on physicochemical properties of surfactant-stabilized emulsions. Food Science. Amherst, MA, University of Massachusetts.

- Weiss, J. and D. J. McClements (2000). "Mass transport phenomena in oil-in-water emulsions containing micelles:solubilization." Langmuir **16**: 5879-5883.
- Wilson, W. W., M. M. Wade, et al. (2001). "Status of methods for assesing bacterial cell surface charge properties based on zeta potential measurements." Journal of Microbiological Methods **43**: 153-163.
- Zottola, E. A. and L. B. Smith (1990). "The microbiology of foodborne disease outbreaks: an update." Journal of Food Safety **11**: 13-29.

Vita

Sylvia Gaysinsky Segura was borne on August 29th 1979 in Mexico City, Mexico. She studied at the Israeli School of Mexico, Mexico City, Mexico, from Kindergarten through High school (1981-1997). . When she was eight years old, she became an active member of her community, joining a Youth Organization that focused on keeping traditions through generations and the forming of leaders. She started as a student and became an instructor later.

She lived in Jerusalem, Israel, during the academic year 1997-1998, where she studied informal education. Sylvia started her B.S. in Food Chemistry in the Department of Chemistry of La Salle University in Mexico City, Mexico in 1998. During the academic period of 2000-2001 she worked in an internship for six months in Elli Lilly and Company, Mexico City, Mexico, in the Good Manufacturing Practices area. During the academic year 2001-2002 she had the opportunity to become an exchange student at the Department of Food Science and Technology at The University of Tennessee, Knoxville, thanks to ISEP (International Student Exchange Program). She also worked at the Department of Food Science and Technology during that summer in the Food Chemistry and Biophysics Laboratories under the supervision of Dr. Jochen Weiss. In the fall 2002 she returned to her home University where she obtained her degree in Food Chemistry. In the fall 2003, she enrolled at the University of Tennessee, Knoxville to pursue her Masters degree in Food Science and Technology under Dr. Weiss' supervision. She was a finalist in the Chemistry Division poster competition at the IFT annual meeting

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