DESIGN AND CHARACTERIZATION OF CAPSAICIN LOADED NANOEMULSIONS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ELİF AKBAŞ

IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

AUGUST 2016

Approval of the thesis:

DESIGN AND CHARACTERIZATION OF CAPSAICIN LOADED NANOEMULSIONS

submitted by **ELİF AKBAŞ** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Alev Bayındırlı
Head of Department, **Food Engineering** _____

Asst. Prof. Dr. Mecit Halil Öztop
Supervisor, **Food Engineering Dept., METU** _____

Dr. U. Betül Söyler
Co-Supervisor, **Food Engineering Dept., METU** _____

Examining Committee Members:

Prof. Dr. Serpil Şahin
Food Engineering Dept., METU _____

Asst. Prof. Dr. Mecit Halil Öztop
Food Engineering Dept., METU _____

Prof. Dr. Servet Gülüm Şumnu
Food Engineering Dept., METU _____

Assoc. Prof. Dr. İlkay Şensoy
Food Engineering Dept., METU _____

Asst. Prof. Dr. Emin Burçin Özvural
Food Engineering Dept., Çankırı Karatekin University _____

Date: 18.08.2016

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Elif Akbaş

Signature :

ABSTRACT

DESIGN AND CHARACTERIZATION OF CAPSAICIN LOADED NANOEMULSIONS

Akbaş, Elif

M.S., Department of Food Engineering

Supervisor: Assist. Prof. Dr. Mecit Halil Öztop

Co-Supervisor: Dr. U. Betül Söyler

August 2016, 197 pages

In recent years, nanoemulsion based systems have been successfully used in food, medical and pharmaceutical applications as effective lipophilic carrier systems for nutraceuticals, drugs, antioxidants and antimicrobial agents. The primary active ingredient of chili pepper, capsaicin is a hydrophobic substance and was proved to be a compound showing good antimicrobial activity against various microorganisms. The aim of the proposed study was to prepare and characterize capsaicin loaded nanoemulsion systems. Nanoemulsions were prepared with emulsifiers Tween 80, lecithin and sucrose monopalmitate (SMP) by using microfluidization and ultrasonication at pH 7.4 and pH 3.8. Effect of glycerol addition and heating the coarse dispersions on nanoemulsion formation were also investigated. Antimicrobial activities of nanoemulsions were evaluated against well-known food pathogens *Escherichia coli* and *Staphylococcus aureus*. In the experiments, 2% capsaicin nanoemulsions decreased *E.coli* population up to 3.4 log after 15 min of contact time by using lecithin and *S. aureus* population up to 5.89 log after 2 hours of contact time by using Tween 80. The smallest particle size of 33.17 nm was obtained using SMP

with microfluidization. Addition of glycerol to the continuous phase showed enhanced effect on the results for both homogenization types. Moreover, nanoemulsions processed by microfluidization exhibited enhanced physical properties and antimicrobial activity. NMR relaxometry technique helped to track changes in nanoemulsions that was associated with the other methods such as particle size, turbidity. In overall, nanoemulsions with improved functionality were obtained using capsaicin.

Keywords: Capsaicin, nanoemulsion, NMR Relaxometry, antimicrobial activity

ÖZ

KAPSAİSİN YÜKLÜ NANOEMULSİYONLARIN TASARLANMASI VE KARAKTERİZASYONU

Akbaş, Elif
Yüksek Lisans, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Yrd. Doç. Dr. Mecit Halil Öztop
Ortak Tez Yöneticisi: Dr. U. Betül Söyler
Ağustos 2016, 197 sayfa

Son yıllarda, nutrasötikler, ilaçlar, antioksidanlar ve antimikrobiyal ajanların taşınmasında etkili lipofilik sistemler olan nanoemülsiyon sistemleri gıda, tıp ve ilaç alanlarında başarıyla kullanılmaktadır. Literatürde, acı biberin birincil aktif bileşeni olan ve hidrofobik bir bileşik olan kapsaisinin çok çeşitli mikroorganizmalara karşı antimikrobiyal aktivite gösterdiği kanıtlanmıştır. Bu çalışmanın hedefi, kapsaisin yüklü nanoemülsiyon sistemlerinin hazırlanması ve karakterizasyonunun yapılmasıdır. Nanoemülsiyon oluşumu için sürfektan olarak Tween 80, lesitin ve sükroz monopalmitat kullanılmış olup; homojenizasyon teknikleri olarak mikroakışkanlaştırıcı (MA) ve ultrasonikasyonla (US) birlikte emülsiyonlar pH 7.4 ve pH 3.8 olmak üzere ki farklı pH'da hazırlanmıştır. Gliserol eklenmesi ve sıcaklığın nanoemülsiyon oluşumuna etkileri de ayrıca incelenmiştir. Antimikrobiyal aktivite deneyleri bilinen gıda patojenleri olan *Escherichia coli* ve *Staphylococcus aureus* ile gerçekleştirilmiştir. %2'lik kapsaisin oleoresin içeren ve lesitin kullanılan nanoemülsiyonların 15 dakika temas süresi sonunda *E. coli* populasyonunda 3.4 log ve benzer şekilde Tween 80 kullanılan nanoemülsiyonların 2 saat temas süresi sonunda *S. aureus* populasyonunda 5.89 log düşüşe sebep olduğu görülmüştür. SMP ile hazırlanan nanoemulsiyonların parçacık boyutu 33.7 nm ile en küçük olarak bulunmuştur. Sürekli faza gliserol eklenmesinin her iki sistem üzerinde (MA ve US)

olumlu etkilerinin olduğu gözlenmiştir. MA yoluyla işlenmiş nanoemülsiyonlar, gelişmiş fiziksel özellikle birlikte antimikrobiyal aktivite göstermiştir. NMR relaksometre ile nanoemülsiyonların yapılarında meydana gelen değişiklikler gözlenip parçacık boyutu, bulanıklık gibi diğer metotlarla ilişkilendirilmiştir. Çalışma sonucunda elde edilen veriler doğrultusunda nanoemülsiyon sistemi ile etkisi zenginleştirilmiş, gıdanın ihtiyacına yönelik kapsaisin içeren nanoemülsiyon sistemlerinin geliştirilebileceği gösterilmiştir.

Anahtar Kelimeler: Kapsaisin, nanoemülsiyon, NMR Relaksometre, antimikrobiyal aktivite

To my beloved mother and sister.

ACKNOWLEDGEMENTS

It is a pleasure to express my deepest thanks and gratitude to my advisor and guide Asst. Prof. Mecit Halil Öztop. I am so grateful for his patience and tremendous attitude to help during my work. It's been an honor and a privilege working with him and be involved in his project. Thank you again for making me a part of your team.

My sincere gratitude is extended to my co-advisor Dr. Betül Söyler for her expert, cordial and valuable guidance and encouragement during my research and lifetime experience. She gave me hope when I had none.

I am extremely thankful to İrem Alaçık for her help and care. She treated me like her sister from the first day in lab meeting until now. I am also thankful to Selen Güner, Simge Tutku Yıldırım, Şirvan Sultan Uğuz, Damla Dağ, Pelin Poçan, Çağrı Helin Karaçam and rest of Öztop Lab members for their supports and endless help. They were more than just friends and made my life at METU unforgettable. I am so glad that distance does not separate our friendship. I wish to thank my business partner Mete Kilercioğlu for his insistent encouragement to keep going on business. I also would like to thank Çağrı Çavdaroğlu for looking after me in late hours in school and the time we spent together.

This study was funded by National and Scientific Technological Council of Turkey (TUBITAK), Short Term R&D Funding Program (1002) with proposal number 214O436. The grant not only provided the research funding but also supported me financially through my study. I would like to thank them for giving me this opportunity to complete my degree.

I would like to express my special thanks to Yiğit Can İşmen for his continuous support and encouragement throughout my life.

Last but not least, I owe a deep sense of gratitude to my family; my mother Benan Koç and my sister Begüm Esra Akbaş. Their support, advice, and limitless patience made me who I am today. I dedicate this work to my family.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

The shift towards fresh and natural food has gained momentum in the past few years as consumers become more interested in preferring minimally processed, physiologically safe and high quality foods free from synthetic ingredients. These consciousness have pushed food manufactures to make radical changes on the products supplied. During processing or storage, food products become susceptible to the physical, chemical and microbiological changes. To improve quality and shelf life of a food product, food manufacturers use different methods for preserving. These methods include heating, cooling, decreasing water activity, curing, salting, pH control, additives such as antimicrobial substances, controlled atmosphere packaging or modified atmosphere packaging (Ayana, 2007). A green and natural alternative to providing long term food safety could be the use of essential oils or other plant extracts as they were shown to exhibit good antioxidant and antimicrobial properties.

The application of nanoemulsions to food, medical and pharmaceutical industries has received great attention among the researchers especially for encapsulation of functional components such as plant extracts for controlled release purposes and ensuring the prevention of degradation of active substances through increased rates of bioavailability and providing antimicrobial efficacy.

1.1 Functional Ingredients Used in Nanoemulsion Formulations

1.1.1. Essential Oils

Aromatic plants include oily liquids on different parts of the plants (i.e. flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). For centuries, aromatic plant materials were used for pharmaceutical purposes but after 19th and 20thcenturies, they were used as additives providing flavors, fragrances and for preservative effects on foods and beverages through their active components (Guenther, 1948). For this reason, scientific interest has increased in these natural, complex substances to investigate the effects and broaden the application areas. Essential oils are obtained from these parts by the commercially mostly steam distillation technique (Van de Braak and Leijten, 1999). Essential oils mainly consist of terpenes (mono-, sesqui- and diterpenes) and aromatic compounds and also alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulfide (Bakkali et al., 2008; Pichersky, Noel, & Dudareva, 2006). Concentration of these compounds changes with extraction methods, harvesting time or what fragment of the plant is used (Fathi&Sefidkon, 2012; Novak, Draxler, Gohler, & Franz, 2005; Olawore, Ogunwande, Ekundayo, & Adeleke, 2005). Several components in the oil are responsible for the antimicrobial activity (Bajpai, Baek, & Kang, 2012). Overall, the phenolic compounds are the crucial components that are related to the antimicrobial activity of essential oils.

1.1.1.1 Mechanisms of microbial inactivation

The inhibition mechanism of these phenolic compounds against microorganisms is not clearly explained but there are possible theories about its action. Mainly, the key roles of essential oils are penetration and also disruption of cell membranes due to their hydrophobicity that cause leakage of ions or vital components of the cell (Burt, 2004). Although relatively slight leakage could be renovated, high amount of solute, ion transport or the crucial contents leakage could result in cell death eventually

(Denyer and Hugo, 1991a; Cox et al., 2001). In addition, according to several studies, phenolic compounds inhibit cellular energy generation system (ATP) and break proton motive force (PMF) (Denyer and Hugo, 1991b; Sikkema et al., 1995; Davidson, 1997). In one study, carvacrol and thymol were used against Gram-negative bacteria and the mode of actions were appeared to be the breakage of the cell membrane, dissociation of lipopolysaccharides and change in the intracellular and extracellular level of ATP (Ultee et al., 2002 ; Helander et al., 1998). Lambert et al. (2001) studied the effect of oregano essential oil against *S. aureus* and *P. aeruginosa* and concluded that dissipation of phosphate ions caused the inhibition of microorganisms. Clove oil is another essential oil containing eugenol as the major component and it was shown in a study that eugenol restricted the generation of the enzymes amylase and proteases of *B. cereus* and inhibited cell wall integrity resulting in cell lysis (Thoroski et al., 1989). Cinnamon oil and its major component cinnamaldehyde affected binding properties of proteins and inhibited the enzyme activity of amino acid decarboxylases in *E.aerogenes* (Wendakoon and Sakaguchi, 1993).

1.1.1.2 Inhibitory effect on Gram-positive and Gram- negative bacteria

The mode of actions of essential oils and its components differ against Gram-positive and Gram-negative microorganisms due to the differences in their cell membrane structures. When essential oils contact with the cell membrane of Gram-positive bacteria, it was reported that they showed more inhibitory effect than Gram- negative bacteria. The main reason behind this was associated with the hydrophobicity differences between cell membranes (Shelef, 1983; Smith-Palmer et al., 1998; Chao & Young, 2000; Cimanga et al., 2002; Sokovic et al., 2010). Gram- negative bacteria contain a hydrophilic cell membrane outside the cell wall that resists the antimicrobial actions of hydrophobic essential oils (Calsamiglia et al., 2007; Ravichandran, Hettiarachchy, Ganesh, Ricke, & Singh, 2011). Smith-Palmer et al. (1998) studied 21 essential oils against five food-borne pathogens, Gram-negative *Campylobacter*

jejuni, Salmonella enteritidis, Escherichia coli and Gram-positive *Staphylococcus aureus* and *Listeria monocytogenes*. Among essential oils, the ones obtained from bay, cinnamon, clove and thyme oil with less than 0.075% showed inhibitory effect on all Gram-positive bacteria. In order to inhibit Gram negative bacteria higher amounts (>0.075%) of essential oils were needed. In another study effect of camphor, carvacrol, 1, 8-cineole, linalool, linalyl acetate, limonene, menthol, α-pinene, β-pinene, and thymol were investigated against *Bacillus subtilis, Enterobacter cloacae, Escherichia coli O157:H7, Micrococcus flavus, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteritidis, S. epidermidis, S. typhimurium,* and *Staphylococcus aureus* and carvacrol showed the highest activity (Sokovic et al. 2010). Hammer et al. (1999) investigated 52 essential oils and their extracts against *Acinetobacter baumanii, Aeromonas veronii biogroup sobria, Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica subsp. enterica serotype typhimurium, Serratia marcescens,* and *Staphylococcus aureus.* Lemongrass, oregano, and bay oils showed an inhibitory effect against all microorganisms at 2% (v/v) concentration. However, apricot kernel, evening primrose, macadamia, pumpkin, sage and sweet almond didn't cause inhibition at the same concentration. On the other hand, thyme oil showed the minimum inhibitory activity of 0.03% (v/v) against *C. albicans* and *E. coli* and also vetriver oil had the minimum inhibitory activity of 0.03 % (v/v) for *S. aureus.*

1.1.1.3 Challenges of using essential oils in food systems

As mentioned above, essential oils have the potential to be used as antimicrobial agents for various microorganisms. However, incorporating essentials as antimicrobial agents to food systems could be challenging due to hydrophobicity difference. There are also other factors that could limit the activity of essential oils in food matrices such as pH, water activity, fat and/or protein content, enzyme activity (Burt, 2004; Firouzi, Shekarforoush, Nazer, Borumand, &Jooyandeh, 2007; Friedly

et al., 2009). It was reported by many researchers that the concentrations of essential oils that were used to inhibit microorganisms in laboratory scale were not enough for food systems which required 100 fold higher amounts (Burt, 2004; Solomakos et al., 2008). Thus, this could cause undesirable taste or odor of the essential oil and induce extra costs. Therefore, a carrier system must be engaged to improve dissolution in an aqueous food system, ensure protection from chemical or physical decomposition, reduce strong taste or odor of essential oils, and help facilitation of transport through microorganisms (Weiss et al., 2009). In that regards, nanoemulsion systems are highly compatible carriers for lipophilic essential oils to fulfill the needs mentioned.

1.1.2. Other plant extracts

There are several active plant extracts other than essential oils used as antimicrobials. Rauha et al. (2000) investigated 29 plant extracts obtained from Finnish plant materials against *Aspergillus Niger, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Staphylococcus aureus* and *Staphylococcus epidermidis*. Among extracts, the ones obtained from chamomile, onion, small cranberry, potato peel, raspberry against *E. coli*; raspberry, pine, small cranberry against *S. aureus*; cloudberry against *S. epidermidis, M. luteus*; purple loosestrife against *Candida albicans* showed inhibitory activity. It was stated that the main active component of these antimicrobial activities came from flavonoids.

Also, polyphenols are widely known antimicrobials and antioxidants extracted from plants. In a work conducted by Bubonja-Sonje et al., (2011), the polyphenol rich rosemary, olive, and cocoa bean were used against *Listeria* spp. The rosemary extract showed the highest antimicrobial activity with minimum inhibitory content (MIC) at 0.083 mg/ mL whereas the olive oil extract had MIC of 0.4 mg/mL. The cocoa extract, on the other hand, could not show bactericidal activity at a concentration of 6.4 mg/mL The antimicrobial activity of rosemary comes from diterpenes, carnosol and carnosic acid (Başer & Buchbauer, 2010). Also, the oleuropein (secoiridoides) is one

of the major phenolic compounds obtained from olive leaves that shows antibacterial activity (Bisignano et al., 1999; Furneri, Piperno, Sajia, & Bisignano, 2004). Recently, it has received great attention due to its antioxidant, anti-tumor and anti-inflammatory properties (Barbaro et al., 2014). In a study, olive leaf extract was used against various bacteria strains. Among them, it showed highest inhibitory activity against *Campylobacter jejuni*, *Helicobacter pylori*, *Staphylococcus aureus* and MRSA (meticillin-resistant *S. aureus*) with MICs ranging from 0.31–0.78% (v/v) (Sudjana et al., 2009). The widely known flavoring spice, cumin extract was used against *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* (Ani, Varadaraj, & Naidu, 2006). Obviously, many substances are responsible for antimicrobial activity such as terpenes, flavonoids, phenolic compounds and also alkaloids. Among alkaloids capsaicin deserves special attention and specifically in this study, it was used to formulate nanoemulsions.

1.1.3. Oleoresin Capsicum

Chili peppers are members of the *Capsicum spp*. under *Solanaceae* family. *Capsicum annuum*, *Capsicum frutescens*, *Capsicum Chinese*, *Capsicum pendulum* and *Capsicum pubescens* are the commonly known species (ASTA, 1995). For industrial applications, mostly *Capsicum annuum* and *Capsicum frutescents* are preferred (Al Othman, Ahmed, Habila, & Ghafar, 2011).

Capsaicin was first isolated from *Capsicum* fruit and named by Tresh in 1876 (Nelson & Dawson, 1923).Then, studies showed that there were other capsaicinoids such as dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicins, caprylic acid vanillyl amide, nonylic acid vanillyl amide and decylicacidvanillyl amide (Figure 1.1). However, capsaicin and dihydrocapsaicin constitute 90% of the total capsaicinoids in chili peppers (De Lourdes Reyes-Escogido, Gonzalez-Mondragon, & Vazquez-Tzompantzi, 2011). White and odorless, lipophilic alkaloid capsaicin (trans-8-methyl-N-vanillyl-6- nonenamide) is in a crystalline state and soluble in alcohol and oil. Its melting point is between 57-66 ^{o}C and has a molecular

weight of 305.40 g/mol. The pungent sense of chili peppers comes from the capsaicinoids which are primarily associated with the capsaicin that is also represented by the Scoville Heat Units (SHU) (Scoville, 1912). Species, growth conditions, harvesting time, extraction method could all affect the pungency level. The Scoville units of various capsaicinoids are illustrated in Table 1.1.

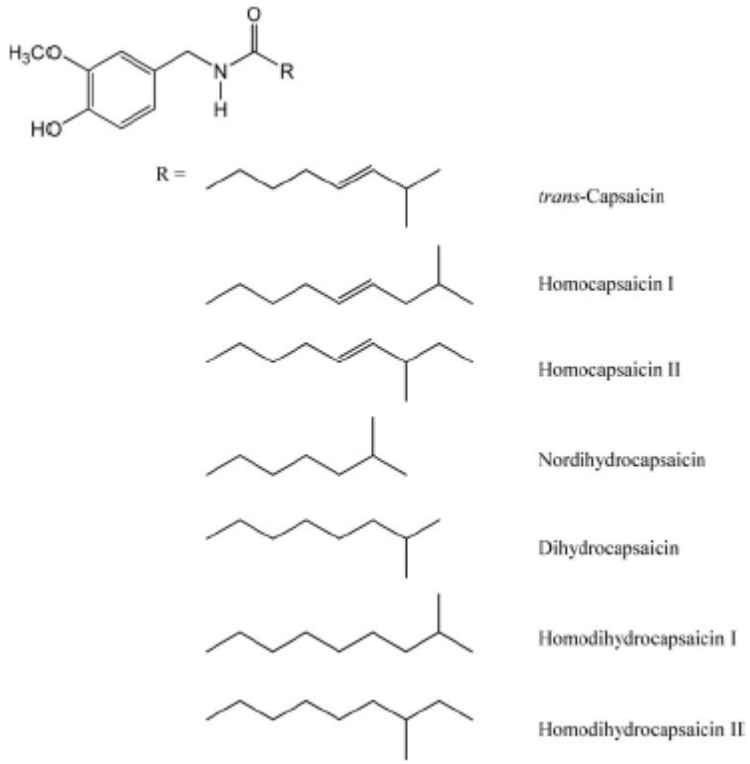

Figure 1.1 The molecular structures of capsaicinoids (Asnin & Park, 2013).

Table 1.1 Pungency level of capsaicinoids according to Scoville (Scoville, 1912).

Capsaicinoids	Scoville Heat Units
Capsaicin	16,000,000
Dihydrocapsaicin	15,000,000
Nordihydrocapsaicin	9,100,000
Homocapsaicin, Homodihydrocapsaicin	8,600,000

Capsaicin is important for food and pharmaceutical applications. It contributes to the aroma, taste, and color of foods. Several studies reported that capsaicin showed analgesic (Deal, 1991; McCarthy and McCarthy, 1991; Derry et al., 2009), antitumor (Anandakumar et al., 2012; Lee et al., 2010), antioxidant (Park et al., 2000; Okada & Okijama, 2001; Prasad et al., 2004), antimicrobial (Cichewicz& Thorpe, 1996; Molina-torres, Garcı, & Ramı, 1999), anti-inflammatory (Reddy and Lokesh, 1994; Joe and Lokesh, 1997; Surh et al., 2005) and ulcers, obesity inhibitory (López-Carrillo et al., 2003;Kawada et al., 1986) properties. The inflammatory effects of capsaicin such as burning and irritating of hands, mouth and temporary blindness of eyes are the basis of self-defense sprays (Al Othman et al., 2011). In this study, it was aimed to examine primarily the antimicrobial properties of capsaicin.

The pure capsaicin is colorless, odorless, crystalline compound (Srinivasan, 2015). Commercially, capsaicin is obtained via extraction from *Capsicum spp.* fruit which also includes pigments, waxes, and resins (Choi, Kim, Cho, Hwang, & Kim, 2009; Kanakdande, Bhosale, & Singhal, 2007). This form is called as oleoresin capsicum. The visual difference between these two forms can be seen in Figure 1.2. In the food

industry, capsaicin is usually used in oleoresin form. Also in this study oleoresin capsicum is used as the capsaicin source.

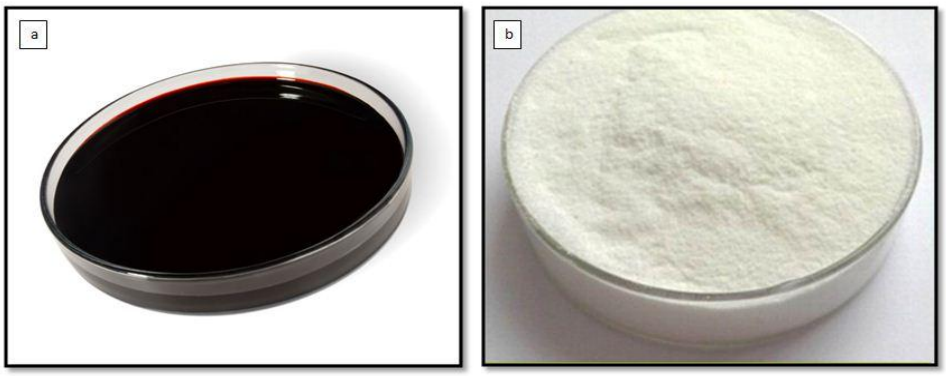

Figure 1.2 a) Oleoresin capsicum; b) Pure capsaicin.

1.1.4. Antimicrobial effect of Oleoresin Capsicum

There are several studies that explored the antiviral, antiparasitic, antifungal and antibacterial properties of capsaicin. A few studies were concluded that herpes simplex virus could be suppressed by capsaicin (Bourne et al., 1999; Ljungdahl et al., 1986; Stanberry et al., 1992). In another study, *Capsicum annum* extracts were used as antiparasitic for the cercariae of *Schistosoma mansoni* (Frischkorn, et al., 1978). Also, it was found that growth of human and animal pathogenic microorganism, *Bacillus subtilis* could be inhibited by capsaicin after 48 hours of incubation (Stephen & Kumar, 2014). In another study that was carried out for well-known microorganisms such as *Staphylococcus aureus, Salmonella enteric* and *Escherichia coli* capsaicin played an important role in inactivation at different levels (Dima, Coman, Cotarlet, Alexe, & Dima, 2013).

However, 300 µg/mL of capsaicin was not able to show antifungal activity against *Saccharomyces cerevisiae* after 24 hours of incubation whereas the same concentration retarded the growth of *E.coli* (Molina-torres et al., 1999).

An alternative food packaging material production study was conducted with 4 different types of hot peppers (*Green Malagueta Salvador, Red Malagueta Salvador, Red Thai Capsicum frutescents* and *Red Cayenne*) to produce antimicrobial films of the extracts, capsaicin and dihydrocapsaicin. Inhibition was observed at 50 g/L, 100 g/L and 150 g/L concentrations against Gram-negative *E. coli*, Gram-positive *Streptococcus and B. subtilis* respectively (Leng, Muhamad, Zaidel, & Khairuddin, 2013).

1.2. Functional delivery systems

As mentioned previously, for functional ingredients to provide bioactivity through microorganisms, they need carriers to eliminate limitation during processing and storage. For instance, microencapsulation of essential oils with different formulations may be a good strategy to ensure protection towards chemical or physical degradation or to increase mass transfer rates through target microorganism.

However, nanoencapsulation techniques serve a better solution through enhancing their bioactivity with depositing on the microorganism's surface due to nanoscale particle sizes and high surface areas (Weiss, Gaysinsky, Davidson, & McClements, 2009). Nanoemulsions, nanoparticles such as nanospheres, nanocapsules, solid lipid nanoparticles, liposomes, and nanofibers can be used as nanocarrier systems (Blanco-Padilla, Soto, Hernández Iturriaga, & Mendoza, 2014) (Figure 1.3). These systems have successfully been utilized in literature and in this study nanoemulsion systems were used.

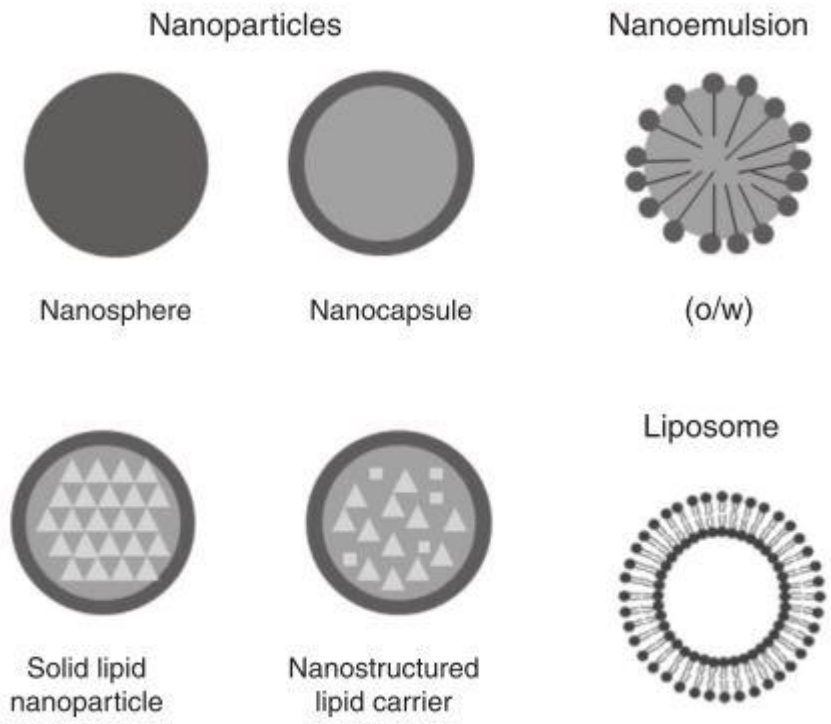

Figure 1.3 Different nano carrier systems (Zorzi et al., 2015).

1.2.1. Properties of nanoemulsions

Emulsions are mixtures of immiscible oil and water phase. In emulsions, the liquid that forms the droplet is called as the dispersed phase while the liquid surrounding the droplet is called as continuous phase (McClements, 1999). Kinetically, emulsions are thermodynamically unstable and tend to phase separate (Israelachvili, 1992). By adding emulsifiers, emulsions become a kinetically stable system without observing phase separation for a period of time. The surface active agents or the so-called emulsifiers in food systems create a barrier between the two phases and provide resistance to flocculation or coalescence due to repulsive forces (McClements, 2012).

Nanotechnology is focused on the production, processing, and characterization of the structures smaller than 100 nm (Quintanilla-Carvajal et al., 2010). Although the general trend to classify particles as nano was set to 100 nm or lower, emulsions which have particle sizes 200 or 500 nm could also be considered as nanoemulsions (Abbaszadeh, Sharifzadeh, Shokri, Khosravi, &Abbaszadeh, 2014; Bouchemal, Briançon, Perrier, & Fessi, 2004; Choi et al., 2011a; Donsì, Sessa, & Ferrari, 2012; McClements, 2012).

As mentioned previously, nanoemulsions are used as carrier systems to boost the durability and bioavailability of hydrophobic substances such as capsaicin. In a pharmaceutical study, capsaicin was used as the oil phase in a nanoemulsion. By using nanoemulsions prepared with 10 mg / kg concentrations of oleoresin capsicum on mice, 131.7 times more bioavailability and long half-life were observed compared to controls that included only oleoresin capsicum (Choi et al., 2013). Also, oleoresin capsicum was used as a therapeutic agent in the treatment of skin diseases in a study. Skin diffusion of capsaicin loaded nanoemulsions was found to be more effective and was confirmed by confocal laser scanning microscopy images (CLSM). The study suggested using the designed system to formulate a transdermal release system (Kim et al., 2014b).

In another study, self-assembly method was used to produce oleoresin capsicum containing nanoemulsions which were stabilized by chitosan and alginate as biopolymers. While the particle sizes of the double layer and triple layer nanoemulsions produced were less than 20 nm, the stability was achieved by the self-assembly method (Choi, Kim, Cho, Hwang, & Kim, 2011a).

1.2.2. Emulsifying Agents (Emulsifiers)

To stabilize an oil and water dispersion, emulsifiers are used generally. By lowering the interfacial tension as well as the surface energy required to form droplet between these two immiscible liquids, emulsifiers promote the dissipation of one phase into

the other. This also lowers the energy input to produce a nanoemulsion. Besides, an emulsifier is able to adsorb to the droplet surface and forms an interfacial barrier like a film around the droplet to protect the droplet from coalescence or aggregation during storage (Karlene and Derick, 2006; Garti, 2002). The type and amount of emulsifier are strongly related with the homogenization approach followed and also the emulsion stability. There are three main categories of emulsifiers that are commonly used in emulsion formation: ionic, nonionic and zwitterionic emulsifiers.

Ionic emulsifiers are capable of providing positive or negative electrical charge to the droplets. The citric acid ester of mono- and diglyceride of fatty acids (CITREM), diacetyl tartaric acid ester of mono- and diglycerides (DATEM), and sodium dodecyl sulfate (SDS) are anionic emulsifiers whereas, lauric arginate is cationic emulsifier (McClements & Rao, 2011).

Nonionic emulsifiers are commonly used emulsifiers both in high and low energy homogenization methods that generate a steric barrier with their bulky molecular sides through the outside of the dispersed phase. Sorbitan monooleate, sucrose monopalmitate are the sugar esters whereas Tweens, Spans are the ethoxylated sorbitan esters included to nonionic emulsifiers (Grigoriev and Miller, 2009).

If the emulsifier has more than two oppositely charged ionizable group, it is called *zwitterionic emulsifier*. Emulsion's pH determines the charge of the emulsifier and it could be negative, neutral or positive. Phospholipids are the most commonly used zwitterionic emulsifiers (Trotta et al., 1996; de Morais et al., 2006). Lecithin, which is a phospholipid, consists of a phosphate group and is esterified with two fatty acids linked to a glycerol backbone. It is a natural emulsifier obtained from egg, soybeans, sunflower kernels, and rapeseed and is widely used in food, cosmetic and pharmaceutical industry (Hoeller, Sperger, & Valenta, 2009; Xue & Zhong, 2014).

Small molecule emulsifiers are widely used in the production of nanoemulsions. Non-ionic emulsifiers such as Tween 80, sucrose monopamitate are mainly used for this purpose due to their high hydrophilic-lipophilic balance (HLB=16, 15, respectively)

(Garti, Clement, Leser, Aserin, & Fanun, 1999; Piorkowski & McClements, 2013; Rao & McClements, 2013). HLB values are determined according to the ratio of hydrophilic and lipophilic groups of the emulsifier molecules and is used to express the behavior of emulsifier in the emulsion. Emulsifiers which have HLB values greater than 10 are mainly water soluble and stabilize O/W emulsions and form micelles (Maali & Mosavian, 2013).

1.2.3. Preparations of nanoemulsions

Breaking the energy barrier between oil and water phase or changing the particle size of the droplets require energy input which are either achieved by additional ingredients such as emulsifiers at high concentrations or mechanical agitation such as homogenizers (McClements, 1999). Nanoemulsions can be obtained by using low and high energy homogenization methods (Acosta, 2009; Leong, Wooster, Kentish, & Ashokkumar, 2009; Piorkowski & McClements, 2013; Tadros, Izquierdo, Esquena, & Solans, 2004).

1.2.3.1. High energy methods

Various oil and emulsifier types can easily integrate with high energy devices to produce small sized nanoemulsions. Although the particle size depends on many factors, droplet disruption by intense energy input drives the system to create small particles (McClements & Rao, 2011). Generally, this process follows three basic steps; 1) droplet production, 2) break down of macro sized droplets into small ones, 3) emulsifier absorption to the interface which stabilizes the final nanoemulsion (Anton, Benoit, & Saulnier, 2008). High pressure homogenization, ultrasonication, and high speed devices could be used to form nanoemulsions (Silva, Cerqueira, & Vicente, 2011).

High speed devices are usually used to disperse system ingredients and thus, create usually micrometer sized coarse emulsions. The energy produced from the device

dissipates and turns to heat. Samples were immersed in the ice-water bath to avoid adverse effects of temperature rise (Anton et al. 2008; Walstra, 1993). High pressure homogenizators such as a microfludizer or ultrasonicators have a number of advantages over high speed devices: higher efficiency, availability to scale up, using without the addition of organic solvent (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2013b).

1.2.3.1.1. Microfluidization

High pressure homogenizer is designed to reduce the particle size significantly by forcing the macro emulsions to pass through a chamber which produces 10-100 MPa pressure and finally reaching high speed levels around 300 m/s. The yield of the system is correlated to the pressure difference between inlet and outlet. Particle size reduction by microfluidizer is obtained first by the separation of the liquid entering the system into two separate flows and after the collision of these two high-speed flow as illustrated in Figure 1.4 (Rodriguez and Xamani, 2003). Using a microfluidizer provides high yield and with this technique, usually small particles could be obtained (Woodle and Papahadjopoulos, 1989). The most important parameters in this techniques are the applied pressure and number of passes that samples are exposed (Lee & Norton, 2013).

The processing time compared to other methods of homogenization in a microfluidizer is shorter and the system can handle both continuous as well as the batch processing operations (Kulshreshth et al. 2009). The main advantage of the microfluidizer is that the designed system at the laboratory scale can be easily transported to the industrial level (higher scale) (Rodriguez and Xamani, 2003; Memoli et al., 1995; Barnadas- Rodriguez and Sabes, 2001; Kulshreshth et al. 2009).

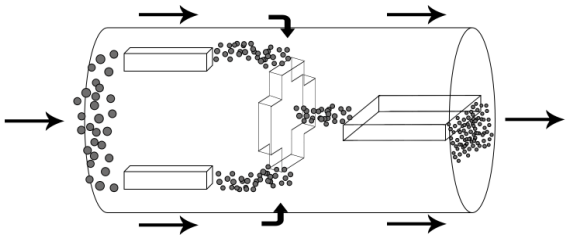

Figure 1.4 Schematic representation of the working principle of microfluidizer (Kırtıl & Öztop, 2014).

In a study, lemongrass oil dispersions were treated through microfludizer with 10 passes at 1,500 bar pressure and particle size decreased from 1410 nm to 6 nm (Salvia-Trujillo, Rojas-Graü, et al., 2013b). This result was also confirmed by using transmission electron microscopy and atomic force microscopy. Even, 3 passes of microfluidization reduced the particle size of β-carotene nanoemulsions from 416 nm to 97.2 nm at 120 MPa pressure and also provided stability during 5 weeks of storage at room temperature (Jo & Kwon, 2013).

1.2.3.1.2. Ultrasonication

Ultrasonication is another commonly used method for preparing nanoemulsions. The principal of sonication is based on the application of sound waves in the frequency range of 16 and 500 kHz and resulting in cavity formation in the sample. Cavitation creates micro-bubbles at the interface of continuous and dispersed phases (Figure 1.5). The formation and collapse of these bubbles induce localized high pressure and temperature rise and a turbulent flow at high speed. This small and temporary turbulence generate high shear that results in the break up of droplets (Abbas et al., 2013; Jiang et al., 2002a).

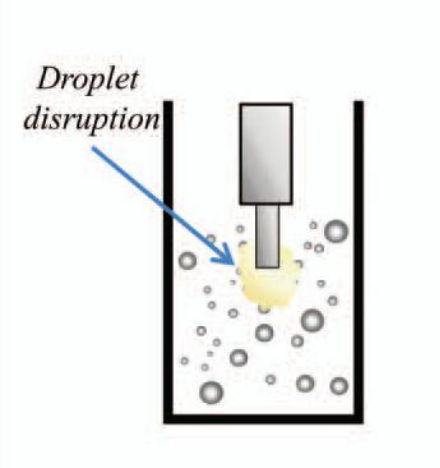

Figure 1.5 Schematic representation of the working principle ultrasound (McClements & Rao, 2011).

In a study, nanoemulsions with average particle size of 135 nm were obtained by using flaxseed oil, water, and emulsifier Tween 40 through ultrasonication process (Kentish et al., 2008). Also, after 60 seconds of ultrasonication process at 24 KHz frequency, the particle size of modified starch, whey protein concentrate, and d-limonene emulsion decreased from 9991 nm to 522 nm (Mahdi Jafari, He, & Bhandari, 2006).

1.2.3.2. Low energy methods

Although low energy methods create long term stability and small particle sizes, high amounts of synthetic emulsifiers (>6%), which are generally non-ionic emulsifiers such as Tween 80 and Span 80, are used. Emulsifier concentration seriously alters the particle sizes of the emulsions. However, high concentrations of emulsifiers could restrict the use of these methods during food processing. Instead, high energy approaches are preferred to use synthetic emulsifier at a low concentration during processing (Anton et al., 2008; Bouchemal et al., 2004; McClements & Rao, 2011;

Tadros et al., 2004). As the low-energy method, spontaneous emulsification and phase-inversion methods are often used (Anton et al., 2008).

1.2.3.2.1. Spontaneous emulsification

Two phases which are organic and aqueous phases are mixed to form nanoemulsions in spontaneous emulsification. The organic phase is composed of oil, hydrophobic emulsifier, and a water-miscible solvent, whereas the aqueous phase is composed of hydrophilic emulsifier and water. The droplets are generated spontaneously due to diffusion of one phase to another (Figure 1.6). Mixing conditions and proper emulsifier/oil/water are required to obtain a stable emulsion. In their research, Yang et al. (2012) compared the microfluidization and spontaneous emulsification to produce nanoemulsion by using medium chain triglycerides and Tweens (Tween 80, Tween 85, and Tween 80/Tween 85). Both processes formed fine droplets with particle sizes less than 100 nm. They concluded that high energy process could use a lower surfactant to oil ratio (SOR< 0.1), but low energy process was very simple and the process needed only mixing even if the surfactant to oil ratio was higher than 0.5 (SOR> 0.5) (Yang et al., 2012).

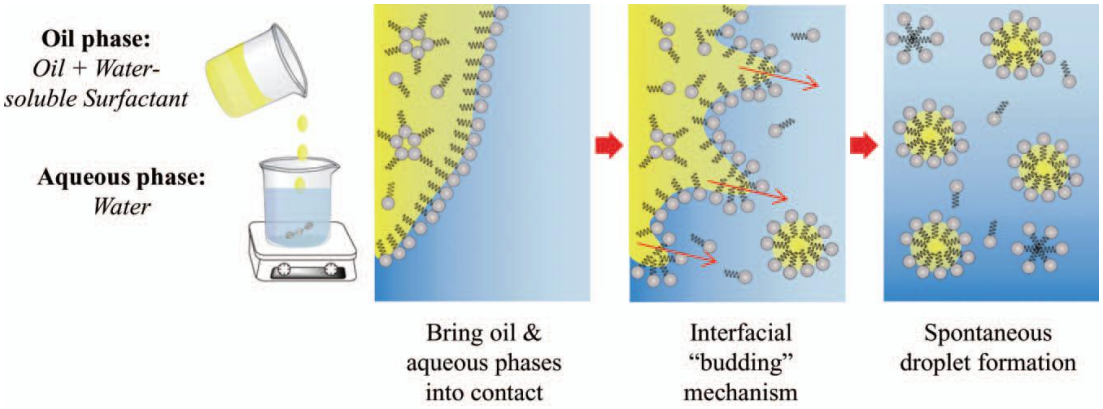

Figure 1.6 Schematic diagram for spontaneous emulsification (Yang et al., 2012).

1.2.3.2.2. Phase-inversion method

In this method, emulsifier solubility is changed by the temperature or the composition at the oil-water interphase during phase-inversion process. The O/W emulsions are changed to W/O emulsions. If the transition occurs with temperature, this is called phase inversion temperature method (PIT) as shown in Figure 1.7-A. This phenomenon is based on the physicochemical changes that occur with temperature. If the transition occurs with changing composition such as adding salt or changing pH, this is called phase inversion composition method (PIC) as in Figure 1.7-B. Roger et al. (2011) compared the PIT and PIC methods. It was found that metastable emulsions with a particle size around 100 nm could be produced by using octa ethylene hexadecyl ether as an emulsifier and hexadecane as the oil phase through PIC method. Also, It was determined that PIT resulted in smaller and narrow sized droplets than PIC method.

1.2.4. Characterization of nanoemulsions

After formulating nanoemulsions, characterization techniques are performed to assess the system properties and emulsion's stability.

1.2.4.1. Zeta potential

Zeta potential represents the electrical charge characteristic of the droplets and also is related to the stability of emulsions. The liquid medium with the emulsifier surrounds the particle and thus creates a layer. This attachment is driven by opposite charges between the droplet and the medium. Zeta potentials higher than ±30 mV define the high stability that resists flocculating or coalescence. The reason is that low charged particles cannot overcome the repulsive force between droplets in the dispersion and begin to aggregate (Silva et al., 2011).

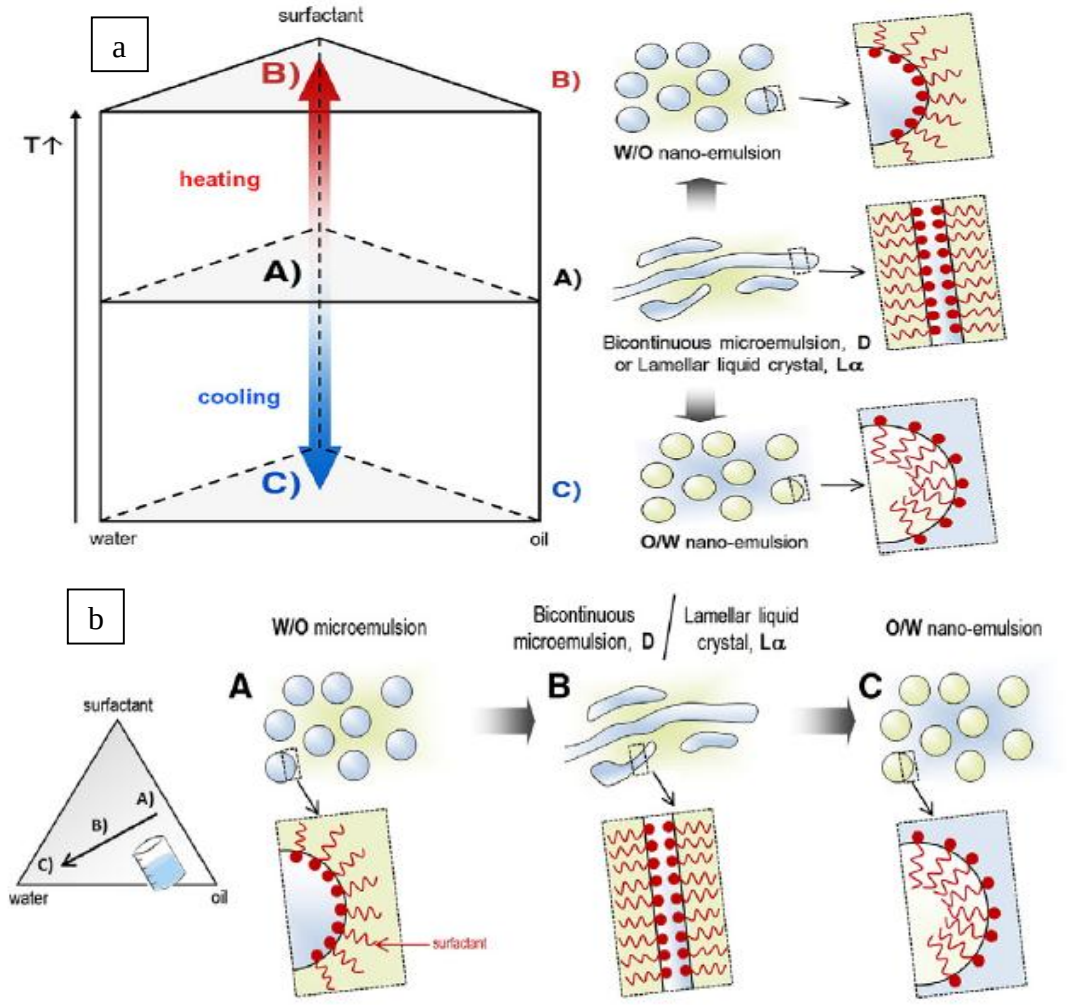

Figure 1.7 Schematic representation of the formation of nanoemulsions by phase inversion methods: a) PIT, b) PIC (Solans & Solé, 2012).

1.2.4.2. Laser Diffraction

This rapid technique is commonly used to measure particle size and distribution of droplets in a suspension or emulsions according to Mie Theory. If a beam of light falls onto a particle, it can absorb, reflect, refract or scatter the light. During

scattering, light leaves in many directions with a broad range of angles. Small sized particles scatter the light widely whereas large sized particles scatter the light at narrow angles. The intensity of light is calculated using the refractive index value of the droplets. Thus, the measured scattering pattern determines the particle size and distribution of the emulsion (Karaca, Nickerson, & Low, 2013; McClements, 1999; Surh, Jeong, & Vladisavljević, 2008). The mean particle size of emulsion is given by surface-weighted mean diameter, D [3, 2] (Eq.1.1) or the volume- weighted mean diameter, D [4, 3] (Eq.1.2) where d_i is the mean diameter and $n_i$is the number of droplet in the i^{th} range of size.

$$D[3,2] = \sum n_i.\ d_i^3 / \sum n_i.\ d_i^2 \qquad \text{(Equation 1.1)}$$

$$D[4,3] = \sum n_i.\ d_i^4 / \sum n_i.\ d_i^3 \qquad \text{(Equation 1.2)}$$

1.2.4.3. Microscopy

Electron beams except than light are used to define particle shape and also size by acquiring an image of the emulsion at smaller wavelengths from the light. Transmission and scanning electron microscopy (TEM and SEM) are the most common techniques to examine the microstructure of emulsions. In the SEM analysis, the surface of the sample is subjected to an electron beam at a particular point and absorbed. After that, some of the electron beams are produced by the sample as secondary electrons and these are recorded by varying intensities at each position and are converted to a topographic image of the sample. On the other hand, in the TEM analysis, directed electron beams are absorbed, scattered or transmitted by the sample at each location of the sample and the transmitted beams are collected by series of magnetic lenses and reflected through the fluorescent screen to produce an image of

the sample. Two dimensional cross sectional image of the sample can be obtained from TEM analysis whereas SEM generates three dimensional of the sample with topography. Both types of equipment work under vacuum. Thus, samples must dry and prepare on a flat surface. Unfortunately, the structure of the sample may be damaged during preparation for analysis. This is the main disadvantage of these systems (Hunter, 1993; McClements, 1999).

Atomic force microscopy is another technique that is used to characterize the morphology of the droplets. In AFM analysis, the nanometer-sized probe is held closer to create a repulsive force from the surface of the sample and bending occurs from the surface. Thus, the degree of bending is collected by the sensitive optical system and used to obtain an image of the sample. During the scanning of the surface, this bending can cause structural changes in the sample. As with the other forms of microscopy, the sample should be dry and prepared on a flat surface with a thin layer (McClements, 1999). Although these instruments are very expensive, they are used as a complementary analysis to other techniques, particularly to confirm the particle size measurements.

1.2.4.4. Nuclear Magnetic Resonance (NMR) Relaxometry

Nuclear magnetic resonance (NMR) is a powerful and noninvasive tool that has begun to be used more often in structural analysis. In determining the hydration and solubility properties of powders (Granizo, Reuhs, Stroshine, & Mauer, 2007), on the design of hydrogel systems (Oztop et al. 2010), in determining the controlled release properties of the active materials (Oztop et al. 2012), NMR relaxometry provides convenience and significant information. It is a technique based on the measurement of T_1 and T_2 relaxation times of the samples. The sample is exposed to a static magnetic field and series of radio-frequency pulses. Once the pulses end, the signal is acquired and the time constants of the signal decay curves are recorded as relaxation times. Different pulse sequences are sued to measure different relaxation times. T_1 time is represented by the recovery of the signal through longitudinal plane

whereas T_2 time is represented the decrease of the signal in the transverse plane. NMR relaxation spectra, which are the output of the NMR relaxometry, is obtained by applying inverse Laplace transform to the signal curve. NMR spectra give information about proton populations in the sample (Ersus et al. 2010, Hills, 1998; Oztop et al. 2010, 2012; Wichikut et al. 2013). Hence, this technique is able to determine the change in the protons of the nanoemulsions system. Also, this technique has a great advantage in terms of durability, easy and being non-destructive technique.

1.2.4.5. Optical properties

The appearance of the emulsions is an important attribute while designing the emulsions for food applications. Basically, the optical properties of emulsions are measured in terms of its turbidity and color. An emulsion can be turbid or opaque which could be quantified by a spectrophotometry analysis. In L*, a*, b* system where L* represents lightness, a*, and b* represents the color coordinates. +a is the red color,-a is the green color while +b is the yellow color, -b is the blue color (McClements, 1999, 2002).

Transparency comes from an object that can permit the light to pass through, whereas opacity comes from an object that scatters or absorbs the light. Most of the emulsions remain between these and thus called as translucent (Clydesdale, 1975). When light directs on an emulsion, it is scattered with different wavelengths through droplets. The degree of scattering changes with oil concentration, droplet size and the refractive index of emulsion (Farinato and Rowell 1983). Natural and appealing looking of a food product could be maintained by changing the turbidity of emulsions due to consumer's choice (Hernandez & Baker, 1991; Hernandez et al, 1991). In a study, to give turbid look to the fruit beverages, oil droplets were used at low concentrations (Tan, 1990; Dickinson, 1994).

1.3. Antimicrobial activity of nanoemulsions

In literature, there are extensive studies about antimicrobial properties of essential oil loaded nanoemulsions. Essential oil type, nanoemulsion formulation or production methods have a significant impact on the antimicrobial properties of the emulsions.

In a study, Salvia-Trujillo, et al., (2014) used nanoemulsion systems prepared using alginate and Tween 80 to enhance the antimicrobial activity of lemongrass essential oil by microfluidization. Nanoemulsions exhibited antimicrobial activity and 1.37, 5.29 and 7.07 log reduction in cell viability of *E.coli* was observed following 5, 10 and 30 min exposure times respectively. In another study conducted by Kim et al., (2014a) it was found that lemongrass oil nanoemulsion coated grapes showed resistance to *S. typhimurium* and *E. coli* O157: H7 at 4 and 28 ° C for 28 days. Nanoemulsion prepared with %16.66 Tween 80, %16.66 eucalyptus oil and % 68.68 water by ultrasonication showed inhibition against *Bacillus cereus, Staphylococcus aureus* and *Escherichia coli* (Sugumar et al., 2013). Another antimicrobial activity study was conducted with *L. delbrueckii* inoculated in orange and pear juice by using terpene mixture and D-limonene loaded nanoemulsions. 1 g/l terpene included nanoemulsion showed retardation of the microbial growth whereas 5 g/l terpene included nanoemulsion showed complete inactivation. Also, it was reported that sensory properties of the fruit juices did not change significantly (Donsì, Annunziata, Sessa, & Ferrari, 2011). Peppermint oil, medium chain triacylglycerol, water was emulsified with a high-pressure homogenizer and assessed antimicrobial activity against Gram-positive *Listeria monocytogenes* Scott A and *Staphylococcus aureus.* The peppermint oil nanoemulsions showed higher inhibitory action than the bulk peppermint oil (Liang et al., 2012).

1.4. Objectives of the study

The aim of this study is to design and characterize capsaicin-loaded nanoemulsion systems and show their potential as antimicrobial delivery systems. Tween 80, lecithin and sucrose monopalmitate were selected as the emulsifying agents in the formulations. To formulate the capsaicin-loaded nanoemulsions, microfluidization and ultrasonication were used as homogenization techniques. Effect of pH, continuous phase composition, and heating before homogenization were investigated on nanoemulsion properties. Antimicrobial activities of these nanoemulsions were examined against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Oleoresin capsicum (OC) was supplied from Alfasol (Gaziantep-Turkey). Its hotness degree was reported by the supplier as 1.000.000 SHU. Tween 80, potassium phosphate monobasic, sodium phosphate dibasic dihydrate, sodium acetate, ethanol, methanol, ethyl acetate, pure capsaicin (≥95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol, Violet Red Bile Agar (VRBA), nutrient broth, peptone from meat was purchased from Merck KGaA (Germany). Baird Parker Agar (BPA) with egg yolk tellurite was supplied by the local firm, Nisan Elektronik Ltd., Ankara, Turkey. Soy lecithin was bought from Smart Kimya (Ankara, Turkey). Sucrose monopalmitate was purchased from Compass Foods Company (Singapore). Distilled water was used for the preparation of all solutions.

2.2. Determination of capsaicin in oleoresin capsicum

Capsaicin amount in the oleoresin capsicum that was used in the study was analyzed by high-performance liquid chromatography (HPLC) that consisted of a Pursuit C18 column Microsorb MV C18 (4, 6 x 250 mm, 5 mm) and UV-VIS (ProStar 330 PDA) detector. The mobile phase was a mixture of methanol: water (70:30 v/v). The flow rate was 0.8 mL/min for 15 min at ambient temperature and detection wavelength

was 280 nm. Analysis was carried out in METU Central Laboratory Facilities. Capsaicin content of the oleoresin capsicum sample was found to be 51.06 mg/mL. HPLC chromatogram is given Appendix A (Figure A. 1).

2.3. Preparation of buffer solutions

Phosphate buffer solution was prepared by dissolving the 4.56 g potassium phosphate monobasic anhydrous and 28.87 g sodium phosphate dibasic dehydrate in distilled water and if necessary adjusting to pH 7.4 using NaOH and/or HCl. Acetate buffer solution was prepared by dissolving 12 mL 0.2 M sodium acetate and 88 mL 0.2 M acetic acid in water and if necessary adjusting to pH 3.8 using NaOH and/or HCl.

2.4. Preparation of emulsions

Emulsion preparation is schematized in the flow chart given in Figure 2.1

2.4.1. Formation of the primary emulsion

Oil-in- water emulsion was prepared by homogenizing 2 wt% oleoresin capsicum as oil phase with 98 wt% aqueous phase. The aqueous phase prepared at two different pH values by using 0.2 M sodium phosphate buffer at pH 7.4 and 0.2 M sodium acetate buffer solution at pH 3.8. Also, emulsifiers which were Tween 80, lecithin and sucrose monopalmitate were added at 2 wt% to that phase. Some samples also included 50 wt% glycerol in the aqueous phase along with the buffer solutions at the two pH values. Thus, the oil phase and aqueous phase was pre-homogenized with Ultraturrax (WiseTis Homogenizer, Witeg Labortechnik GmbH, Germany) at 20,000 rpm for 2 min for emulsifier Tween 80. When lecithin and sucrose monopalmitate were used as emulsifiers, to investigate the effect of heating in the pre-homogenization period, the coarse emulsion was mixed with a magnetic stirrer and heated to 60 ^{0}C for 30 min. After it was cooled to ambient temperature, the resulting mixture was then homogenized with Ultraturrax at 15,000 rpm for 3 min.

Then, the coarse emulsion was further homogenized with microfluidization and ultrasonication to form nanoemulsions.

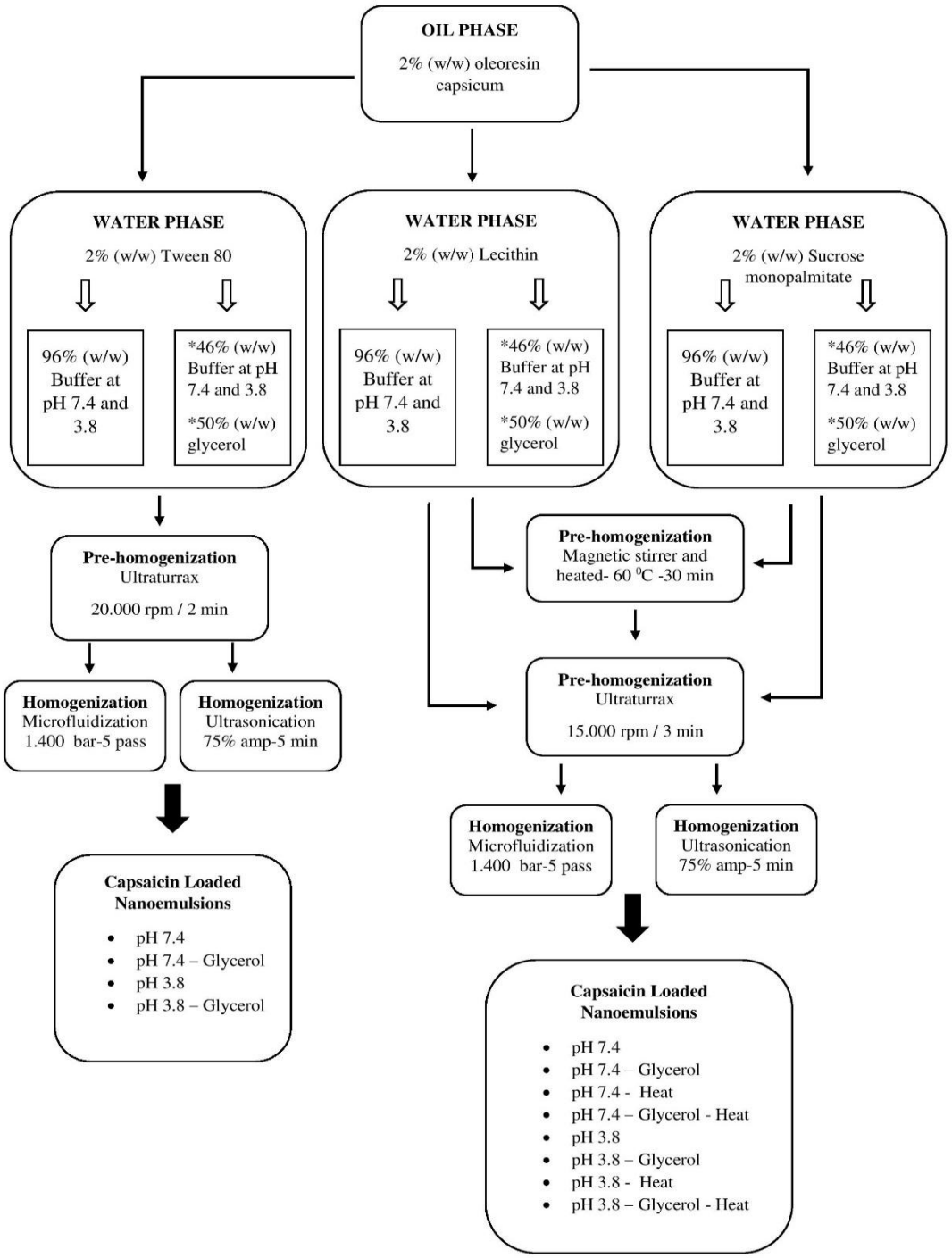

Figure 2.1 Flow chart of nanoemulsion preparation.

2.4.1.1. Microfluidization

The coarse emulsion was fed in a reservoir of microfluidizer (Nano Disperser - NLM 100, South Korea) with 0.3 L and pumped through an interaction chamber for 5 passes at 1.400 bar pressure. During the experiment, the cooling unit that was assembled to interaction chamber of the microfluidizer was run to keep the outlet sample temperature around 25 ^{o}C.

2.4.1.2. Ultrasonication

Following pre-homogenization, the coarse emulsion was also subjected to sonication using an ultrasonic probe (Bandelin Sonoplus HD 3100, Bandelin electronic GmbH & Co. KG, Berlin, Germany) (sonotrode: MS72) for 5 min at 75% amplitude. The 20 mL of each emulsion was kept in a beaker filled with ice to avoid excessive heating. The maximum temperature of the emulsion was recorded to at 33^{0}C. An example of ultrasonication treatment of the emulsion scheme is shown in Appendix Figure B.1.

2.5. Particle size measurements

Particle sizes of capsaicin-loaded emulsions were measured with laser diffraction technique by the Malvern Mastersizer 3000 system (Malvern Instruments Limited, Worcestershire, U.K.). As stated before, the technique relies on the principle of using the intensity of light scattered as the laser beam passes through nanoemulsion particles and then analyzing the signal to calculate the size of the particles by the surface area-based mean diameter D [3, 2]. The refractive index of 1.52 that was for the oleoresin capsicum was used to calculate particle size distributions. These experiments were conducted at 25 ^{o}C.

2.6. Turbidity measurements

The turbidity of all emulsions was measured using a UV-Visible spectrophotometer (UV–VIS spectrophotometer, Optizen, Mecasys, Korea) at 600 nm according to the method of Rao & McClements (2013).

2.7. Color

The color of emulsions was analyzed by a bench-top CM-5 spectrophotometer (Konica Minolta, Inc., Japan) with illuminant D65 and angle of 10̊ at 740 nm. The parameters of color measurement were L (brightness), a (red/green ratio), b (yellow/blue ratio). Pure water with values of L*ref =100.0, a*ref =0.0, b*ref =0.0 was used as reference to make white calibration for the instrument standardization. The emulsions were filled in quartz cells and L*, a*, b* color space was used for the measurements.

2.8. NMR (Nuclear Magnetic Resonance) Relaxometry experiments

NMR experiments were conducted using a 0.5 T (22.35 MHz) bench top low-resolution NMR system (SpinCore Technologies, Inc., Gainesville, USA). The T_2 relaxation times of capsaicin-loaded nanoemulsions were obtained by using Carr, Purcell, Meiboom and Gill (CPMG) pulse sequence with a 90–180 pulse gap (τ) of 1.0 ms, spectral width of 300 kHz, 32 scans, 512 points, repetition delay of 3 s, and 1000-5000 number of echoes. All T_2 measurements were performed at room temperature. Samples were measured in glass tubes with 10 mm sample size.

2.9. Encapsulation efficiency of nanoemulsions

The amounts of capsaicin trapped in nanoemulsions were analyzed according to the method of Surassmo et al. (2010) with minor modifications using UV–visible spectrophotometer (UV–VIS spectrophotometer, Optizen, Mecasys, Korea). 0.3 mL of capsaicin-loaded nanoemulsion was added to 4.2 mL of ethyl acetate and the

mixture was vortexed for 5 min, then centrifuged for 10 min at 348xg. The absorbance of the supernatant was recorded by UV-VIS spectrophotometer at 451 nm (A1). Ethyl acetate was used as the blank. The calculations of encapsulation efficiency were done in the following way;

$$\text{Encapsulation efficiency}(\%) = \left[\frac{\text{total amount of capsicum oleoresin content-C1}}{\text{total amount of capsicum oleoresin content}}\right] \times \%100$$

The total amount of capsicum oleoresin content is in g and C1 is the free capsicum oleoresin amount (g) in which A1 values were converted to concentration by using calibration curve. Calibration was carried out with different oleoresin capsicum concentrations in ethyl acetate of 20 mg/mL, 15 mg/mL, 12 mg/mL, 10 mg/mL, 5 mg/mL, 2 mg/mL, 0 (Appendix Figure C.1). Before the measurement began, maximum absorbance was detected using 20 mg/mL capsaicin- ethyl acetate solution between 200-500 nm with the UV-VIS spectrophotometer and the maximum UV absorbance was observed at 451 nm.

2.10. Transmission electron microscopy (TEM)

The morphology of the selected capsaicin-loaded nanoemulsion was examined by using TECNAI G2 Spirit BioTwin transmission electron microscope (Philips-FEI, Eidenhoven, and Holland) operated at 80 kV. For TEM analyze approximately 5 μL of diluted capsaicin-loaded nanoemulsion (1/50) was dropped onto 3 mm carbon film coated copper grid and left dried at room temperature for 3 hours. The bright film imaging mode was used to obtain TEM images. All TEM experiments were conducted at METU Central Laboratory Facilities.

2.11. Atomic force microscopy (AFM)

After capsaicin-loaded nanoemulsions were prepared with microfluidization, the image of nanoemulsions was captured using Veeco Multimode V atomic force microscope (Veeco, Santa Barbara, CA) equipped with a j-type scanner (ca. 125×125×5 μm3 scan range) scanned with a tapping mode at a speed of 1 Hz. Nanoemulsions were diluted to 1:100 with distilled water. A 5 μL diluted drop of nanoemulsion was placed onto smooth and a dry glass surface that was dried in the air. Experiments were conducted at METU Central Laboratory Facilities.

2.12. Determination of nanoemulsion stability

The physical stabilities of nanoemulsions were tested during 28 days storage. Tween 80 and lecithin containing nanoemulsions were kept at 4 ^{o}C and SMP containing nanoemulsions was kept at 20 ^{o}C due to instability problem. To assess stability during storage, particle size, NMR relaxometry, turbidity and color measurements were conducted for all nanoemulsion formulations.

2.13. Determination of antimicrobial activity

2.13.1. Microorganisms and growth conditions

Capsaicin loaded nanoemulsions were tested against two different bacteria strains. The Gram-positive *Staphylococcus aureus* (ATCC 43300) and the Gram-negative *Escherichia coli* (ATCC 11229) were provided by Public Health Institution of Turkey, Ankara from culture collection and preserved at the Department of Food Engineering, METU. Before use, 0.2 mL bacteria cultures were inoculated in 20 mL nutrient broth, shaken with agitated incubator (New Brunswick Scientific, Edison, N.J.,USA), incubated at 37 ^{o}C overnight for *E. coli* and 35 ^{0}C 48 h for *S. aureus* to reach final concentrations of 10^{8}- 10^{9} colony forming units/ milliliter (CFU/mL) for both bacteria strains. The number of cells in the culture was measured by reading the

absorbance at 600 nm (OD600) by UV–VIS spectrophotometer (UV–VIS spectrophotometer, Optizen, Mecasys, Korea) (data not shown). To ensure the exact number of cells in culture, bacteria were inoculated in agar media that was VRBA for *E. coli* and BPA for *S. aureus*. After that, the working cultures of bacteria was prepared by centrifuging at 3600 ×g for 10 min and washing twice with sterile saline (0.85% NaCl)-Tween 80 (0.1%) solution and adding to nutrient broth with a known inoculum number (*E. coli*, $7.3x10^{8}$; *S. aureus*, $1.2x10^{9}$), and finally putting 1 mL into each sterile eppendorf tubes that were stored at 4 ^{o}C. Bacterial stocks were maintained in cryotubes with beads and stored at -80 ^{o}C in nutrient broth at the Department of Food Engineering, METU.

2.13.2. Spread plate technique

The antimicrobial activity of capsaicin-loaded nanoemulsions was performed according to the method of Al-Adham et al. (2000), Salvia-Trujillo et al. (2014) and Abbaszadeh et al. (2014) with few modifications. Inhibition of microbial growth was tested by using spread plate method. 1% v/v -aliquot of sub-cultures of each bacteria strain was mixed with 0.5 mL of the capsaicin-loaded nanoemulsion and 4.5 mL of sterile phosphate buffer solution (PBS at pH 7.4). This mixture was left at 37 ^{o}C during 15 min for *E.coli*. Then 1 mL sample was taken from the mixture and diluted with 9 ml of sterile peptone water. After that 0.1 mL of diluted sample was spread on VRBA. Incubation was conducted at 37 ^{o}C for 24 h and colonies were counted. For *S. aureus*, a similar procedure with *E.coli* was followed. This time, the only difference was the contact time that the microbes were exposed to. Following the contact for 2 hours at 35 ^{o}C, 1 mL mixture was taken and diluted several times. 0.1 mL of the dilution was spread on BPA with egg yolk tellurite and colonies were counted after incubation at 35 ^{o}C for 48 h. These experiments were performed in duplicate both for *E. coli* and *S. aureus*. To understand if some antimicrobial effect was present on the individual components of the nanoemulsions, all ingredients of the nanoemulsions were tested against the microorganisms as controls.

2.14. Statistical analysis

All nanoemulsion samples were prepared in triplicate and also data from the two duplicates from each nanoemulsion were recorded to obtain the overall mean. The differences between these mean values were tested with Analysis of Variance (ANOVA). If the difference was detected, to assess the significance of differences in means Tukey test at 5% significance level was employed using Minitab (ver.16.2.0.0, Minitab Inc., United Kingdom).

2.15. Experimental design

Considering the literature studies which are described previously, factors and levels studied are summarized as in Table 2.1.

Table 2.1 Experimental factors and levels used in the study.

Factors	Levels	Responses
Oil concentration	2 %	1. Particle size ^{AP,EW} 2. Turbidity ^{AP,EW} 3. Color values ^{AP,EW} 4. T_2 times ^{AP,EW} 5. Encapsulation efficiency^{AP} 6. Antimicrobial activity^{AP} 7. Emulsion stability^{EW} 8. Transmission electron microscopy (TEM) ^{AP} 9. Atomic force microscopy (AFM) ^{AP}
Homogenization Techniques	Microfluidization, Ultrasonication	
Microfluidization parameters	@ 1.400 bar with 5 pass	
Ultrasonication parameters	75 % amplitude for 5 min	
Emulsifier Type	Tween 80, Lecithin, Sucrose Monopalmitate	
Emulsifier concentration	2 %	
pH	7.4, 3.8	
Continuous phase composition : Glycerol %	0 , 50%	
Heating before homogenization (for emulsifier - **lecithin** and **sucrose monopalmitate**-SMP)	Yes, No	

*^{AP} denotes ‘right after preparation’ and ^{EW} means ‘every week’.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Preliminary experiments for determination of oleoresin capsicum content for nanoemulsion composition

When the studies in the literature were examined it was observed that different concentrations of capsaicin were used in different formulations. In the skin penetration study that was also mentioned before the best formulation was determined using a ternary phase diagram and it was found that a stable nanoemulsion could be formulated with more than 50 % of water, less than 18 % of oleoresin capsicum and 15.4 %- 33.3 % of emulsifier mix (Kim et al., 2014b). In another study where spontaneous emulsification was used, nanoemulsions were prepared with 2% oleoresin capsicum and emulsifier concentrations of 2% (Dima et al., 2013) . Capsaicin content of emulsions prepared with alginate and chitosan using spontaneous emulsification had one-third of the Tween 80 content (Choi et al., 2013). An important point to be noted here is that in all studies mentioned oleoresin capsicum was used as the active agent and the main oil phase. Taking into account the previous studies, oleoresin capsicum concentration was determined to be 2 % by weight in this study.

Moreover, in order to verify the concentration in terms of antimicrobial activity, without using a surfactant, capsaicin (2%) + buffer mixtures were applied against

E.coli and S. aureus. At 2% concentration, reasonable antimicrobial activity was detected so considering the studies in the literature, 2% (w/w) oleoresin capsicum was decided to be used in all nanoemulsion formulations.

Also contact time determination experiments, which is an important parameter for antimicrobial tests were also conducted.

For *E.coli* strain, at 5, 15, 30, 60 min and 2 hours after mixing the culture and nanoemulsions, aliquots of samples were collected and antimicrobial activity test that was explained in Section 2.13 was conducted. After the colonies were counted, 15 min contact time was shown to have the higher antimicrobial activity (Fig. 3.1). Besides, it was interesting to observe that after 15 min of contact time in nanoemulsions, *E.coli* did not contribute any further decrease on the population. The utilization of capsaicin from various species such as *Variovorax* species was observed in literature. These species used capsaicin as sole carbon source (Flagan and Leadbetter, 2006). Also, *E.coli* was capable of using different materials such as acetate as carbon source (O'Beirne & Hamer, 2000). In that regard it was hypothesized that after adaption to the capsaicin containing medium following 15 min contact time, *E.coli* could utilize capsaicin as the carbon source and continue to grow up (Figure 3.1). So, capsaicin could not be considered as a perfect antimicrobial additive for *E. coli*.

For *S. aureus* strain, at 5, 15, 30, 60 min and 2, 24 hours after mixing the culture and nanoemulsions, aliquots of samples were collected and antimicrobial activity tests were conducted. After the colonies were counted, 24 hours contact time was shown to have highest antimicrobial activity, but this was too long to be feasible, so with a similar effect, 2 hours contact time was chosen. These results are shown in Figure 3.1.

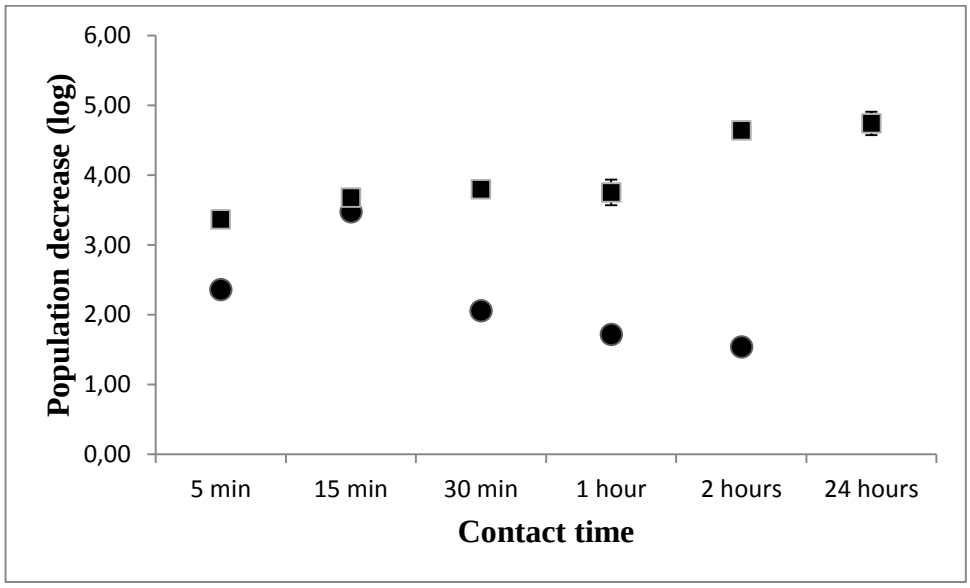

Figure 3.1 Effect of contact time of 2% (w/w) capsaicin-loaded nanoemulsions against *Escherichia coli* (●) and *Staphylococcus aureus* (■).

3.2. Nanoemulsions produced by microfluidization

3.2.1. Mean particle sizes of nanoemulsions

Mean particle sizes of nano-sized emulsions obtained by microfluidization are shown in Figure 3.2. The smallest particle sizes were obtained with emulsions prepared with sucrose monopalmitate (SMP) whereas the largest particle sizes were obtained with the one prepared with lecithin. Tween 80 resulted in slightly higher particle size nanoemulsions than SMP ($p \leq 0.05$).

3.2.1.1. Emulsifier type- Tween 80

In literature, Tween 80 was widely used in nanoemulsion formulations due to its ability to produce small sized and higher stability nanoemulsions (Ariyaprakai & Tananuwong, 2015; Porras, Solans, González, & Gutiérrez, 2008; Rao &

McClements, 2011b, 2012; Saberi, Fang, & McClements, 2013; Sari et al., 2015; Zhang et al., 2009). It was reported that nonionic emulsifiers such as Tween 80 could adsorb to oil droplet surface, avoiding the droplets to form aggregates and also easing the droplet break up during microfluidization process (Kralova & Sjöblom, 2009).

According to the results of this study, pH had a significant effect on the mean particle sizes of nanoemulsions prepared with Tween 80 ($p \leq 0.05$). The mean particle size decreased as the pH of the nanoemulsions decreased. The main reason was that decreasing the pH of the nanoemulsion might have increased the steric repulsion forces between the particles in the colloidal dispersion.

The influence of glycerol addition to the continuous phase on the particle size of nanoemulsions containing Tween 80 is shown in Figure 3.2-A. In the presence of glycerol, relatively smaller particles were observed with a diameter 63.77 and 50.30 nm for pH 7.4 and 3.8 respectively. Glycerol was added to the nanoemulsions as a water-soluble co-solvent that changed the physicochemical properties such as emulsion viscosity, refractive index, interfacial tension, solubility of nonionic or ionic emulsifiers and played an important role in maintaining the stability of the emulsion (Saberi et al., 2013). Furthermore, dielectric constant (εR) of glycerol is 42.5 while dielectric constant of water is 78.5 (Lide, 2003; D'Errico, Ciccarelli, & Ortona, 2005). Therefore, electrostatic interaction might have been enhanced by adding glycerol to the colloidal dispersion. According to this approach, the decrease of particle size can be explained.

3.2.1.2. Emulsifier type- Lecithin

The particle size of the nanoemulsions prepared with lecithin was found to be larger compared to other emulsifiers. The particle size of these emulsions varied from 215 to 615 nm as seen in Figure 3.2-B.

The opposite relation was observed between pH and particle size and lecithin nanoemulsions at pH 7.4 had smaller particle size than the ones prepared at pH 3.8.

At pH 3.8, the electrostatic repulsion could not be effective in overcoming attractive interactions between droplets like van der Waals and hydrophobic which caused them to produce aggregation. Lecithin contains ionizable anionic phospholipids group in acidic pH (Surh et al., 2008). As the pH decreased, lecithin could have lost this group as well as the repulsive force between oil droplets. Besides, Shchipunov & Schmiedel (1996) stated that emulsifier lecithin was able to maintain stability by creating an interfacial film between oil and water interface. As a result, all these possible effects could have increased the particle size of lecithin containing capsaicin-loaded nanoemulsions prepared by microfluidization.

The influence of glycerol in lecithin nanoemulsions was found to be different than Tween 80 nanoemulsions and adding glycerol increased the particle size at pH 7.4 whereas decreased the particle size at pH 3.8 significantly ($p \leq 0.05$). Well, these results for the effect of pH were consistent with the literature (Comas, Wagner, & Tomas, 2006). Researchers observed that decreasing the pH from 6.2 to 2 caused an increase in the mean particle sizes of soybean lecithin and sunflower oil containing emulsions. This situation was explained with the diminishing of the swelling behavior of phospholipids in lecithin with acid addition. The interfacial film created by lecithin became less resistant to prevent coalescence of particles and its emulsifying ability decreased. However, a mechanism on how glycerol affected the particle size in such a way was not stated elsewhere. It is believed that glycerol containing samples contained 50% less buffer solution. Thus, swelling of lecithin could have decreased and lecithin could have maintained its emulsifier ability at a higher degree. On the other hand, at pH 7.4 lecithin molecules remained at the interface and formed thicker film layers which could also have increased in thickness due to viscosity enhancing the effect of glycerol.

Capsaicin loaded nanoemulsions prepared with lecithin were heated to 60 ^{o}C before high-pressure homogenization. Heating helped to dissolve the lecithin and started to create small particles in the colloidal dispersion during continuous stirring in pre-homogenization. Also by heating, the molecular structure of the emulsifier had

changed and that could result in smaller droplet size (Rao & McClements, 2013; Shinoda and Friberg, 1986). Thus, heating decreased significantly the mean particle size of the emulsions in the presence of glycerol and pH of 3.8 ($p \leq 0.05$) (Appendix Table D.2). Rao& McClements, (2011a) showed that heating formed smaller particle size emulsions. Moreover, Ozturk, et al. (2014) found that to achieve smaller particle size of nanoemulsions by using surfactants as lecithin and quillaja saponin, a higher amount of lecithin should be used relative to the quillaja saponin. Thus, to achieve smaller particle size as with Tween 80 or SMP higher amounts of lecithin which would still be lower than the micellar concentration might be needed.

3.2.1.3. Emulsifier type- SMP

By using SMP as an emulsifier, capsaicin-loaded nanoemulsion were produced successfully using microfluidization and the lowest particle size emulsions were obtained by using SMP in nanoemulsion formulations ($d < 40$ nm). There are a number of other studies about SMP containing nanoemulsions (Choi et al., 2011b; Henry et al., 2009; Rao & McClements, 2012) showing similar results to our study. In a study, Tween 80 and SMP were used with lemon oil and Tween 80 containing emulsions were shown to have larger particle sizes. This was associated with a hydrophilic head group of Tween 80 being bigger than that of SMP. Thus, after the oil and emulsifier were mixed, they formed bigger nanoemulsion droplets in the colloidal dispersion (Rao & McClements, 2012). The same situation was observed in this study as well. Further, under acidic conditions (at pH 3.8) SMP containing nanoemulsions showed poor stability and right after microfluidization, phase separation occurred within an hour. Two possible (chemical and physical) reasons for the phase separation were hypothesized. Chemically, SMP could have been hydrolyzed into sucrose and palmitic acid under acidic pH and while sucrose units dissolved in the aqueous phase, palmitic acid units remained in the oil-water interface. The splitting of sucrose head from the molecule reduced the steric repulsion between droplets. Moreover, an increase in the hydrolysis of sucrose from the

emulsifier molecule increased acidity due to increase in free ionized palmitic acid. When the pH of the solution reaches or becomes closer to the palmitic acid's pKA (4.9), the concentration of charged of palmitic acid decreases and the particles begins to aggregate due to attractive interactions (van der Waals) which are stronger than repulsive interactions (steric and electrostatic). In this case, the hydrophilic head group becomes insufficient to provide steric repulsion. Physically, hydrolysis could be triggered by the protonated free fatty acid impurities present in SMP (Choi et al., 2011a; Rao & McClements, 2011a, 2012).

Since pH 3.8 emulsions were not stable and characterization tests were not able to be conducted for those samples, the balance of the experimental design for ANOVA for SMP samples was disrupted and thus evaluating the individual effects of pH, glycerol and heating could not be possible due to imbalance design. That is why, while evaluating SMP emulsions, the formulation itself was treated as a level and the treatment type itself was considered as the factor. In that regard, there were 7 formulations that were compared with each other:

- pH 7.4
- pH 7.4 + Glycerol
- pH 3.8 + Glycerol
- pH 7.4 + Heat
- pH 3.8 + Heat
- pH 3.8 + Glycerol + Heat
- pH 7.4 + Glycerol + Heat

Although glycerol addition or heating before homogenization did not cause significant difference at pH 3.8 ($p > 0.05$), these effects caused significant decrease at pH 7.4 of particle sizes of nanoemulsions with SMP ($p \leq 0.05$) Also, in the presence of glycerol, particle sizes were not changed significantly ($p > 0.05$) (Appendix Table D.3).

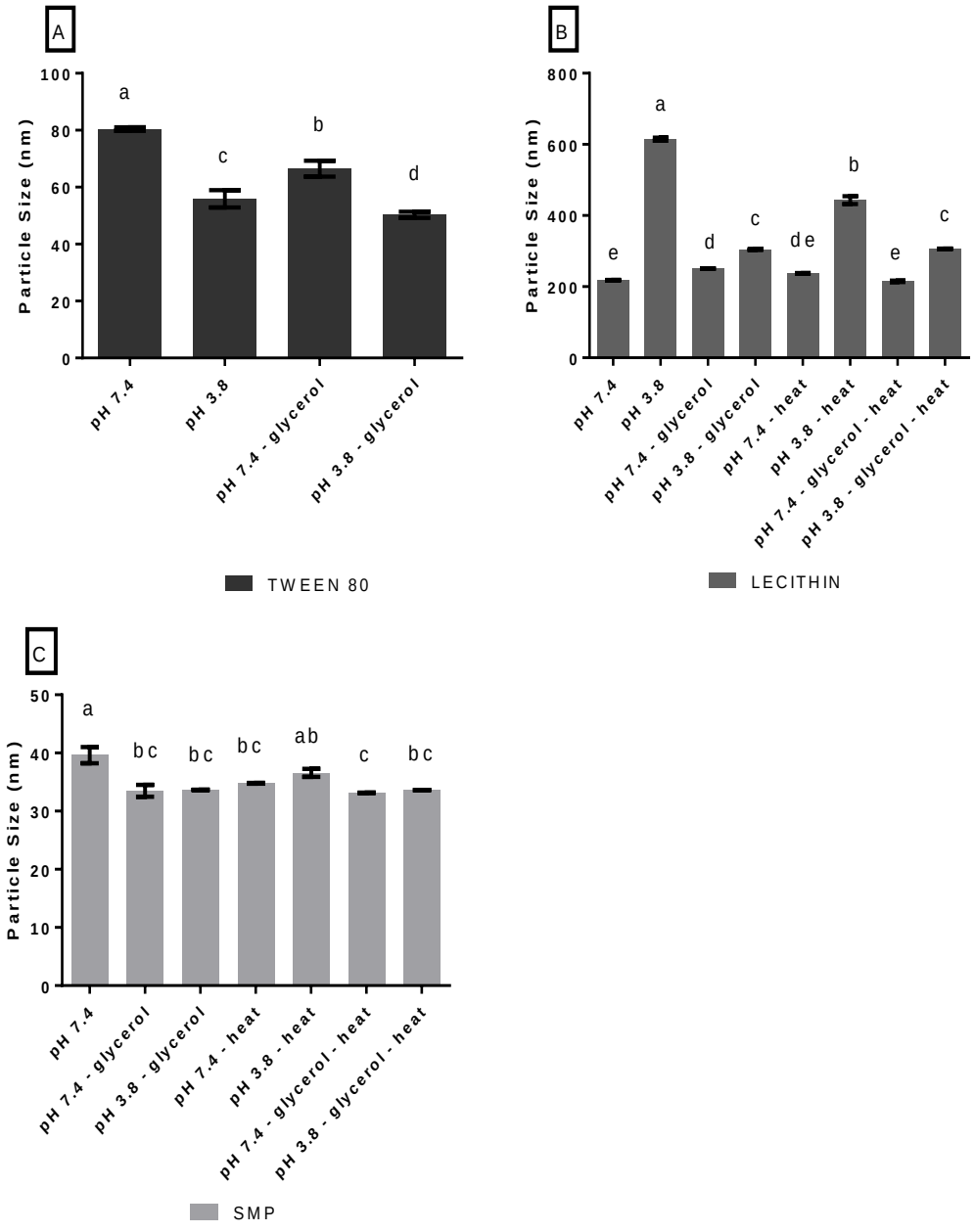

Figure 3.2 Particle sizes of different formulations of capsaicin-loaded nanoemulsions prepared with microfluidization: (A) Tween 80, (B) Lecithin, and (C) SMP. Means in the same graph indicated by different letters are significantly different ($p \leq 0.05$).

3.2.2. NMR measurements

In this study, NMR relaxometry was used to characterize the structural properties of capsaicin-loaded nanoemulsions by relating the information obtained from relaxation curves with other analysis techniques to understand the effects of emulsifier type, pH, glycerol addition or heating before homogenization on nanoemulsion formation. As NMR Relaxometry experiments, T_2 relaxation times of capsaicin-loaded nanoemulsions were measured. NMR Relaxation times gives information about the proton populations and how it is affected by its environment. In an emulsion, water, oil and surfactant are all proton providers and thus their presence effect relaxation times. Rate and mobility of water in a sample is largely reflected on T_2 relaxation times. As stated, the mobility of water molecules is strongly related to the interactions with the other components such as oils and emulsifiers. These interactions could restrict the water molecules but still free water bulk remains present in the colloidal dispersion. These molecular motions reflect the properties of dispersions by changing the T_2 relaxation times. While the restricted water shows fast relaxation and lower T_2 values, the free water shows slow relaxation and higher T_2 values (Granizo et al., 2007). Likewise, the oil molecules have short relaxation times and emulsifiers decrease the molecular mobility by solubilizing in the dispersion (Jenning, Ma, & Gohla, 2000).

Figure 3.3 shows the T_2 relaxation times of microfluidized capsaicin-loaded nanoemulsions. In all nanoemulsion formulations, the addition of glycerol decreased the T_2 times. Glycerol acts as a co-solvent in nanoemulsions and it lowers the mobility of water molecules. Thus, glycerol added samples showed fast relaxations in the magnetic field. In a study, polymorphism of triglycerides was monitored through NMR and glycerol containing parts of the triacylglycerol were found motionless (Hagemann, 1988).

3.2.2.1. Emulsifier type- Tween 80

As the pH decreased, T_2 times of nanoemulsions containing emulsifier Tween 80 didn't change significantly in the absence of glycerol ($p \geq 0.05$) whereas T_2 times increased in the presence of glycerol (Figure 3.3-A). It was possible to conclude that that in the absence of glycerol at lower pH, the interactions of water/oil phase with Tween 80 was higher. Results also showed the only factor influencing the T_2 times was the addition of glycerol. Effect of pH was not found significant on T_2 relaxation times ($p \geq 0.05$). As Tween 80 being a nonionic surfactant this result was not surprising.

3.2.2.2. Emulsifier type- Lecithin

ANOVA results (Appendix Table D.2) showed that pH, glycerol, and heat are all significant on T_2 relaxation times of lecithin emulsions ($p \leq 0.05$). Except the interaction between pH and heat all other interactions were also found to be significant ($p \leq 0.05$) which are shown in Figures 3.3-B. T_2 times of glycerol added nanoemulsions decreased by decreasing pH. Heating also decreased the T_2 times indicating that lecithin was more solubilized and interacted with water more which resulted in restriction of the mobility of water (Capitani, Segre, & Sparapani, 1991). All glycerol free lecithin containing nanoemulsions were found to have lower T_2 times than other glycerol-free nanoemulsions prepared with either SMP or Tween 80.

3.2.2.3. Emulsifier type- SMP

T_2 relaxation times are shown both in Figures 3.3-C and statistical results are given in Appendix Table D.3. Using heating before homogenization caused significant changes in the T_2 values. Due to instability after an hour T_2 measurements for the pH 3.8 samples in the absence of glycerol with no heating was not recorded. However, it was visually obvious that heating had an effect on the nanoemulsions. Even though SMP was supposed to be hydrolyzed at lower pH, emulsions were stable and T_2

values were recorded. A notable change observed in the absence of glycerol with heating was the increase in T_2 times. At pH 7.4, glycerol as being a co-solvent might have diminished the effect of heating and did not change the T_2 values, but at pH 3.8 there was a slight but significant decrease on T_2 with heating. Possible reason for this observation was that sucrose units were split from the molecule due to acidity at pH 3.8 and induced interactions with free water resulting in restricted water mobility and lower T_2 values (Fabri, Williams, & Halstead, 2005). Heating could also have accelerated the hydrolysis.

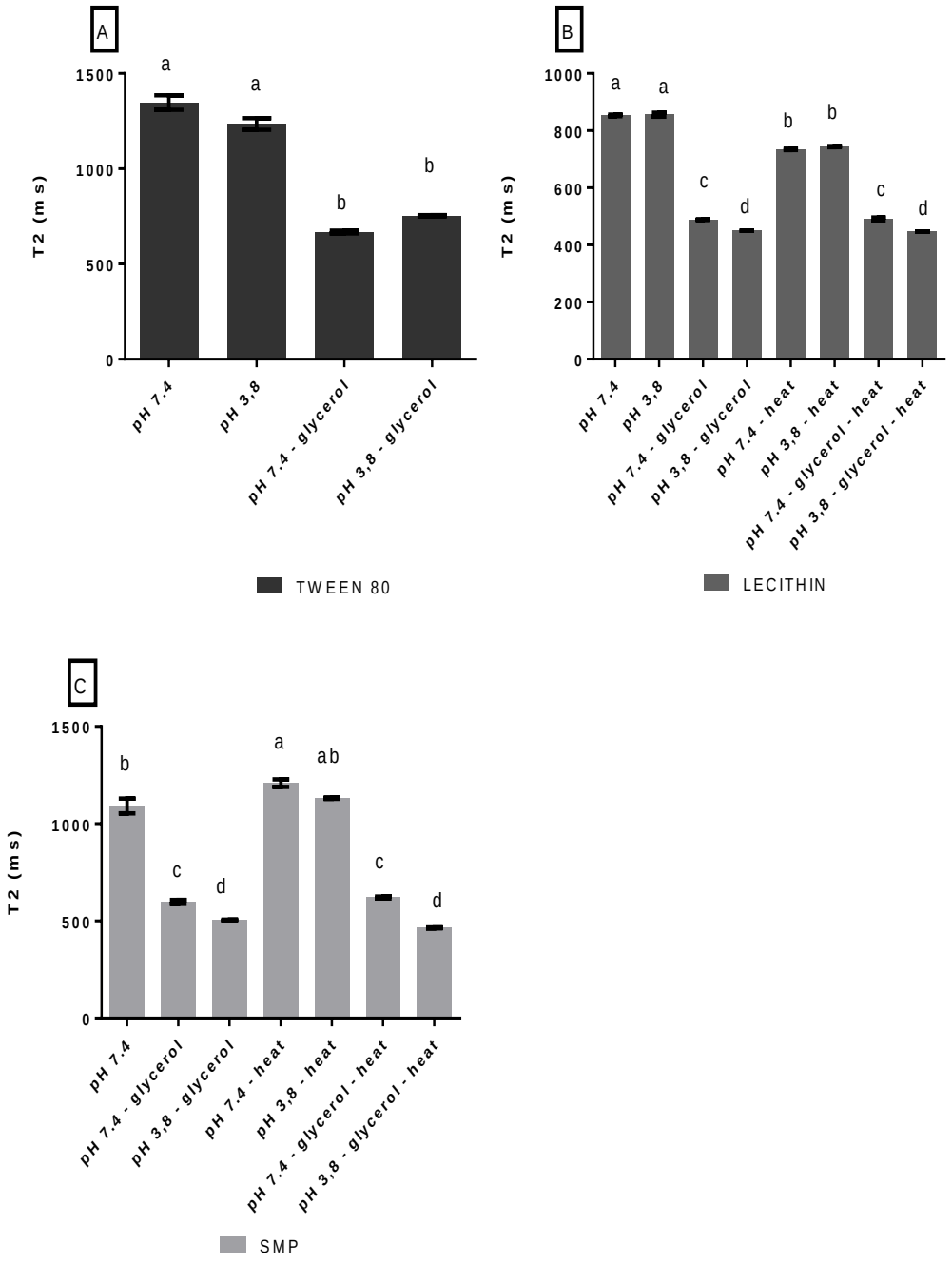

Figure 3.3 T_2 values of different formulations of capsaicin-loaded nanoemulsions prepared with microfluidization: (A) Tween 80, (B) Lecithin, and (C) SMP. Means in the same graph indicated by different letters are significantly different ($p \leq 0.05$).

3.2.3. Turbidity

3.2.3.1. Emulsifier type- Tween 80

The light scattering was measured by using spectrophotometer at 600 nm for all emulsion formulations as in Figure 3.4. Effect of pH and glycerol addition was significant on turbidity ($p \leq 0.05$) (Appendix Table D.1). Turbidity values of nanoemulsions decreased with the addition of glycerol and decreasing pH. Also, changes in droplet size affect the turbidity of emulsion (McClements, 2002). When Pearson correlation analysis was conducted between turbidity values and mean particle sizes a positive correlation of 0.86 was obtained with emulsifier Tween 80 ($p \leq 0.05$). Additionally, glycerol addition provided a lower turbidity value. A similar result was observed in 5 wt % octadecane nanoemulsions emulsified with 2.5wt % sodium dodecyl sulfate (SDS) containing glycerol by Qian & McClements, (2011) and opaque emulsion turned to slightly turbid with increasing glycerol content from 0 to 50% in the aqueous phase. This was an expected result for two reasons. Firstly, glycerol affects the refractive index contrast between the two phases in emulsion and turbidity changes. Secondly, mean particle sizes changes when glycerol is added resulting in the different scattering behavior of the emulsions (McClements, 2002).

3.2.3.2. Emulsifier type- Lecithin

Lecithin containing nanoemulsions showed opaque and had higher turbidity values than other emulsifiers, possibly because of the low solubility of lecithin in the aqueous phase. Effect of pH and glycerol addition was found to be significant ($pH \leq 0.05$) Lecithin solubility is high in alcohol medium such as ethanol (Kahlweit, Busse, & Faulhaber, 1995) and even though the particle sizes of the prepared nanoemulsions were small, this might have caused the opaque view of the nanoemulsions.

3.2.3.3. Emulsifier type- SMP

In a similar way, there was a significant change in turbidity when glycerol was added to nanoemulsions or emulsions were pre-heated before the main homogenization. Transparent emulsions were obtained by using glycerol. Possibly, heating affected the turbidity by promoting solubility of SMP except for pH 3.8 ones. In acidic media SMP was not good at preventing the droplet aggregation and thus with heating, the droplets gained kinetic energy accelerating the aggregation. In that regard, turbidity analysis could be used to point out the structural changes such as flocculation or aggregation similar to the NMR experiments. The bigger particle caused turbidity rising to the highest value (1.137) for SMP nanoemulsions. A positive correlation of 0.66 was found between mean particle sizes and turbidity results ($p \leq 0.05$). When heating was combined with glycerol, there was no significant difference at the same pH ($p > 0.05$) (Appendix Table D.3).

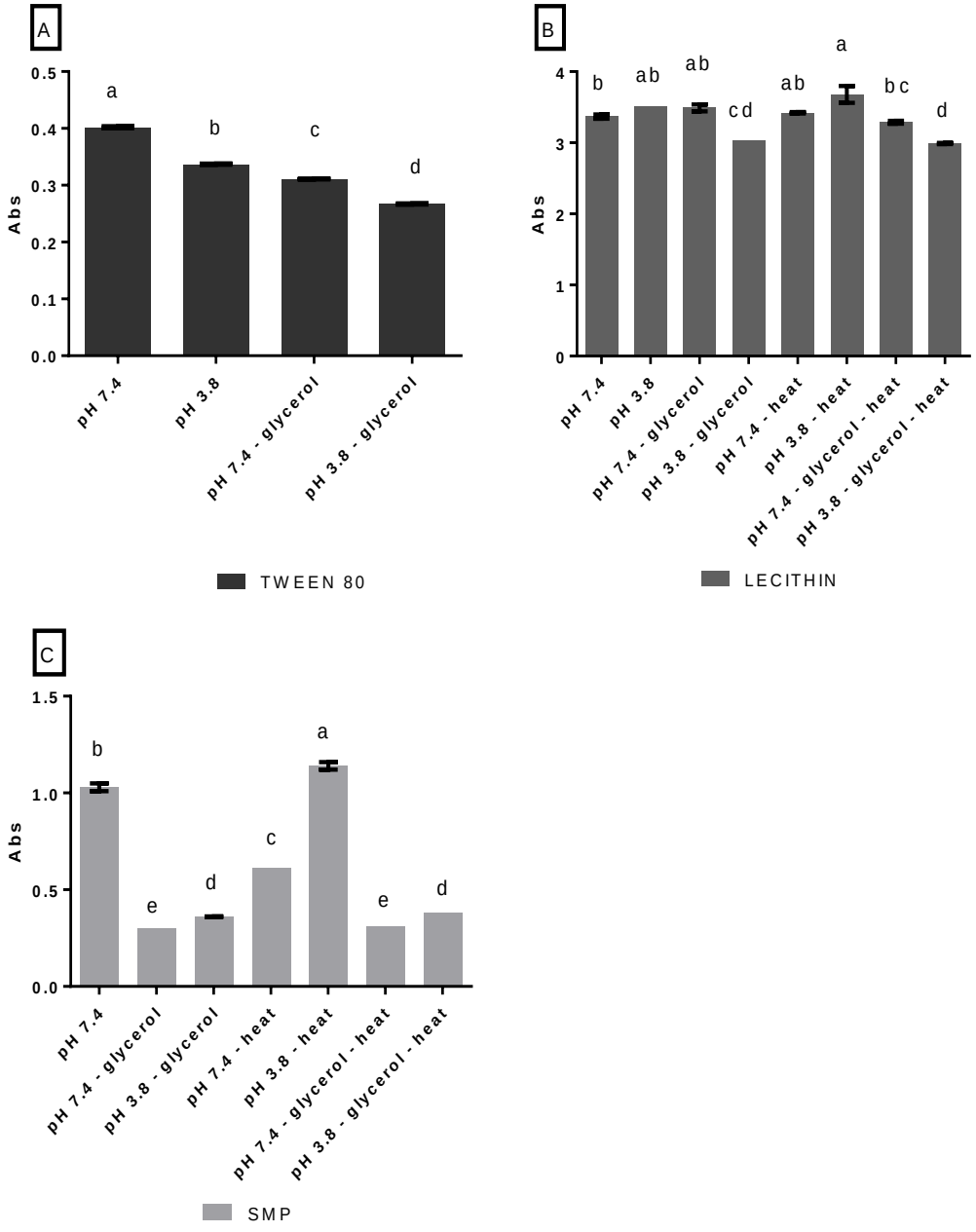

Figure 3.4 Turbidity results of different formulations of capsaicin-loaded nanoemulsions prepared with microfluidization: (A) Tween 80, (B) Lecithin, and (C) SMP. Means in the same graph indicated by different letters are significantly different ($p \leq 0.05$).

3.2.4. Color of nanoemulsions

Visual observations are important for food product design. The color is another important feature of emulsions. Several methods were developed to quantify the color values of samples such as the degree of redness, blueness, greenness or yellowness by using light scattering or absorption properties of samples (Chanamai & McClements, 2001). The color of an emulsion is mainly determined by absorption of light through the droplets that change with the presence, concentration, and type of the chromophores (McClements, 1999). Commission International de l'Eclairage CIE created a system which was the tristimulus coordinates such as XYZ or L*a*b to specify the color values of samples. For example, mathematically the L* value represents the degree of lightness and a* and b* values represent the degree of redness (+a*), greenness (-a*), yellowness (+b*) and blueness (-b*) respectively (McClements, 2002). The degree of absorption, thus emulsion color, could also be affected by droplet size (Chanamai & McClements, 2001; Meleson, Graves, & Mason, 2004; Tadros et al., 2004).

Whereas the pure capsaicin was white, the oleoresin capsicum used in this study had very dark red color (almost black) in which L*a*b* values were found to be 0.18, +0.25, -0.11 respectively. Carotenoid pigments of capsanthin, capsorubin, and capsanthin 5,6-epoxide are reasons for the red, orange, yellow color of emulsions (Hornero-Méndez & Mínguez-Mosquera, 2001). The color values of microfluidized capsaicin-loaded nanoemulsions are given in Table 3.1.

All the L*a*b* values increased due to dilution of oleoresin capsicum in formulations. Thus, the strong color of oleoresin capsicum which could have resulted in significant color changes diminished. Tween 80 containing nanoemulsions had very bright and red-colored as well as SMP containing nanoemulsions, but lecithin gave a yellowish color and decreased the L*a*b* values of nanoemulsions compared with other emulsifiers. Also, opaque lecithin nanoemulsions had very low L*a*b* values as seen in Table 3.1. This wasn't surprising since these emulsions had large

particle sizes, too. Pearson correlation analysis result revealed that higher than 0.84 and 0.92 of correlation were observed with particle size results and L* b* values of Tween 80 and SMP containing nanoemulsions respectively ($p \leq 0.05$). As the largest particle size of lecithin nanoemulsion was 614.5 nm at pH 3.8, the L*a*b* values were found the lowest as 0.86, 4.09, 1.42 respectively. Similarly, as turbidity values decreased, color values increased. At pH 7.4 turbidity value of glycerol containing lecithin nanoemulsion was 3.49 and the L*a*b* values were 3.43, 15.71, 5.77 respectively, while at pH 3.8 turbidity value of glycerol containing lecithin nanoemulsion decreased to 3.03 and the L*a*b* values increased to 8.22, 27.15, 14.15 respectively. The negative correlation of 0.87 was observed between turbidity and L*a* values of lecithin containing nanoemulsions ($p \leq 0.05$).

As, both the turbidity values and particle sizes of SMP containing nanoemulsions were very low than other emulsifiers, bright, red color nanoemulsions were obtained. This situation reflected itself on the color values being higher. In the presence of glycerol, heating affected were not affected significantly the lightness (L*) and yellowness (b*) values ($p > 0.05$) whereas pH changes did ($p \leq 0.05$). However, redness (*) values were not significantly affected by pH ($p > 0.05$) (Appendix Table D.3)

Table 3.1 Effect of the different emulsifiers on the color values of capsaicin-loaded nanoemulsions prepared by microfluidization.

Samples		L* value[#]	a* value[#]	b* value[#]
Oleoresin capsicum		0.18 ± 0.0	0.25 ± 0.11	-0.11 ± 1.00
Tween 80	pH 7.4	54.40 ± 0.03^{a}	54.0 ± 0.40^{a}	93.90 ± 0.05^{a}
	pH 3.8	52.00 ± 0.3^{b}	54.70 ± 0.13^{a}	89.7 ± 0.46^{b}
	pH 7.4- glycerol	53.40 ± 0.4 ^{ab}	54.20 ± 0.29^{a}	92.1 ± 0.36^{ab}
	pH 3.8- glycerol	52.60 ± 0.14^{b}	54.10 ± 0.54^{a}	82.10 ± 0.02^{c}
Lecithin	pH 7.4	7.65 ± 0.02^{c}	26.50 ± 0.02^{c}	13.10 ± 0.03^{c}
	pH 7.4- glycerol	3.43 ± 0.01^{f}	15.70 ± 0.02^{f}	5.80 ± 0.01^{f}
	pH 7.4 - heat	4.98 ± 0.03^{e}	21.70 ± 0.13^{e}	8.40 ± 0.07^{e}
	pH 7.4- glycerol- heat	5.18 ± 0.04^{d}	22.20 ± 0.08^{d}	8.90 ± 0.05^{d}
	pH 3.8	0.86 ± 0.02^{g}	4.10 ± 0.03^{g}	1.40 ± 0.01^{g}
	pH 3.8- glycerol	8.22 ± 0.06^{b}	27.20 ± 0.1^{b}	14.2 ± 0.13^{b}
	pH 3.8- heat	0.83 ± 0.00^{g}	3.90 ± 0.1^{g}	1.30 ± 0.02^{h}
	pH 3.8- glycerol- heat	8.93 ± 0.05^{a}	27.80 ± 0.1^{a}	15.4 ± 0.11^{a}
SMP	pH 7.4	39.10 ± 0.04^{e}	47.90 ± 0.02^{c}	66.4 ± 0.99^{d}
	pH 7.4- glycerol	49.80 ± 0.01^{a}	54.80 ± 0.0^{a}	85.5 ± 0.33^{a}
	pH 7.4 - heat	44.40 ± 0.04^{d}	51.50 ± 0.01^{b}	76.6 ± 0.07^{c}
	pH 7.4- glycerol- heat	49.70 ± 0.03^{a}	53.80 ± 1.0^{a}	85.70± 0.05^{a}
	pH 3.8- glycerol	47.60 ± 0.02^{b}	54.80 ± 0^{a}	82.10± 0.04^{b}
	pH 3.8- heat	45.00 ± 0.01^{c}	53.40 ± 0^{a}	77.60± 0.01^{c}
	pH 3.8- glycerol- heat	47.60 ± 0.00^{b}	55.20 ± 0^{a}	82.10± 0^{b}

[#]Data are the mean ± standard error results. Means in the same column indicated by different letters are significantly different ($p \leq 0.05$). ANOVA was conducted for each surfactant type.

3.2.5. Encapsulation efficiency of nanoemulsions

Encapsulation efficiencies of capsaicin-loaded nanoemulsions were determined using the method explained in Chapter 2. The results are shown in Table 3.2. Encapsulation efficiencies of nanoemulsions changed for different formulations. In literature, rather than emulsifier type, the amount of emulsifier within the colloidal dispersion was found to be an important factor on encapsulation efficiency (Xing, Cheng, Yi, & Ma, 2005). However, the effect of emulsifier content was not investigated in this study and fixed concentrations were used in all formulations. It was seen that lecithin containing nanoemulsions showed maximum efficiency with 96% in the presence of glycerol and with heating at pH 3.8. Similarly, glycerol containing nanoemulsion at pH 3.8 without heating showed also the higher efficiency of 89% with the presence of lecithin as shown in Table 3.2. It was concluded that the structure builder glycerol was responsible for higher efficiencies. In overall, pH, glycerol addition, and heating were all found be significant on efficiency (Appendix Table D.1-2-3) ($p \leq 0.05$).

When Tween 80 was used as an emulsifier, the overall efficiency results were higher with an average of 83% that again the higher efficiency obtained by pH 3.8 nanoemulsion. Possible reason for the observed efficiency rises at pH 3.8 is that solubility of the emulsified oil, oleoresin capsicum, may be higher at this pH both for emulsifiers Tween 80 and lecithin.

For capsaicin-loaded nanoemulsions prepared with SMP, encapsulation efficiency results did not change between formulations with an average of 67%. Only the heat included pH 3.8 nanoemulsion was significantly different from others ($p \leq 0.05$) (Appendix Table D.3).

Table 3.2 Effect of the different emulsifiers on the encapsulation efficiency of capsaicin-loaded nanoemulsions prepared by microfluidization.

Samples		Encapsulation efficiency (%)#
Tween 80	pH 7.4	81.92 ± 0.00 ^{c}
	pH 3.8	88.06 ± 0.00 ^{a}
	pH 7.4- glycerol	82.80 ± 0.00 ^{b}
	pH 3.8- glycerol	79.47 ± 0.00 ^{d}
Lecithin	pH 7.4	57.61 ± 0.00 ^{e}
	pH 7.4- glycerol	57.78 ± 0.00 ^{e}
	pH 7.4 - heat	62.44 ± 0.01 ^{c}
	pH 7.4- glycerol- heat	59.15 ± 0.00 ^{d}
	pH 3.8	56.65 ± 0.00 ^{f}
	pH 3.8- glycerol	89.01 ± 0.00 ^{b}
	pH 3.8- heat	62.95 ± 0.00 ^{c}
	pH 3.8- glycerol- heat	96.25 ± 0.00 ^{a}
SMP	pH 7.4	70.69 ± 0.00 ^{a}
	pH 7.4- glycerol	67.83 ± 0.01 ^{ab}
	pH 7.4 - heat	68.31± 0.01 ^{a}
	pH 7.4- glycerol- heat	68.17 ± 0.00 ^{a}
	pH 3.8- glycerol	65.44 ± 0.01^{ab}
	pH 3.8- heat	61.89 ± 0.03^{b}
	pH 3.8- glycerol- heat	64.92 ± 0.01^{ab}

#Data are the mean ± standard error results. Means in the same column indicated with different letters are significantly different ($p \leq 0.05$). ANOVA was conducted for each emulsifier type.

3.2.6. Stability of nanoemulsions

As mentioned in Chapter 1, the stability of nanoemulsions is an important issue and it shows the oil/water balance and applicability of the designed nanoemulsion system for further applications. Stability is affected by many environmental conditions such as temperature, pH or ionic strength (Rao & McClements, 2012). In this study, nanoemulsions were evaluated for 28 days. Since the overall aim of the nanoemulsions is to add them to a food product, Tween 80 and lecithin based nanoemulsions were kept at 4 ^{o}C to represent refrigeration temperature. However, SMP based nanoemulsions caused phase separation at this temperature after 1-day storage so these nanoemulsions were kept at 20 ^{o}C throughout the storage period. The stabilities of nanoemulsions were evaluated separately for each homogenization technique as microfluidized nanoemulsions and ultrasonicated nanoemulsions.

3.2.6.1. Emulsifier type- Tween 80

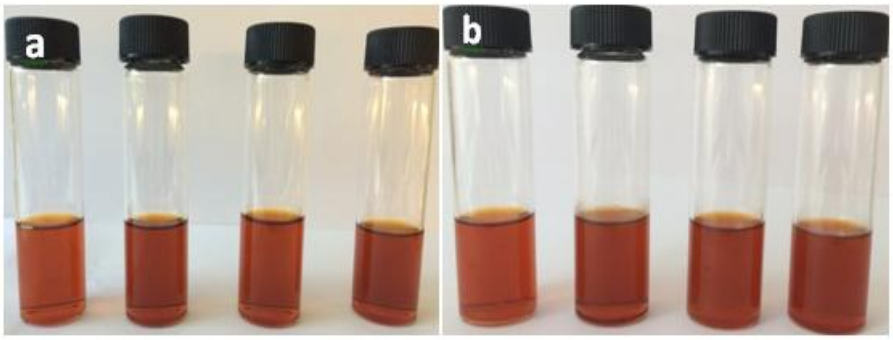

Figure 3.5 Photographs of microfluidized Tween 80 containing capsaicin loaded nanoemulsions stored for 0 or 28 days: a) 0 th, pH 7.4- pH 7.4, glycerol- pH 3.8- pH 3.8, glycerol; b) 28 th, pH 7.4-pH 7.4, glycerol-pH 3.8- pH 3.8, glycerol.

After 28 days of storage, visually there was no change in Tween 80 containing microfluidized nanoemulsions as shown in Figure 3.5. Physical analysis results (T_2, mean particle size, turbidity and color values) are also shown in Figure 3.6. Overall, mean particle sizes and turbidities of Tween 80 nanoemulsions increased whereas T_2 and color values decreased significantly with time ($p \leq 0.05$) (Appendix Table D.4). Despite particle grown up, the highest particle size was remained lower than 50 nm. While, the L*, a* and b* values of glycerol containing nanoemulsions tended to decrease, other nanoemulsions were not affected by color changes during storage significantly ($p>0.05$), (Appendix Table D.4). As mentioned in Section 3.2.2.2. Molecular motion of larger particles became slower in colloidal dispersion and it caused faster relaxation with low T_2 as in the pH 7.4 nanoemulsion (Figure 3.6 A-F). In the presence of glycerol, T_2 times were not affected by pH. Glycerol could be tolerated the droplet grow up. Thus, T_2 times of glycerol containing samples did not change T_2 times significantly ($p> 0.05$). Previous studies showed that Tween 80 is a good emulsifier to promote stability of nanoemulsions. At the end of 28 days, significant instability problems were not detected in the samples. The reason for maintaining stability was associated with the large head group size of Tween 80 that produced great repulsive interaction between droplets (Rao & McClements, 2012; Terjung et al., 2012).

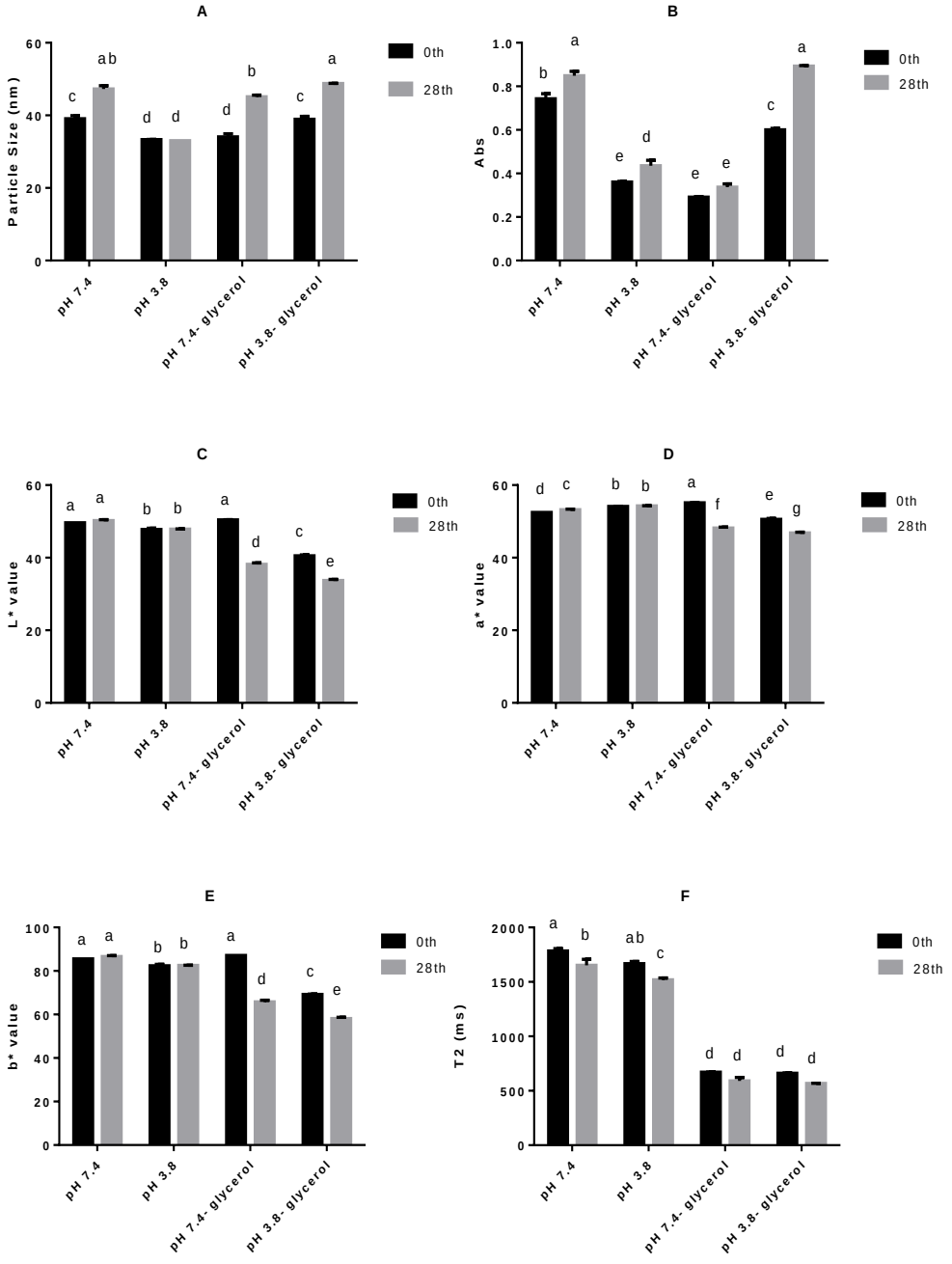

Figure 3.6 Stability results of microfluidized Tween 80 containing capsaicin-loaded nanoemulsions for 0 and 28 days: **A)**Particle sizes, **B)**Turbidities, **C)**Color- L* values, **D)** Color- a* values, **E)** Color- b*values, **F)**T2 times.

3.2.6.2. Emulsifier type- Lecithin

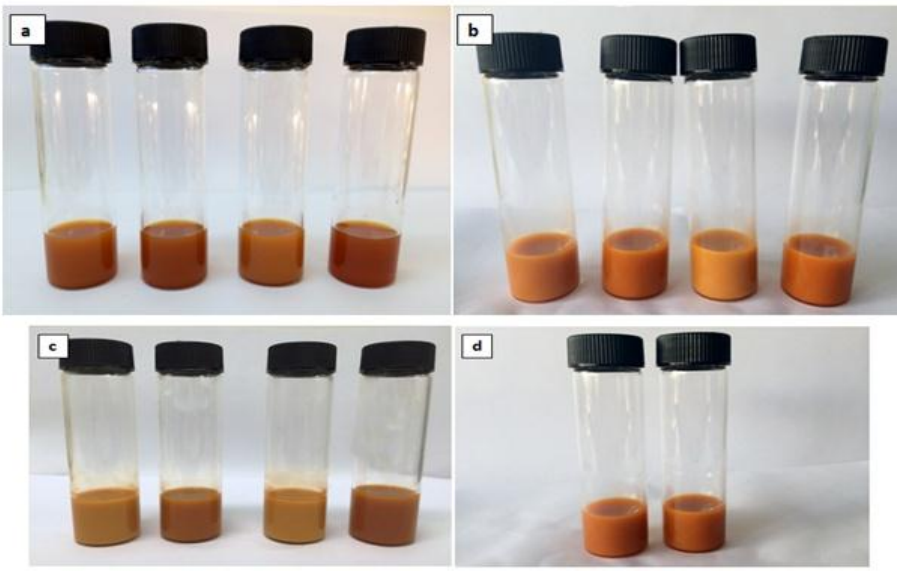

Figure 3.7 Photographs of microfluidized lecithin containing capsaicin loaded nanoemulsions stored for 0 or 28 days: **a) 0th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **b) 28th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **c)0th,** pH 3.8- pH 3.8, glycerol-pH 3.8, heat- pH 3.8, glycerol, heat; **d) 28th**, pH 3.8, glycerol- pH 3.8, glycerol, heat.

Lecithin based nanoemulsions were less stable than Tween 80 based nanoemulsions. The appearances of nanoemulsions were opaque both at the 0th and 28th days and also there were instability observations (Figure 3.7). Nanoemulsions which were named as pH 3.8 and pH 3.8- heat, phase separated after 7 days of storage and could not be further analyzed. As mentioned previously, emulsifier lecithin could lose its anionic phospholipids group in acidic pH. This situation was driven to the particles to form

bigger particles that resulted with oiling off. Also, heating might have caused emulsifier head group to become dehydrated. Droplet coalescence could occur also after dehydration (Saberi et al., 2013). Acidity along with the heating caused particles to grow up in size and during storage further increase in droplets ended up with phase separation.

As mentioned in Chapter 1, larger particles are more prone to coalescence compared to small particles due to their higher Laplace pressure. Hydrodynamic interactions, colloidal forces, and surface charge become important for larger particles. During the storage time of 28 days, dramatic increases in the particle sizes of lecithin containing nanoemulsions were observed whereas Tween 80 containing nanoemulsions showed little growth over time as shown in Figure 3.8-B. As the particles get smaller, their energy becomes higher. However, in that period small particles tended to reduce their energies with temperature drop around 4 ^{o}C during storage and this might have been driven to the particles merged. In the presence of glycerol, particle sizes were not changed significantly at pH 3.8 ($p > 0.05$).

Overall, mean particle sizes and turbidity values were increased while T_2 and color values were decreased significantly with time ($p \leq 0.05$) (Appendix Table D.5). The coalescence of particles decreased the color values of the nanoemulsions by absorbing much light and reflecting little. The coalescent of particles increased the turbidity values. However, there are decreases in turbidities as shown in Figure 3.8-E. Further coalescence can cause oiling-off but in these samples, particles are prone to coalescence locally and not ending with oiling-off.

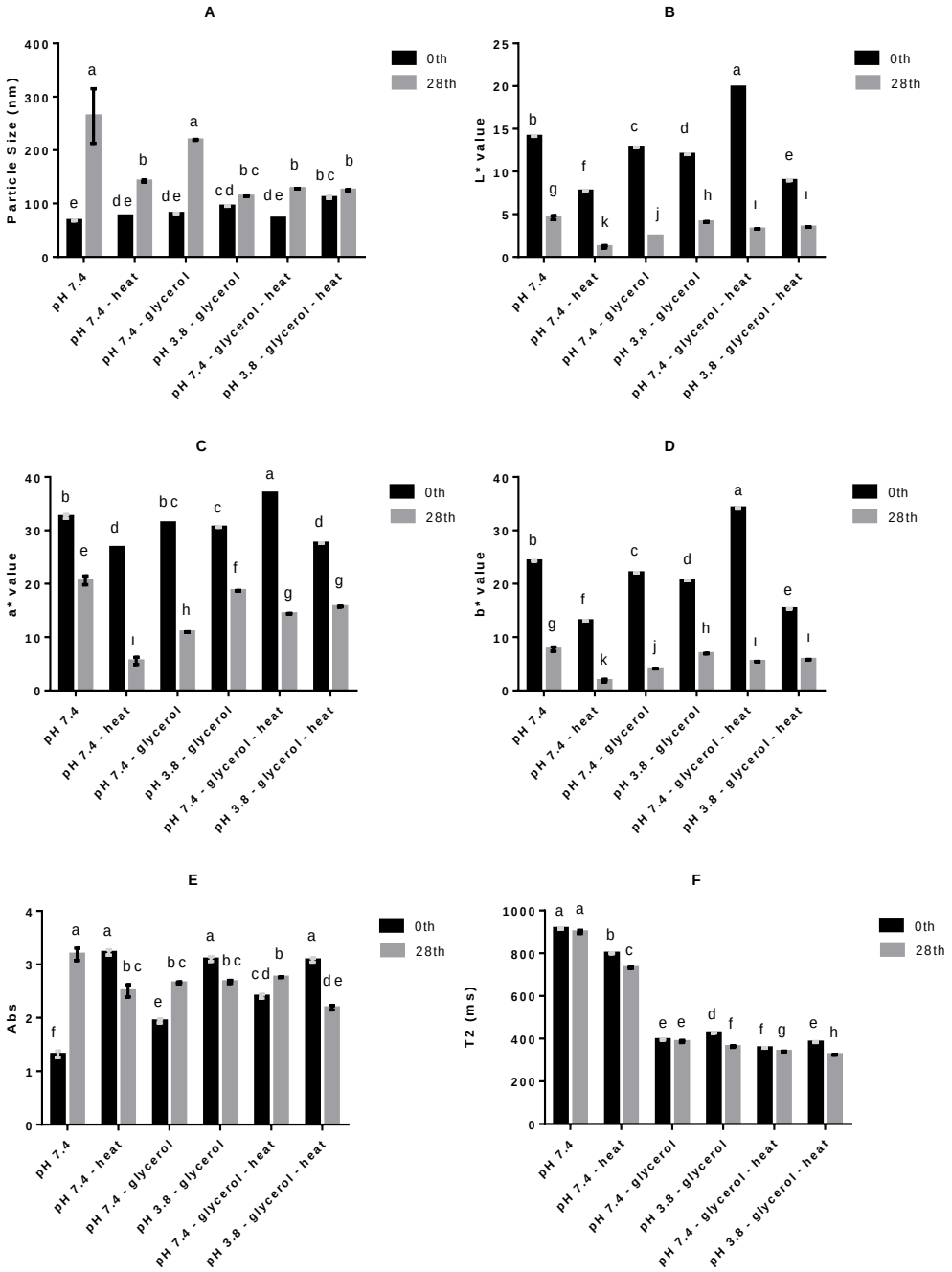

Figure 3.8 Stability results of microfluidized lecithin containing capsaicin-loaded nanoemulsions for 0 and 28 days: **A)** Particle sizes, **B)** Color- L* values, **C)** Color- a* values, **D)** Color- b* values, **E)**Turbidity, **F)**T2 times.

3.2.6.3. Emulsifier type- SMP

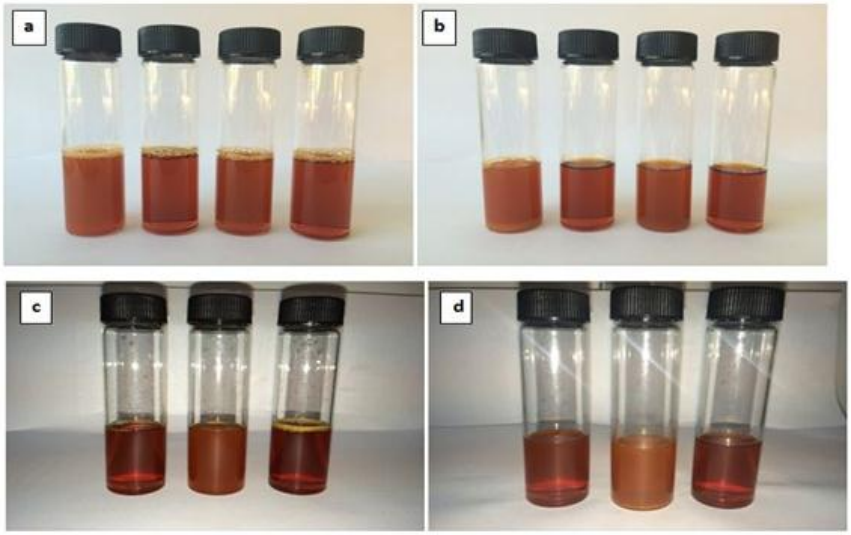

Figure 3.9 Photographs of microfluidized SMP containing capsaicin loaded nanoemulsions stored for 0 or 28 days: **a) 0th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **b) 28th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **c)0th,** pH 3.8, glycerol-pH 3.8, heat- pH 3.8, glycerol, heat; **d) 28th**, pH 3.8, glycerol- pH 3.8, heat- pH 3.8, glycerol, heat.

Transparent and slightly opaque microfluidized nanoemulsions were prepared with SMP as shown in Figure 3.9. After microfluidization, pH 7.4, pH 7.4-heat, pH 3.8, pH 3.8- heat nanoemulsions were prone to phase separation and could not be analyzed. On the other hand, glycerol containing nanoemulsions showed good stability and even after 28 days of storage. It was hypothesized that the structure enhancer glycerol could have retarded the hydrolysis of SMP by creating a barrier around the emulsifier and showed greater stability during storage. Overall, after the

28 days mean particle size, turbidity, L* and b* values were increased but T2 and a* values decreased significantly with time ($p \leq 0.05$) (Appendix Table D.6). Although the increase in particle size with time was found significant in the presence of heat ($p \leq 0.05$) at pH 3.8, the average particle size remained small (<43 nm) (Appendix Table D.6). The increases in turbidities of nanoemulsions at pH 3.8 were found significant ($p \leq 0.05$). Also, T_2 times did not change significantly ($p > 0.05$) between treatments. Unlike lecithin based nanoemulsions, microfluidization facilitated droplet break up and produced small sized and monomodal distributed nanoemulsions with SMP. Throughout the storage period, the repulsive force between droplets remained and particle grown up occurred possibly via Ostwald ripening. As mentioned in Chapter 1, small particles are more prone to Ostwald ripening or coalescence rather than flocculation. Then, droplet grown up of microfluidized SMP nanoemulsions might be due to Ostwald ripening which was different from lecithin based nanoemulsions. Also, bigger particles resulted in increase turbidity values. Glycerol containing SMP based nanoemulsions looked still transparent at the end of 28 days and might be considered as one of the most promising nanoemulsion formulation for further studies with oleoresin capsicum.

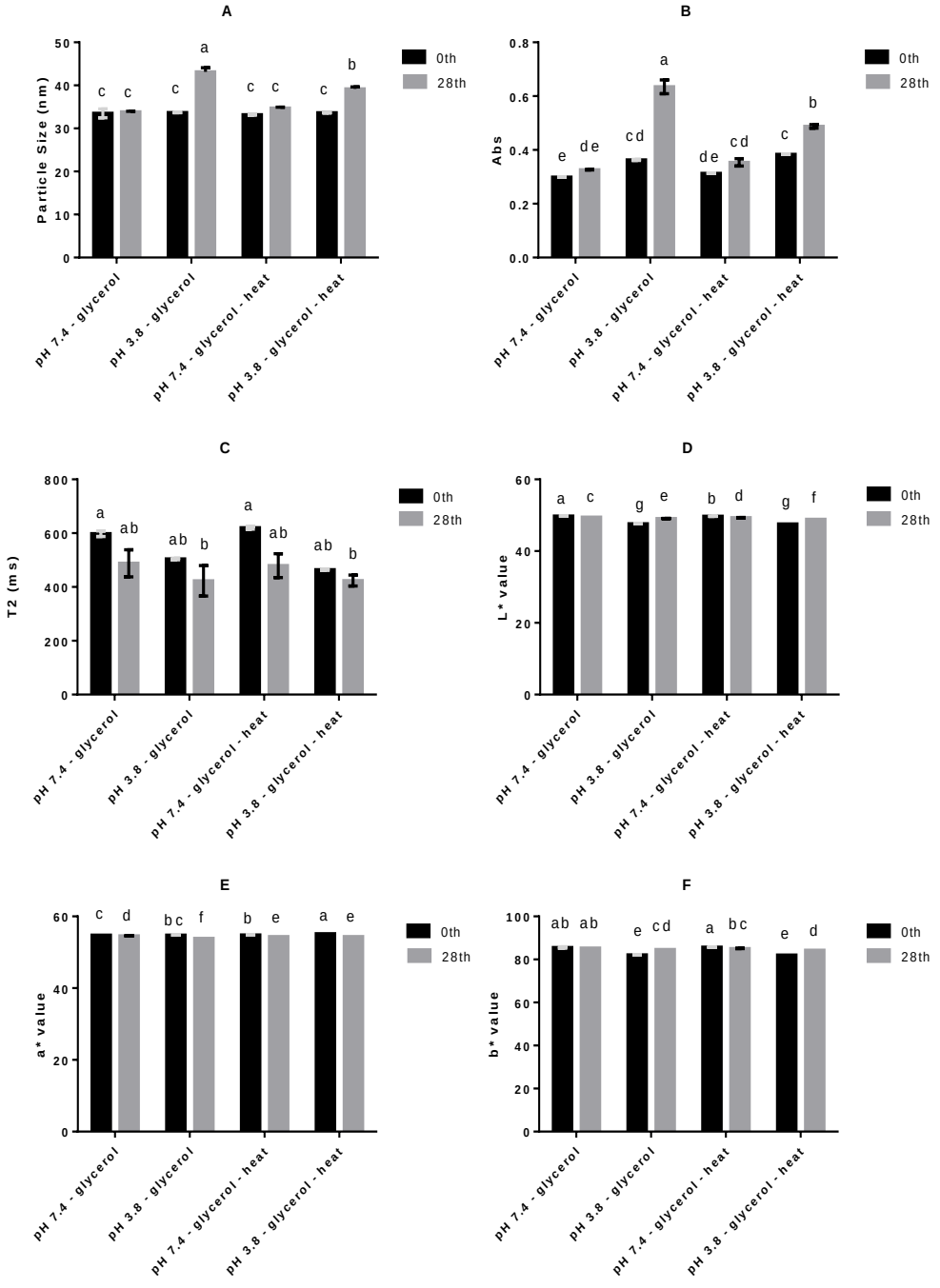

Figure 3.10 Stability results of microfluidized SMP containing capsaicin-loaded nanoemulsions for 0 and 28 days: **A)**Particle sizes, **B)**Turbidities, **C)**T2 times, **D)**Color- L* values, **E)** Color- a* values, **F)** Color- b* values.

3.2.7. Antimicrobial activity of nanoemulsions

3.2.7.1. Emulsifier type- Tween 80

The antimicrobial activities of prepared nanoemulsions are shown in Figure 3.11. Both *S. aureus* and *E.coli* populations decreased with different ratios when exposed to different nanoemulsions. Namely, the decrease in pH decreased the antimicrobial activity of the nanoemulsions towards *E.coli* for emulsifier Tween 80 ($p \leq 0.05$). *E.coli* is a tough microorganism which can survive harsh environmental conditions such as pH fluctuations, high temperature (Lee & Kang, 2016). Therefore, decreasing the pH may not be effective to kill the population along with the capsaicin addition as expected. Freidman et al., (2004) examined several essential oils against *E. coli* O157:H7 and *Salmonella* Hadar. In apple juice which had pH 2.8-3.0 that acidity couldn't contribute to the inhibition of *E.coli*. Moreover, in the literature acetate was described to be effective in the growth of *E. coli.* In a study, Shimizu et al., (1992) found that unlike propionate, succinate, and formate, acetate could make a discernible inhibitory effect on *E.coli*. Also, it was stated that logarithmic decrease was achieved by changing acetate concentration. In addition, the inhibitory level of acetate was reported lower than 1.25 g/L and *E.coli* could continue to grow in it by doing a glyoxylate bypass (O'Beirne & Hamer, 2000). Based on these studies it was concluded that the 96% and 48% (glycerol containing ones) of acetate buffer concentrations in nanoemulsion formulations couldn't result in a desirable reduction in *E.coli* population.

On the contrary, the pH change didn't significantly affect antimicrobial activity of capsaicin-loaded nanoemulsions against *S. aureus*. Also, the addition of glycerol in formulation didn't affect antimicrobial activity of nanoemulsions significantly when Tween 80 was used as emulsifier against *E.coli* as it did against *S. aureus*. Almost 1.5 times higher inhibition was observed when glycerol was added to the formulations. The possible reason of this could be explained by the increase in solubility of capsaicin through the addition of glycerol that acted like a co-solvent.

Also, the mechanism of action of same materials against *S .aureus* and *E. coli* can be different as in this situation. Capsaicin loaded nanoemulsions showed highest antimicrobial activity against *S. aureus*. Namely, glycerol containing capsaicin-loaded nanoemulsions prepared with emulsifier Tween 80 caused 5.89 log reduction in *S. aureus* population. As mentioned in Chapter 1, the differences in hydrophobicity differences between cell membranes of microorganisms could give different results under the same conditions.

3.2.7.2. Emulsifier type- Lecithin

When lecithin was used as an emulsifier in capsaicin-loaded nanoemulsions, the microbial load reduction ranged from 2.34 to 3.40 log for *E.coli* and 3.57 to 4.43 log for *S. aureus.* Again, *S. aureus* showed slightly higher reduction than *E.coli*. However, in lecithin containing formulations *E.coli* showed more reduction than Tween 80 containing formulations. It is likely that phospholipid parts of membranes eased the entrance of lecithin covered droplets which had also phospholipid parts that lead to a higher decrease on the cell hydrophobicity by capsaicin. Based on this explanation, higher antimicrobial activity was expected from these lecithin containing nanoemulsions but it was important to mention that all lecithin molecules could not integrate with capsaicin droplets. Gill et al., (2002) stated that bacteria cells could be expected to repair themselves in nutrient-rich media such as lecithin. Thus, free lecithin molecules might have been used in repairing the damaged cell membrane by *E.coli* which might have limited the antimicrobial activity. On the other hand, the maximum inhibitory activity for all formulations among the microfluidized samples was obtained by lecithin and glycerol containing heated nanoemulsions prepared at pH 7.4 with 3.40 log reduction against *E.coli*. Effect of pH and glycerol addition caused a significant decrease of the *S. aureus* population ($p < 0.05$). It was also found that heating the nanoemulsions before homogenization increased antimicrobial effect on *S. aureus* compared to unheated nanoemulsions. The maximum reduction was

obtained using lecithin containing heated nanoemulsions at pH 7.4 with a value of 4.43 log against *S. aureus*.

3.2.7.3. Emulsifier type- SMP

At low concentrations, sucrose generally doesn't show the inhibitory effect as it is a carbohydrate source and moreover it is used to contribute microorganism growth. On the contrary, higher concentrations of sucrose showed inhibitory activity due to water binding ability which limits the available water for the growth of microorganisms (Artz & Hansen, 1994). Remarkably, the antimicrobial activities of SMP nanoemulsions were found much lower than other emulsifier containing nanoemulsions. One possible reason for differences in results may be that sucrose created proper growth conditions for the microorganisms and capsaicin droplets couldn't exhibit its hydrophobicity. In the work conducted by Thomas et al., (1998) inhibitory effect of nisin several Gram-positive and Gram-negative bacteria strains were explored. Sucrose fatty acid esters were used to enhance the incorporation of the nisin in the study. It was observed that Gram-positive bacteria were affected more than Gram-negative ones. The same result was demonstrated in this study. *Staphylococcus aureus* showed more inhibition than *Escherichia coli* as shown in Figure 3.11. Besides, SMP might have masked the antimicrobial activity of capsaicin while forming higher stability and appealing nanoemulsions. Also, the pH drop to the 3.8 increased the log reduction by increasing the acidity of nanoemulsions especially for *E.coli* due to the increase in the amount of hydrolyzed sucrose units that bound more water. Thus, the antimicrobial activities of pH 3.8 nanoemulsions prepared with SMP were found to be higher than that of pH 7.4 nanoemulsions for *E.coli*. Also, there is no significant difference between treatments at pH 3.8 as shown in Figure 3.11-C ($p > 0.05$). The highest log reduction observed was 1.55 log for *E.coli* and 2.75 log for *S. aureus* with SMP containing microfluidized nanoemulsions.

There is no significant difference between treatments for *S. aureus* inhibition except that in the presence of glycerol. Heating affected at pH 7.4 significantly ($p \leq 0.05$) (Appendix Table D.3).

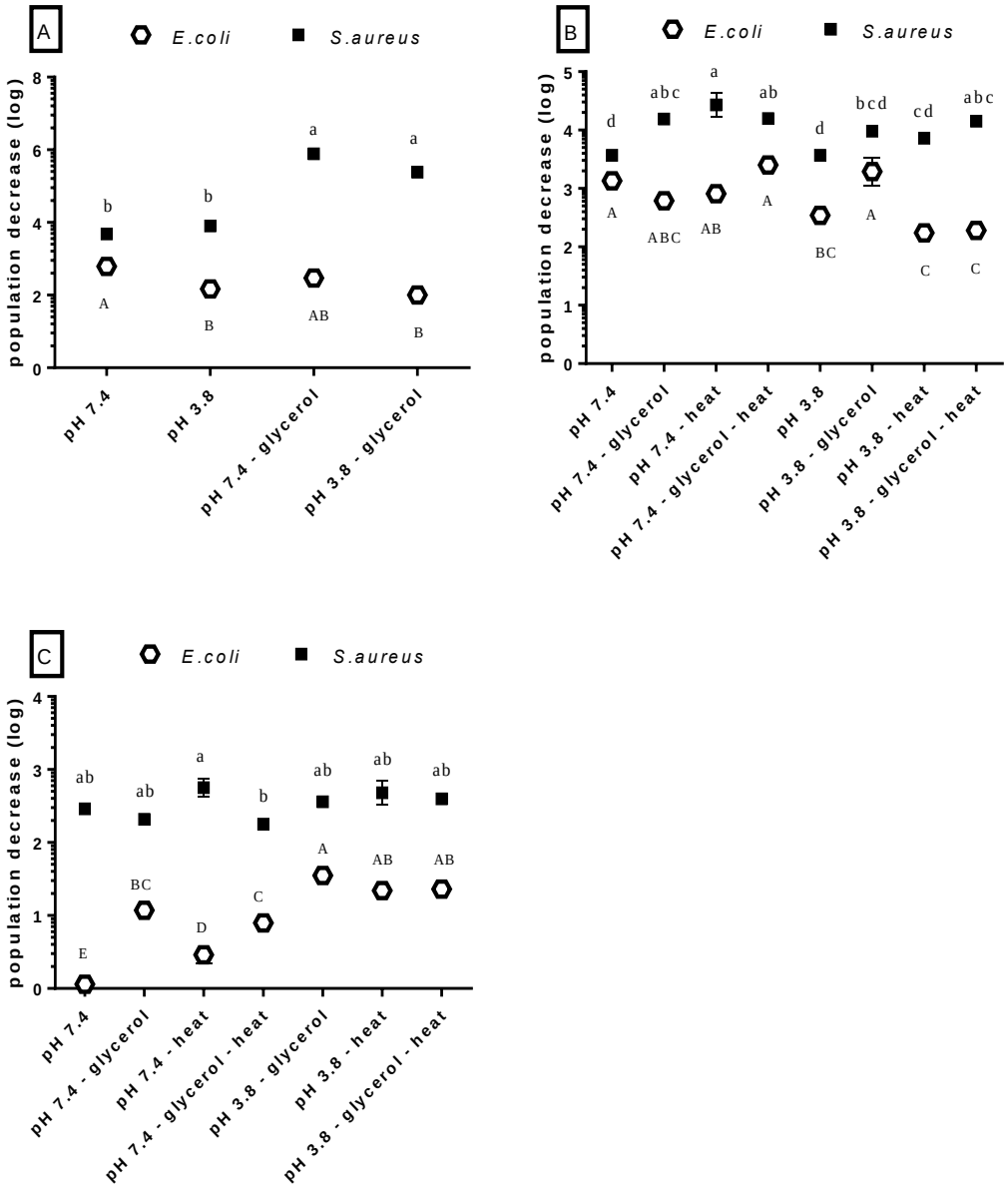

Figure 3.11 Effect of capsaicin-loaded nanoemulsions on *Escherichia coli* and *Staphylococcus aureus* prepared with microfluidization. Population decrease means the ratio of survived microorganisms (CFU/mL) to inoculum culture (CFU/mL). ANOVA was conducted for each microorganism and emulsifier type.

3.3. Nanoemulsions produced by ultrasonication

3.3.1. Mean particle sizes of nanoemulsions

3.3.1.1. Emulsifier type- Tween 80

Mean particle sizes of ultrasonicated capsaicin-loaded nanoemulsions are given in Figure 3.12. Effect of pH and glycerol was found to be significant on the mean particle ($p \leq 0.05$, Appendix Table D.7). The droplet sizes of Tween 80 based nanoemulsions were found to be lower than 65 nm. Effect of glycerol was somehow interesting. At pH 7.4 mean particle size decreased but at pH 3.8 it increased with glycerol addition. Salvia-Trujillo et al., (2013a) reported that droplet break up was enhanced with ultrasonication by forming electrical charge around the droplets and increased the surfactant adsorption. In their work, the minimum droplet size was found 4.31±0.18 nm for lemongrass oil nanoemulsions containing aqueous sodium alginate solution (1 %w/v) and Tween 80 (1 %v/v) prepared with ultrasound at 100 μm amplitude for 180 s. Leong et al. (2009) used Tween 80 and sunflower oil to make nanoemulsions with ultrasonication at 30 μm amplitude for 20 min and average droplet sizes were found to be 40 nm.

3.3.1.2. Emulsifier type- Lecithin

Statistic evaluation of the results showed that effect of pH was significant (Appendix Table D.5) and decreasing the pH to 3.8 significantly increased the droplet sizes of lecithin based nanoemulsions ($p \leq 0.05$). Moreover effect of heating was found insignificant (Appendix Table D.5). It is probable that ultrasonication could have already achieved the desired structural change, increased the dissolution. Even, without adding glycerol droplet size increased almost 5 times (Figure 3.12-B) when the pH decreased from 7.4 to 3.8. On glycerol added samples, 40 or 50 nm increase was observed when the pH decreased from 7.4 to 3.8 but in overall the results with the presence of glycerol were found lower than 192 nm.

3.3.1.3. Emulsifier type- SMP

At the previous sections, unstable characteristic of SMP nanoemulsions at low pH was mentioned. The same behavior observed with ultrasonicated nanoemulsions and phase separation was observed at pH 3.8 and pH 3.8-heat nanoemulsions within 1 hour after preparation. The droplet size results of SMP nanoemulsions are shown in Figure 3.12. While very small particle sizes were observed with microfluidized SMP based nanoemulsions (<40 nm), to achieve same sizes heating and glycerol were required significantly with ultrasonication treatment ($p \leq 0.05$) (Appendix Table D.9). Without adding glycerol, heating caused higher droplet sized emulsions due to flocculation. It was very obvious that glycerol addition was essential in the emulsion formulation in order to achieve smaller droplet size.

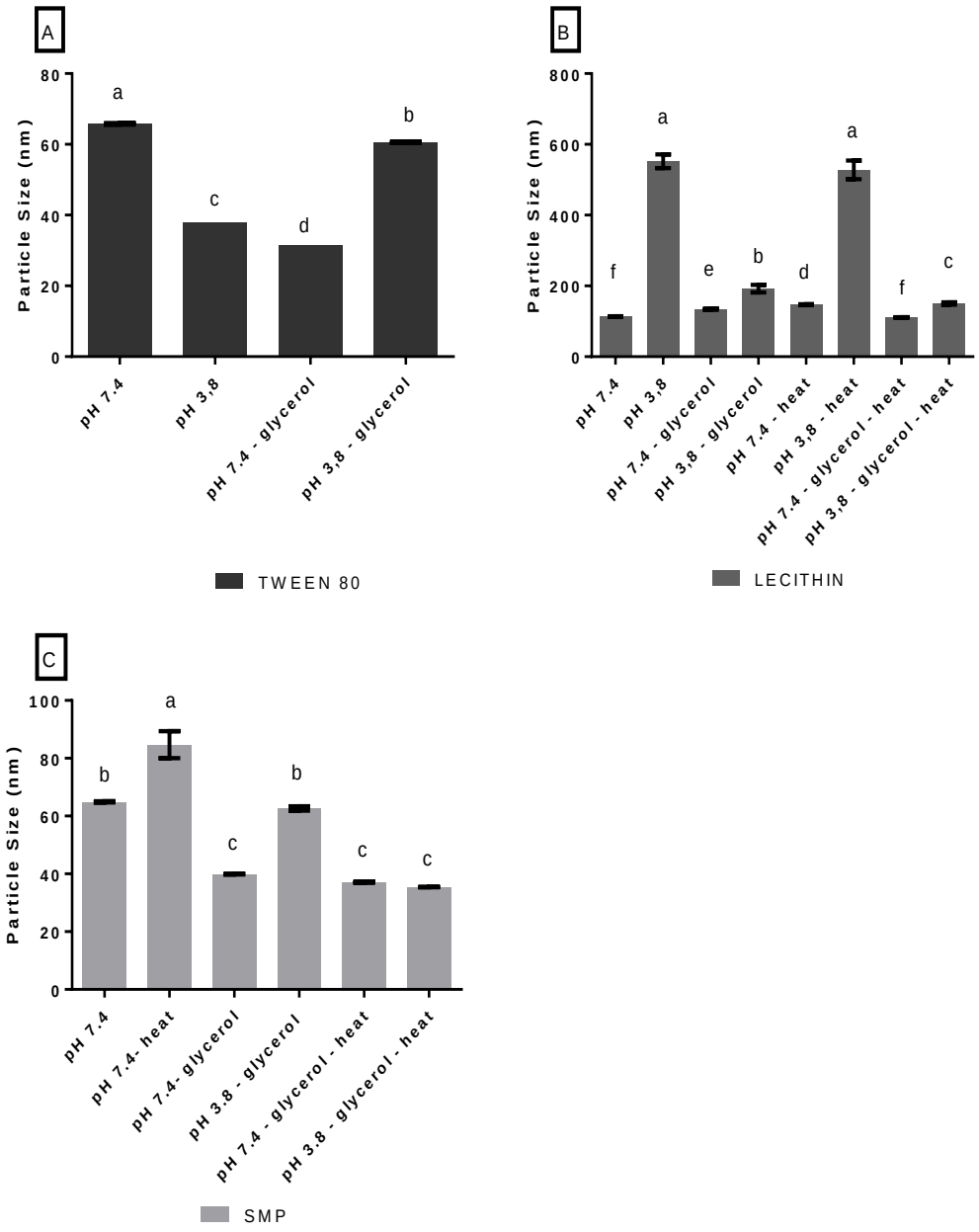

Figure 3.12 Particle sizes of different formulations of capsaicin-loaded nanoemulsions prepared with ultrasonication: (A) Tween 80, (B) Lecithin, and (C) SMP. Means in the same graph indicated by different letters are significantly different ($p \leq 0.05$).

3.3.2. Turbidity

3.3.2.1. Emulsifier type- Tween 80

The turbidity results are given in Figure 3.13. The appearances of the sonicated nanoemulsions containing Tween 80 were found optically opaque and had relatively high turbidities (abs>> 0.7) than that of microfluidized nanoemulsions with Tween 80. Effect of pH was found to be insignificant on the turbidity ($p \geq 0.05$) (Appendix Table D.7). Although the particle sizes of sonicated nanoemulsions were almost 65 nm, higher turbidity values might be due to the presence of larger particles after ultrasonication or rapid flocculation till measurement time. The presence of co-solvent, glycerol, decreased the turbidities of nanoemulsions and slightly transparent nanoemulsions were obtained with glycerol addition to the formulations.

3.3.2.2. Emulsifier type- Lecithin

Decreasing pH, adding glycerol and heating before homogenization resulted in slightly transparent nanoemulsions prepared with lecithin through ultrasonication as given in Figure 3.13-B. Regardless of the droplet sizes, lecithin gave turbid appearances to the nanoemulsions both for microfluidization and ultrasonication processes.

3.3.2.3. Emulsifier type- SMP

As with the Tween 80 results, more transparent nanoemulsions were obtained using emulsifier SMP by sonication. The turbidity values of SMP nanoemulsions are given in Figure 3.13-C. In the absence of glycerol, turbidity values very high and this decreased almost by half by adding glycerol whereas at pH 3.8 turbidity values increased to the high levels as in the absence of glycerol. As mentioned previously, the unstable feature of SMP at pH 3.8 was associated with increasing turbidity values.

In overall considering the turbidity values, homogenization affected particle size distribution and resulted in monomodal distributed colloidal dispersions that were reflected in the appearance. Transparent capsaicin-loaded nanoemulsions were obtained successfully with using ultrasonication.

Also, after ultrasonication, some particles tended to aggregate causing optically opaque appearances. Commonly, adding glycerol to the nanoemulsion formulations enhanced the transparent look that might be due to its ability to change the refractive index contrast between oil and water phase as mentioned before.

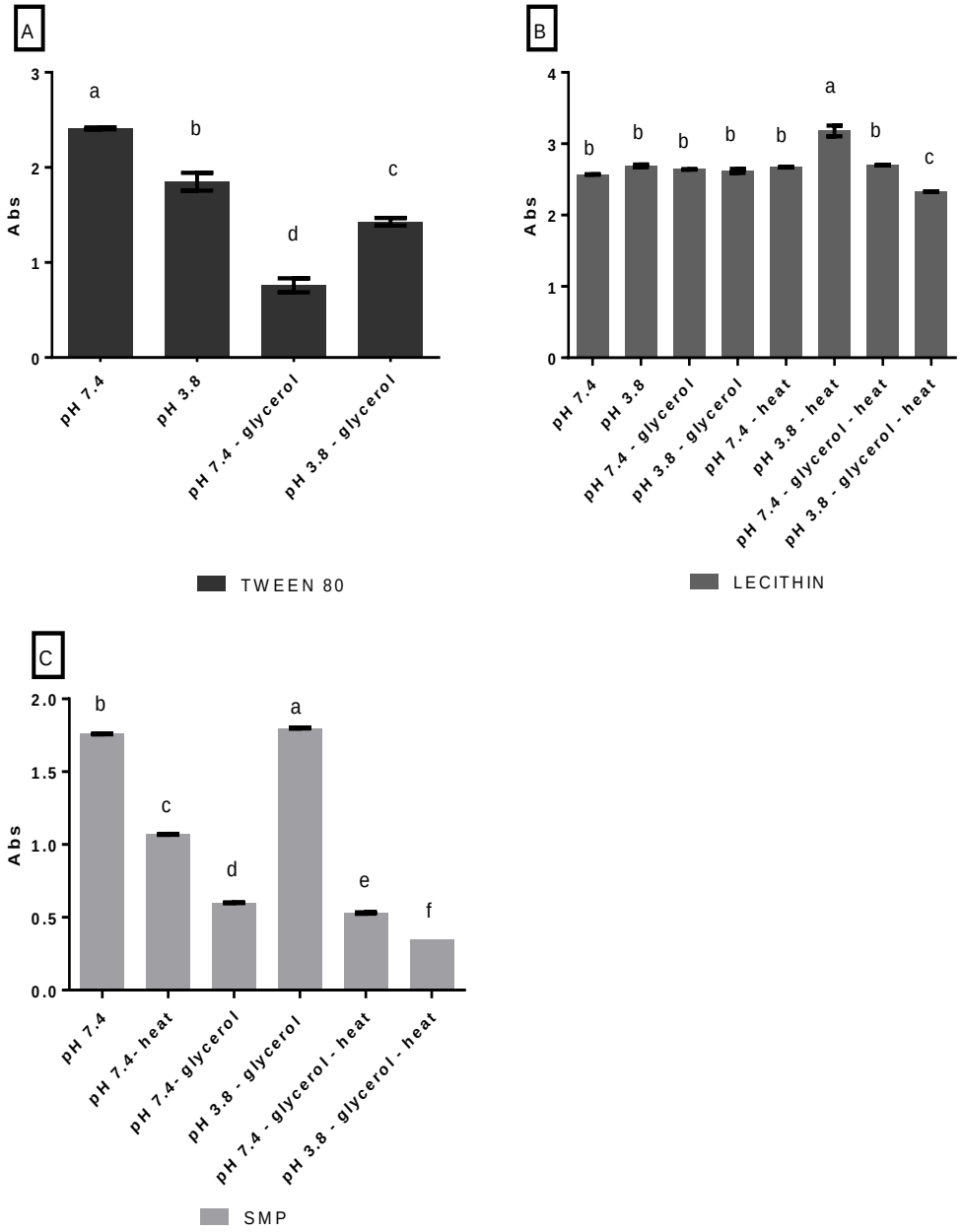

Figure 3.13 Turbidity values of different formulations of capsaicin-loaded nanoemulsions prepared with ultrasonication: (A) Tween 80, (B) Lecithin, and (C) SMP. Means in the same graph indicated by different letters are significantly different ($p \leq 0.05$).

3.3.3. Color of nanoemulsions

L*a*b* values of sonicated capsaicin-loaded nanoemulsions are summarized in Table 3.3. The ultrasound process positively affected the Tween 80 containing nanoemulsion color and lightness (L*) and yellowness (b*) values increased relative to the microfluidization process. However, redness (a*) values decreased. A considerable increase was observed again with the lecithin based sonicated nanoemulsions especially for lightness (L*) values compared with microfluidization. The other values a* and b* were similar or slightly higher than that of microfluidized samples. The pH decrease affected the color values and at pH 3.8 they were all found to be lower than the values at pH 7.4. Also, glycerol addition enhanced color values for both lecithin and SMP containing nanoemulsions. Redness (a*) values increased almost two times relative to microfluidized SMP containing nanoemulsions. Generally, oil concentration, droplet size, and distribution created the differences in color values and enhanced the appearance of nanoemulsions. It was hard to state a single reason on why ultrasound resulted in high color value nanoemulsions. By changing the homogenization process, the characteristics of nanoemulsions changes even if the formulations are same. It all depends on size and distribution of capsaicin oil droplets that created the difference in the refractive index within the nanoemulsions. As a result, lighter, red and transparent nanoemulsions might have been obtained.

Table 3.3 Effect of the different emulsifiers on the colors of capsaicin-loaded nanoemulsions prepared by ultrasonication.

Samples		L* value[#]	a* value[#]	b* value[#]
Oleoresin capsicum		0.18 ± 0.0	0.25 ± 0.11	-0.11 ± 1.0
Tween 80	pH 7.4	72.04 ± 0.00^{b}	41.06± 0.01^{b}	117.72 ± 0.01^{a}
	pH 3.8	72.28 ± 0.01^{a}	40.41 ± 0.01^{c}	116.92 ± 0.01^{b}
	pH 7.4- glycerol	70.55 ± 0.11^{c}	39.58± 0.14^{d}	114.15 ± 0.04^{c}
	pH 3.8- glycerol	62.36 ± 0.01^{d}	42.98 ± 0.00^{a}	105.73 ± 0.03^{d}
Lecithin	pH 7.4	35.91 ± 0.02^{e}	40.99 ± 0.02^{e}	61.84 ± 0.03^{e}
	pH 7.4- glycerol	47.23 ± 0.05^{b}	43.44± 0.02^{b}	81.6 ± 0.31^{b}
	pH 7.4 - heat	35.12 ± 0.07^{f}	40.73 ± 0.03^{f}	60.59 ± 0.11^{f}
	pH 7.4- glycerol- heat	48.96 ± 0.02^{a}	43.34 ± 0.01^{c}	84.15 ± 0.04^{a}
	pH 3.8	28.27 ± 0.01^{g}	37.8 ± 0.0 ^{h}	48.6 ± 0.02^{g}
	pH 3.8- glycerol	42.54 ± 0.34^{d}	42.85± 0.00^{d}	73.8 ± 0.02^{d}
	pH 3.8- heat	27.55 ± 0.01^{h}	37.88± 0.01^{g}	47.38 ± 0.01^{h}
	pH 3.8- glycerol- heat	45.94 ± 0.00^{c}	44.18 ± 0.00^{a}	79.15 ± 0.00^{c}
SMP	pH 7.4	16.36 ± 0.02^{e}	33.14 ± 0.01^{e}	28.14 ± 0.03^{e}
	pH 7.4- glycerol	43.51 ± 0.00^{d}	52.07± 0.00^{b}	75.02 ± 0.00^{d}
	pH 7.4 - heat	13.88 ± 0.01^{f}	30.97 ± 0.02^{f}	23.86 ± 0.01^{f}
	pH 7.4- glycerol- heat	44.63 ± 0.03^{c}	52.83 ± 0.02^{a}	76.94 ± 0.04^{c}
	pH 3.8- glycerol	69.17 ± 0.01^{b}	41.72 ± 0.01^{c}	115.34 ± 0.05^{b}
	pH 3.8- glycerol- heat	72.88 ± 0.02^{a}	39.88 ±0.01^{d}	117.58 ± 0.06^{a}

[#]Data are the mean ± standard error results. Means in the same column indicated by different letters are significantly different ($p \leq 0.05$). ANOVA was conducted for each emulsifier type.

3.3.4. Encapsulation efficiency of nanoemulsions

The encapsulation efficiency results of sonicated capsaicin-loaded nanoemulsions are given in Table 3.4. The similar trend but lower results were observed in ultrasonic homogenization compared to microfluidization. The average encapsulation efficiencies are 73%, 66%, and 62% respectively for Tween 80, lecithin and SMP based nanoemulsions. In the absence of glycerol, the highest efficiency was observed at pH 3.8 nanoemulsions prepared with Tween 80. As the particle size of the Tween 80 based nanoemulsions decreased, the encapsulation efficiencies increased. A negative correlation of -0.70 was obtained between particle size and efficiency values with Tween 80 ($p \leq 0.05$). Decreasing pH, adding glycerol and heating before homogenization increased the encapsulation efficiency of lecithin nanoemulsions ($p \leq 0.05$) (Appendix Table D.8). The highest result was obtained at pH 3.8 in the presence of glycerol and heating for lecithin. At pH 7.4 interaction of glycerol addition and heating increased the efficiency of SMP nanoemulsions significantly ($p \leq 0.05$) (Appendix Table D.9). Also, in the presence of glycerol, decreasing pH from 7.4 to 3.8 increased efficiency from 61% to 70% of SMP nanoemulsions.

Table 3.4 Effect of the different emulsifiers on the encapsulation efficiency of capsaicin-loaded nanoemulsions prepared by ultrasonication.

Samples		Encapsulation efficiency (%)[#]
Tween 80	pH 7.4	68.99 ± 0.00^{d}
	pH 3.8	77.35 ± 0.00^{a}
	pH 7.4- glycerol	73.35 ± 0.00^{b}
	pH 3.8- glycerol	73.16 ± 0.00^{c}
Lecithin	pH 7.4	66.65 ± 0.01^{b}
	pH 7.4- glycerol	63.58 ± 0.01^{cd}
	pH 7.4 - heat	66.31 ± 0.01^{bc}
	pH 7.4- glycerol- heat	64.43 ± 0.01^{bc}
	pH 3.8	66.59 ± 0.00^{b}
	pH 3.8- glycerol	65.45 ± 0.00^{bc}
	pH 3.8- heat	61.48 ± 0.01^{d}
	pH 3.8- glycerol- heat	78.64 ± 0.00^{a}
SMP	pH 7.4	58.47 ± 0.00^{c}
	pH 7.4- glycerol	59.83 ± 0.00^{bc}
	pH 7.4 - heat	59.26 ± 0.00^{bc}
	pH 7.4- glycerol- heat	61.76 ± 0.01^{bc}
	pH 3.8- glycerol	63.01 ± 0.01^{b}
	pH 3.8- glycerol- heat	70.17 ± 0.02^{a}

[#]Data are the mean ± standard error results. Means in the same column indicated by different letters are significantly different ($p \leq 0.05$). ANOVA was conducted for each emulsifier type.

3.3.5. Stability of nanoemulsions

3.3.5.1. Emulsifier type- Tween 80

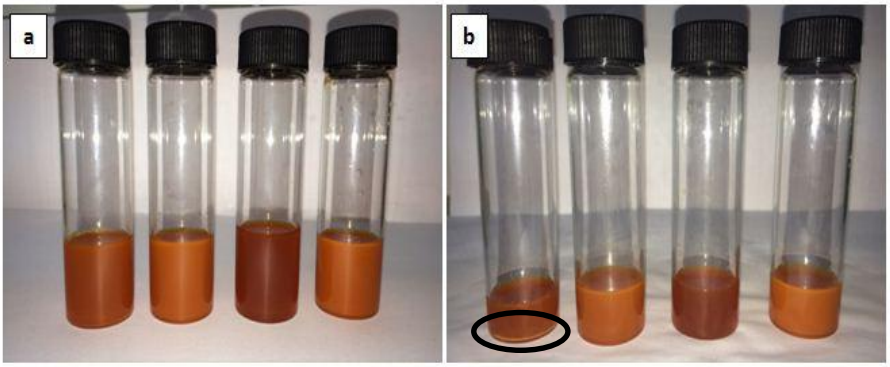

Figure 3.14 Photographs of sonicated Tween 80 containing capsaicin loaded nanoemulsions stored for 0 or 28 days: **a) 0th**, pH 7.4- pH 7.4, glycerol- pH 3.8- pH 3.8, glycerol; **b) 28th**, pH 7.4- pH 7.4, glycerol- pH 3.8- pH 3.8, glycerol.

As with the microfluidized nanoemulsions, sonicated nanoemulsion which contained Tween 80 as the emulsifier showed visually good stability without observing phase separation or creaming (Figure 3.14) except for the pH 7.4 one. The 28 days stabilities of sonicated nanoemulsions formed with Tween 80 are given in Figure 3.15. Ultrasound promotes the droplet disruption by helping adsorption of the emulsifier to the oil surface and prevents instability during storage. In a study, Salvia-Trujillo et al.(2013a) stated that Tween 80 and alginate containing lemon oil nanoemulsions showed higher stability with ultrasonication process. It was claimed that by creating an electrical charge on the surface of nanoemulsion ultrasound increased the stability. Also, small particles helped to maintain stability.

Increase in particle sizes values were observed for glycerol containing nanoemulsions significantly with time ($p \leq 0.05$) (Appendix Table D.10). Glycerol separated from the dispersion and formed clumps. This was not observed in the storage period of 28 days visually due to the rate of separation being slow. There was settling in pH 7.4 nanoemulsions that were visually observable (Highlighted in Figure 3.14). Also, this caused a decrease in particle size and turbidity values of the nanoemulsion. Turbidity values of glycerol containing nanoemulsions tended to increase due to the formation of larger particles. Also, droplets packed more favorably when the dispersion show polydisperse characteristic. The span values for the Tween 80 nanoemulsions were range between 2.80 ± 0.01 and 1.95 ± 0.0. This might be the reason to see separation from the nanoemulsion.

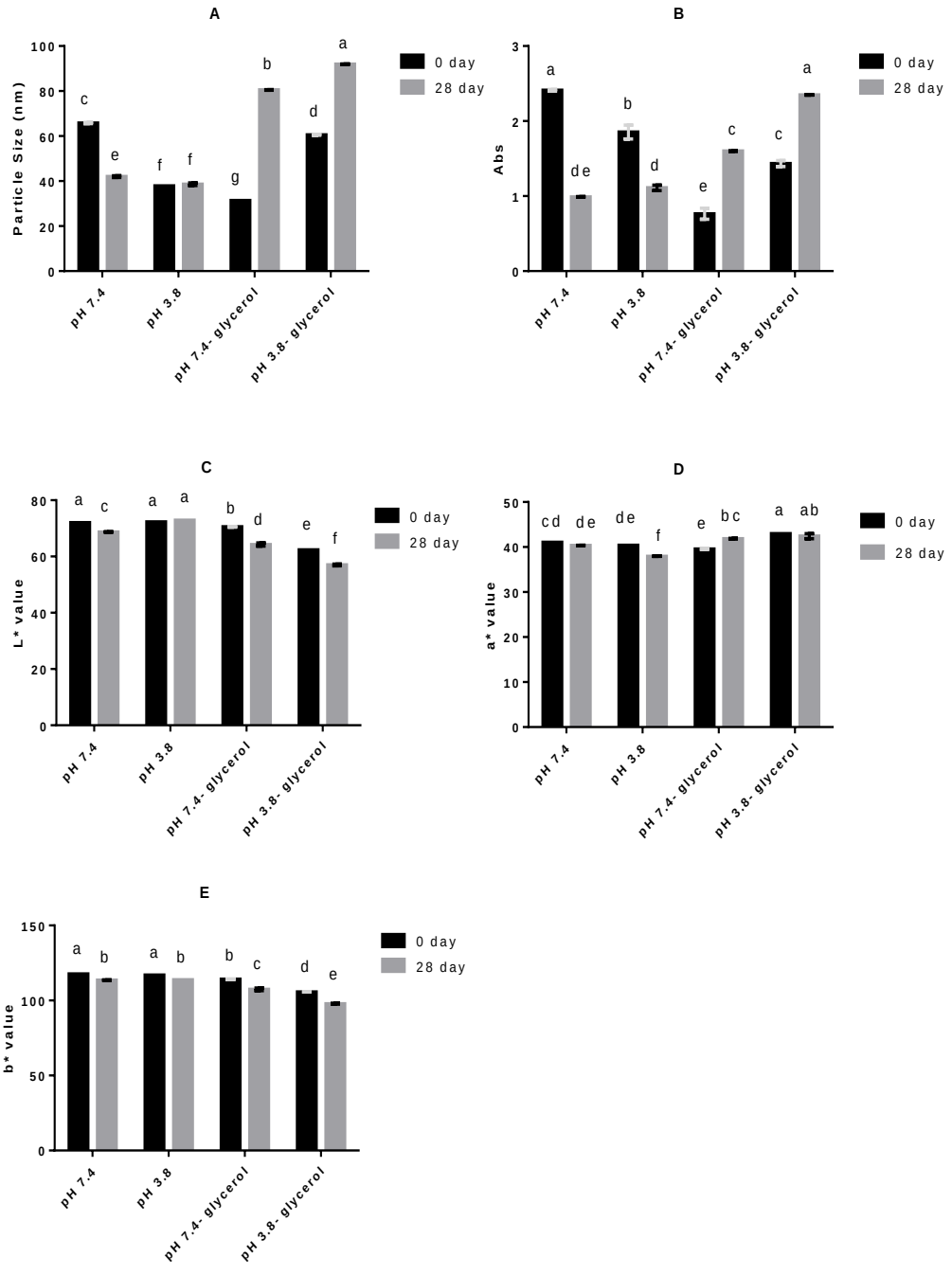

Figure 3.15 Stability results of sonicated Tween 80 containing capsaicin-loaded nanoemulsions for 0 and 28 days: **A)**Particle sizes, **B)**Turbidities, **C)**Color- L* values, **D)** Color- a* values, **E)** Color- b*values.

3.3.5.2. Emulsifier type- Lecithin

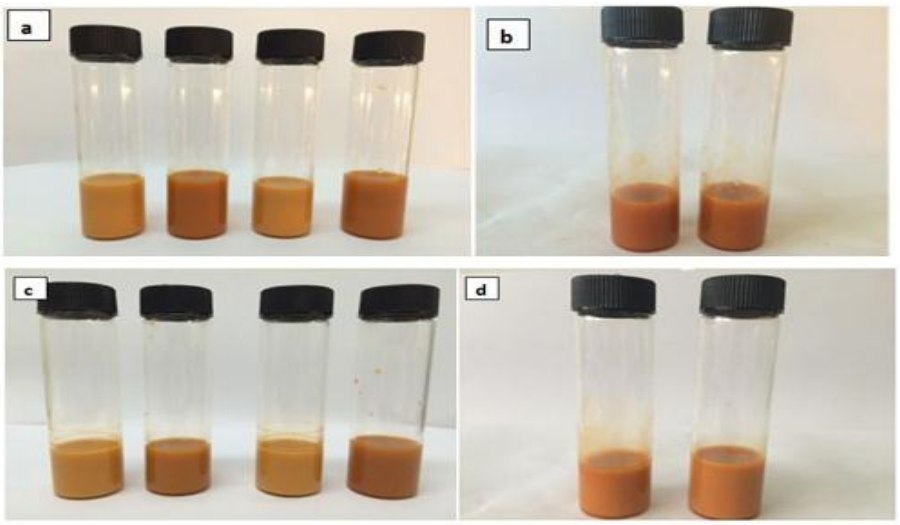

Figure 3.16 Photographs of sonicated lecithin containing capsaicin loaded nanoemulsions stored for 0 or 28 days: **a) 0th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **b) 28th**, pH 7.4, glycerol- pH 7.4, glycerol, heat; **c) 0th,** pH 3.8- pH 3.8, glycerol-pH 3.8, heat- pH 3.8, glycerol, heat; **d) 28th**, pH 3.8, glycerol- pH 3.8, glycerol, heat.

The opaque appearances were shown again in sonicated lecithin based nanoemulsions as in Figure 3.16 during 28 days of storage. It was confirmed that opaque appearance could not be overcome neither with microfluidization nor with ultrasonication when 2% of lecithin were used. On the other hand, sonicated nanoemulsions the so-called pH 7.4, pH 7.4-heat, pH 3.8 and pH 3.8- heat ones phase separated after 7 days of storage and could not further be analyzed. On the other hand, glycerol addition affected the interfacial properties thus it established stable nanoemulsions.

The results of particle size, turbidity and color measurements for the 28 days storage period are given in Figure 3.17. Particle sizes and turbidities of lecithin nanoemulsions were increased whereas color values decreased significantly with time ($p \leq 0.05$) (Appendix Table D.11). The biggest particle grown up was observed at pH 7.4- glycerol-heat nanoemulsion. On the other hand, the dramatic decrease was observed on color values of samples at pH 3.8. These results suggested that after preparation with ultrasonication, droplets easily overcame the steric repulsion and Ostwald ripening or further droplet grown up occurred upon storage. In fact, these nanoemulsions had already opaque appearances and larger particle sizes since the first day. In particle size measurement, the sample was subjected to continuous stirring in the sample dispersion unit containing pure water. As the sample was added, flocculate but not coalescent particles are dispersed with stirring while measuring and the results could be misleading.

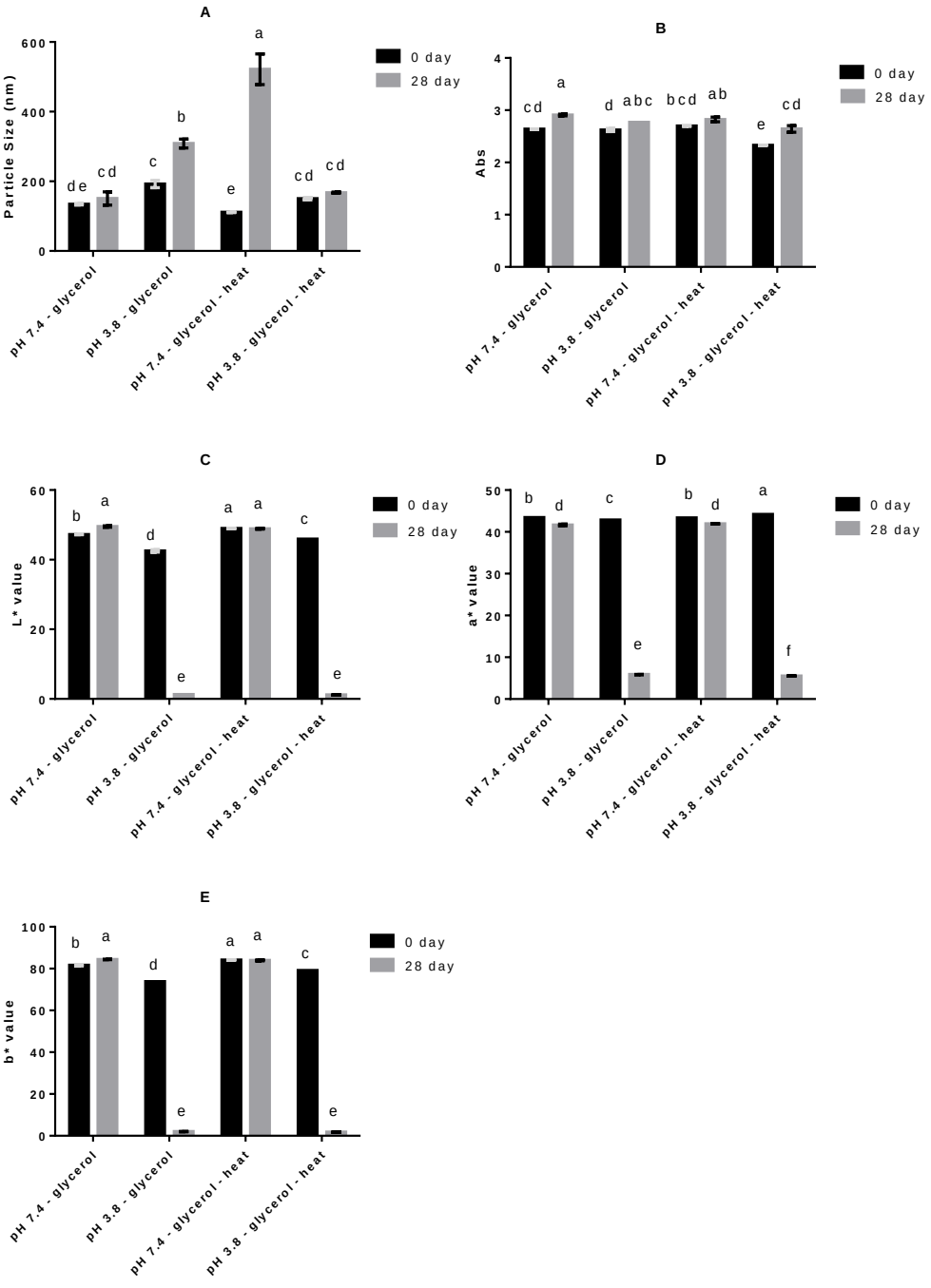

Figure 3.17 Stability results of sonicated lecithin containing capsaicin-loaded nanoemulsions for 0 and 28 days: **A)**Particle sizes, **B)**Turbidities, **C)**Color- L* values, **D)** Color- a* values, **E)** Color- b*values.

3.3.5.3. Emulsifier type- SMP

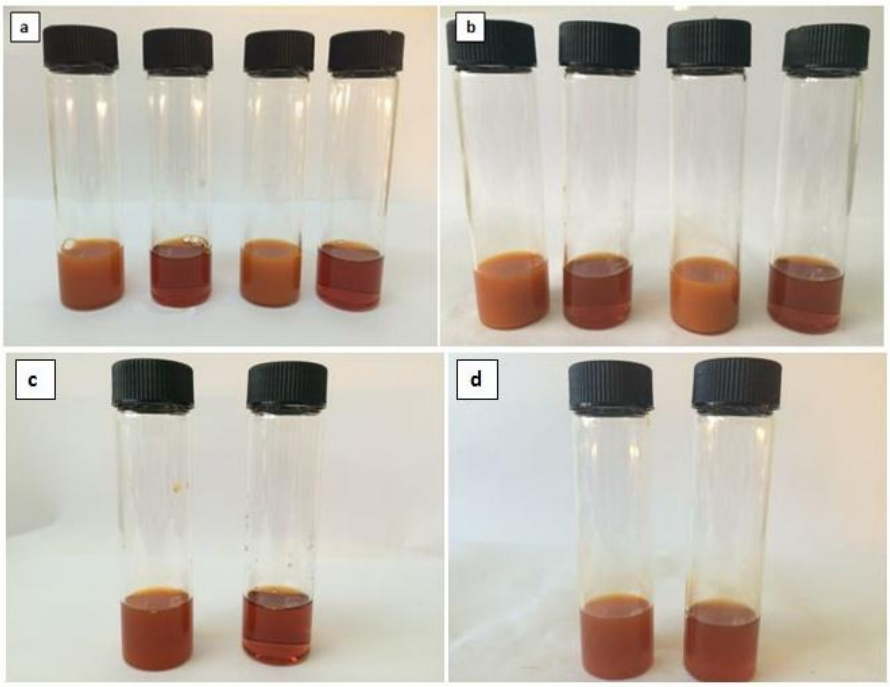

Figure 3.18 Photographs of sonicated SMP containing capsaicin loaded nanoemulsions stored for 0 or 28 days: **a) 0th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **b) 28th**, pH 7.4- pH 7.4, glycerol- pH 7.4, heat- pH 7.4, glycerol, heat; **c)0 th,** pH 3.8, glycerol-pH 3.8, glycerol, heat; **d) 28th**, pH 3.8, glycerol- pH 3.8, glycerol, heat.

The storage stabilities of nanoemulsion are shown in Figure 3.18. The sonicated SMP based nanoemulsions which were named as pH 3.8 and pH 3.8- heat phase separated even after ultrasonication process and could not further be analyzed. This result was not surprising since the low acid stability of SMP was indicated previously. The difference between microfluidized and sonicated nanoemulsions during storage and

ultrasonication helped to keep the stabilities of especially pH 7.4 and pH 7.4-heat nanoemulsions which were unstable after microfluidization. However, locally bigger particles formed during storage at pH 7.4-heat nanoemulsion that showed a decrease in particle size as seen in. Figure 3.19-B. On the other hand, particle size increased significantly but remained lower than 97nm after 28 days ($p \leq 0.05$) (Appendix Table D.12). There were increases in turbidity values which were observable both visually and instrumentally at the end of storage. Rather than heating the nanoemulsions, glycerol resulted with transparent and good stability nanoemulsions by using SMP as an emulsifier. Color values decreased due to increase in particle size with time.

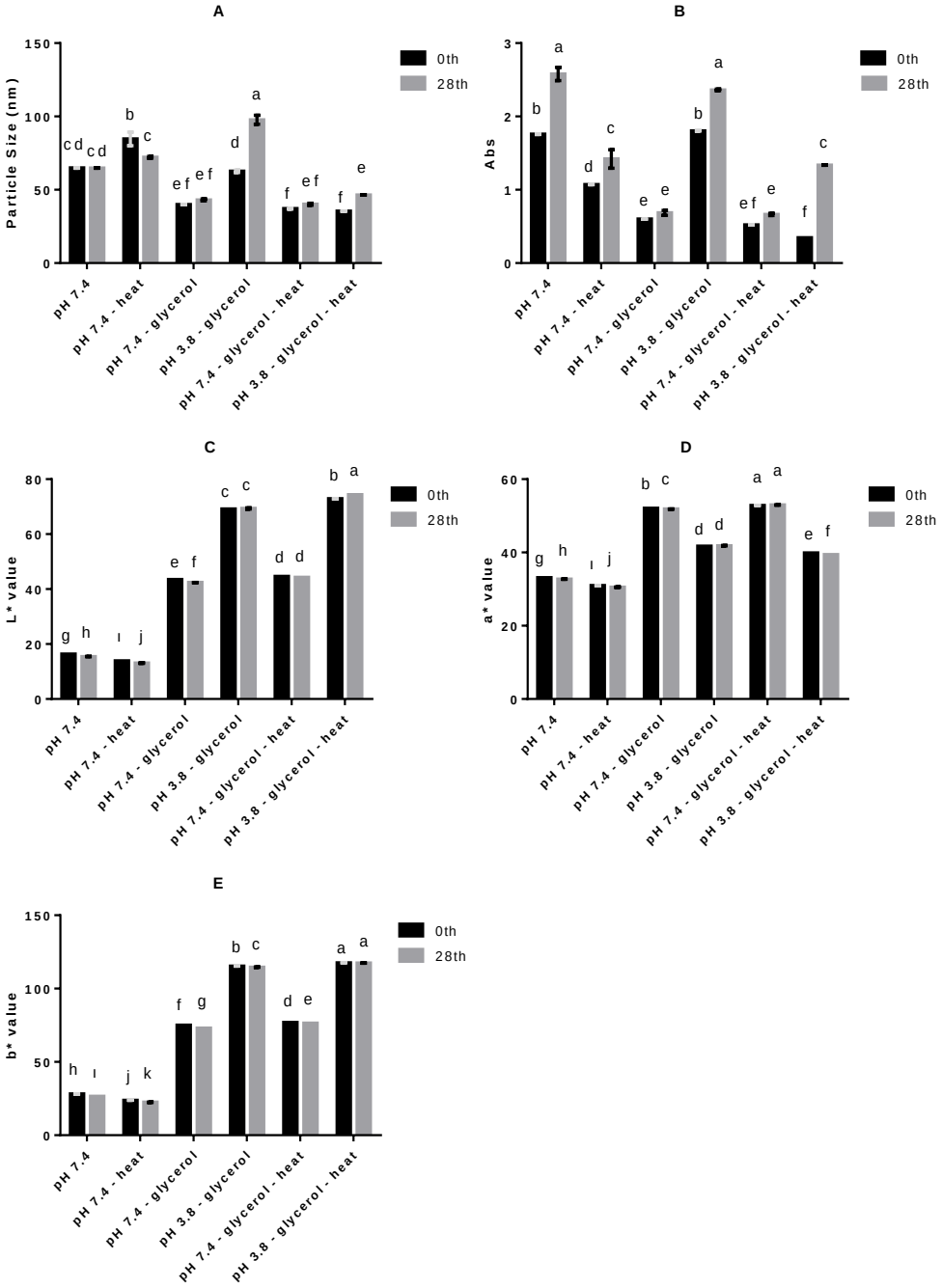

Figure 3.19 Stability results of sonicated SMP containing capsaicin-loaded nanoemulsions for 0 and 28 days: A)Particle sizes, B)Turbidities, C)Color- L* values, D) Color- a* values, E) Color- b*values.

3.3.6. Antimicrobial activity of nanoemulsions

After ultrasonication treatment, antimicrobial activity results of capsaicin-loaded nanoemulsions decreased relative to microfluidized nanoemulsions. Thanks to cavitation phenomena, air bubbles were produced and collapsed within the fluid sample during ultrasonication process and local pressure and temperature increase could be observed. Hydrophobic volatile essential oils are sensitive to instantaneous temperature fluctuations that may lose their antimicrobial activity. Also, this process could trigger to produce hydroxyl radicals, hydrogen atoms and even hydrogen peroxide that could lead to a change in the essential oil functionality (Jiang, Pétrier, & Waite, 2002a,2002b; Kidak & Ince, 2006; Nanzai, Okitsu, Takenaka, Bandow, & Maeda, 2008). A study revealed that sunflower oil droplets oxidized during homogenization with ultrasonication and reduced desirable properties of sunflower oil (Chemat, et al., 2004, 2011). In another study, Salvia-Trujillo et al., (2014) found that microfluidized lemongrass oil loaded nanoemulsions were more active to inhibit *E.coli* than sonicated nanoemulsions. Similar results were observed in this study as well.

3.3.6.1. Emulsifier type- Tween 80

The effect of ultrasound processing on antimicrobial activity of capsaicin-loaded nanoemulsions is displayed in Figure 3.20. Statistical analysis showed that glycerol addition clearly enhanced inactivation of *E. coli* and *S. aureus* of nanoemulsions prepared with emulsifier Tween 80. The highest population decrease values are 2.25 log for *E.coli* and 3.01 log for *S. aureus* obtained through glycerol addition.

3.3.6.2. Emulsifier type- Lecithin

In contrast, pH changes increased the antimicrobial action of lecithin based nanoemulsions rather than glycerol addition. While low inhibition was observed at pH 3.8 glycerol added nanoemulsions (1.53 log), this value increased to the 2.28 log

at pH 7.4 for *E.coli*. Similar results observed were for *S. aureus* and at pH 3.8 population decrease was 2.47 log but at pH 7.4 it was 2.72 log.

3.3.6.3. Emulsifier type- SMP

In a similar way, at pH 7.4 SMP containing nanoemulsions the population decrease for *E.coli* was found higher than that of microfluidized glycerol added nanoemulsions with a value of 2.69 log. It was 1.07 log for microfluidized nanoemulsions. Statistically, there were no differences between SMP containing nanoemulsion prepared with ultrasonication for *S. aureus* log decrease values ($p > 0.05$) (Appendix Table D.9). The highest inhibition was 2.92 log and the lowest was 2.63 log. These results suggested that glycerol as a co-solvent have a good impact on the antimicrobial activity of nanoemulsions prepared with ultrasonication.

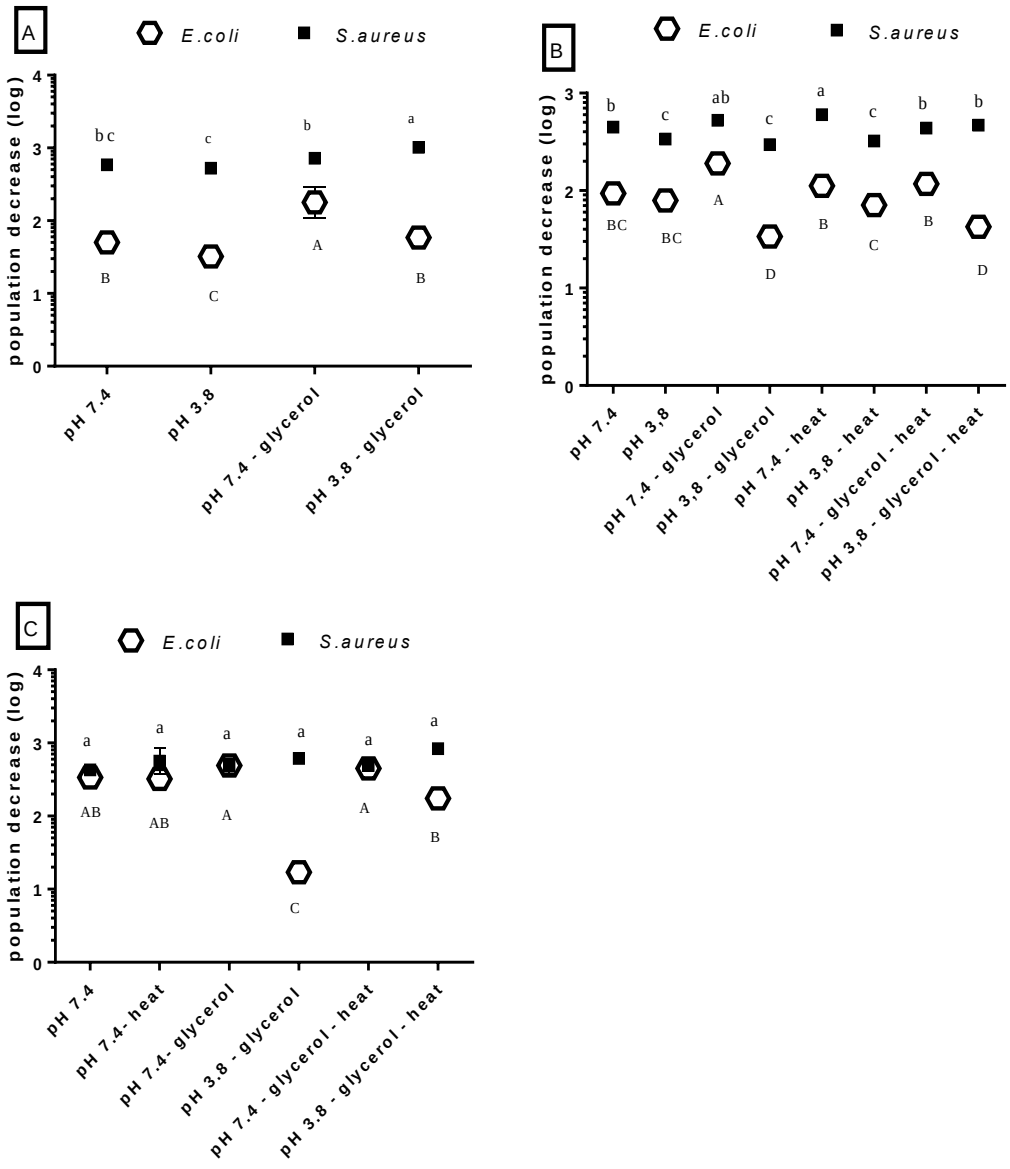

Figure 3.20 Effect of capsaicin-loaded nanoemulsions on *Escherichia coli* and *Staphylococcus aureus* prepared with ultrasonication. Population decrease means the ratio of survived microorganisms (CFU/mL) to inoculum culture (CFU/mL). ANOVA was conducted for each microorganism and emulsifier type.

3.4. Transmission Electron Microscopy and Atomic Force Microscopy

AFM and TEM analysis were carried to investigate the morphologies of selected capsaicin-loaded nanoemulsions. Samples were prepared with only distilled water, emulsifier, and oil in order to avoid interference of other compounds such as ions by microfluidizer The observations of microfluidized nanoemulsions prepared with Tween 80, lecithin and sucrose monopalmitate revealed that oil droplets had spherical in shape. However, depending on the type of emulsifier, oil droplets in the emulsions showed different distributions. According to TEM observations, particle sizes were around 50, 200, 100 nm for nanoemulsions prepared with Tween 80, lecithin and sucrose monopalmitate respectively as showed in Figure 3.21. These results were in agreement with dynamic light scattering results.

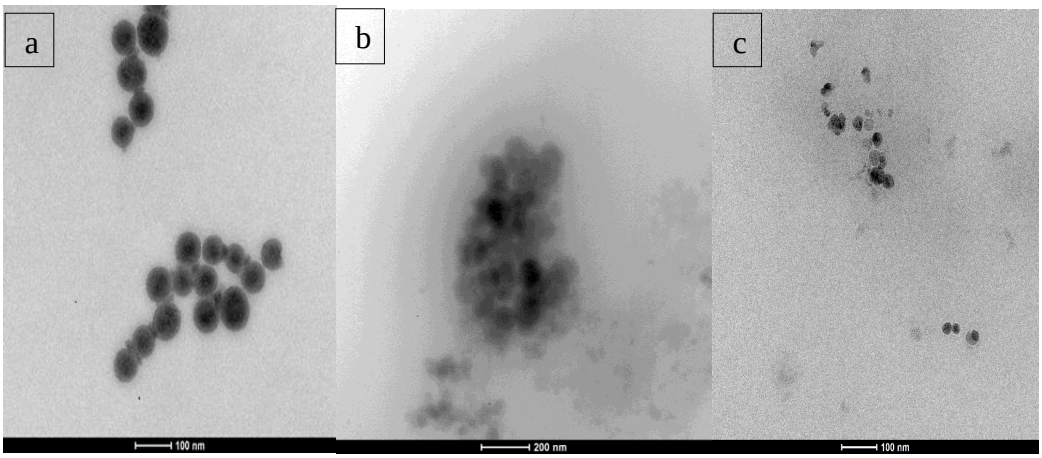

Figure 3.21 Transmission electron micrograph of capsaicin-loaded nanoemulsion (prepared with microfluidizer). a) emulsifier Tween 80; b) emulsifier lecithin; c) emulsifier sucrose monopalmitate.

On the other hand, based on AFM observations, the particle sizes were range between 118-189 nm, 96 nm- 1.07 µm and 20 nm-1µm for nanoemulsions prepared with Tween 80, lecithin and sucrose monopalmitate respectively as showed in Figure 3.22. Besides, by using dynamic light scattering techniques on same nanoemulsion samples, the particle sizes were measured as 70 nm, 257.5 nm, and 40.5 nm, respectively. The reason why the dynamic light scattering and AFM result might be was the tip broadening effect. This situation caused when the tip is in contact with a sticky particle and thus widened throughout the surface of the particle during measurement. Also, surface homogeneity of the nanoemulsion sample also might have been lost during drying, and the result could be obtained. In the same way, Salvia-Trujillo et al. (2013b) indicated that AFM results gave higher particle size results than dynamic light scattering in their study. In contrast to these observations, Surassmo et al. (2010) found similar results in their study.

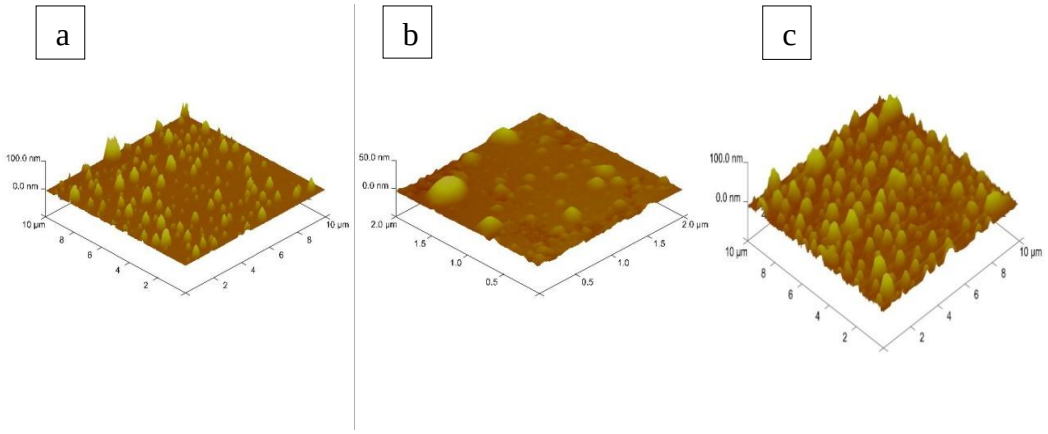

Figure 3.22 AFM images of capsaicin-loaded nanoemulsions prepared with microfluidizer. a) emulsifier Tween 80; b) emulsifier lecithin; c) emulsifier sucrose monopalmitate.

CHAPTER 4

CONCLUSION

In this work, oleoresin capsicum loaded nanoemulsions were prepared by using three emulsifiers (Tween 80, lecithin, sucrose monopalmitate) at two different pH's (7.4 and 3.8) with two different homogenization techniques (microfluidization and ultrasonication). Also, glycerol addition to continuous phase composition and effect of heating the coarse emulsion was examined. Furthermore, nanoemulsions were stored for 28 days to analyze the storage stability. Along with the general tools for characterization such as particle size, turbidity, and color measurement, NMR relaxometry was used to characterize the different formulations of nanoemulsions. In order to verify the structural differences, TEM and AFM images were obtained for selected nanoemulsions. To understand the main aim of the nanoemulsion, antimicrobial activity assays were done for two food-borne microorganisms *Escherichia coli* and *Staphylococcus aureus*. The results obtained from these experiments are summarized below:

- Nano-sized emulsions were prepared with both homogenization techniques. Capsaicin loaded nanoemulsions obtained at 1.400 bars for 5 passes with microfluidization showed minimum average particle size of 35 nm by using sucrose monopalmitate. On the other hand, 75 % amplitude for 5 min with ultrasonication resulted in minimum average particle size of 49 nm by using Tween 80. The minimum average particle size of nanoemulsions by using

lecithin was higher than other emulsifiers such as 323 and 241 nm for microfluidization and ultrasonication respectively. The TEM and AFM results confirmed the nano-sized particles of nanoemulsions.

- Microfluidized capsaicin-loaded nanoemulsions achieved mostly 3.40 log reduction on *E.coli* population with lecithin whereas 5.89 log reduction on *S. aureus* population with Tween 80. On the other hand, sonicated capsaicin-loaded nanoemulsions achieved mostly 2.69 log reduction on *E.coli* population with sucrose monopalmitate whereas 3.01 log reduction on *S. aureus* population with Tween 80.
- The most efficient capsaicin-loaded nanoemulsions for microfluidization technique was pH 7.4-glycerol added-heated nanoemulsions prepared with lecithin with particle size 215 nm for *E. coli* whereas for *S. aureus* pH 7.4-glycerol added nanoemulsions prepared with emulsifier Tween 80 with particle size 63.77 nm. In the same way, for ultrasonication technique, these were pH 7.4-glycerol added nanoemulsions prepared with sucrose monopalmitate with a particle size of 39.90 nm for *E. coli* whereas for *S. aureus* pH 3.8-glycerol added nanoemulsions prepared with Tween 80with particle with a size 60.57 nm.
- The nanoemulsions formulated with Tween 80 showed higher stability during 28 days with particle sizes ranges from 33 to 49 nm by microfluidization.
- By adding glycerol to the continuous phase exhibited clear, almost transparent, bright red colored nanoemulsions due to refractive index changes.
- Microfluidization was proved to be a good homogenization technique which improved physical properties such as color, turbidity along with the enhanced antimicrobial activity.
- Low-resolution NMR relaxometry used successfully to characterize nanoemulsions and also the results were compared with the widely used techniques in literature such as particle size or turbidity.

Consequently, results obtained in this thesis showed that capsaicin can be used in the form of a nanoemulsion systems to improve its antimicrobial activity against certain microorganisms. However, more work need to be done about its influence in real food systems. By using the parameters obtained from this work, industrial based packaging materials, biofilms can be designed as well as the research may be expanded to other pathogens to deepen the antimicrobial activity.

REFERENCES

Abbas, S., Hayat, K., Karangwa, E., Bashari, M., & Zhang, X. (2013). An Overview of Ultrasound-Assisted Food-Grade Nanoemulsions. *Food Engineering Reviews*, *5*(3), 139–157. http://doi.org/10.1007/s12393-013-9066-3

Abbaszadeh, S., Sharifzadeh, A., Shokri, H., Khosravi, A. R., & Abbaszadeh, A. (2014). Antifungal efficacy of thymol, carvacrol, eugenol and menthol as alternative agents to control the growth of food-relevant fungi. *Journal de Mycologie Medicale*, *24*(2), e51–e56. http://doi.org/10.1016/j.mycmed.2014.01.063

Acosta, E. (2009). Bioavailability of nanoparticles in nutrient and nutraceutical delivery. *Current Opinion in Colloid and Interface Science*, *14*(1), 3–15. http://doi.org/10.1016/j.cocis.2008.01.002

Al Othman, Z. A., Ahmed, Y. B. H., Habila, M. A., & Ghafar, A. A. (2011). Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using high performance liquid chromatography. *Molecules (Basel, Switzerland)*, *16*(10), 8919–29. http://doi.org/10.3390/molecules16108919

Anandakumar, P., Kamaraj, S., Jagan, S., Ramakrishnan, G., Asokkumar, S., Naveenkumar, C., Raghunandhakumar, S., and Devaki, T. (2012). Capsaicin inhibits benzo(a)pyrene-induced lung carcinogenesis in an in vivo mouse model. *Inflamm. Res.*, 61: 1169−1175.

Ani, V., Varadaraj, M. C., & Naidu, K. A. (2006). Antioxidant and antibacterial activities of polyphenolic compounds from bitter cumin (Cuminum nigrum L.). *European Food Research and Technology*, *224*(1), 109–115. http://doi.org/10.1007/s00217-006-0295-z

Anton, N., Benoit, J. P., & Saulnier, P. (2008). Design and production of nanoparticles formulated from nano-emulsion templates-A review. *Journal of Controlled Release*, *128*(3), 185–199. http://doi.org/10.1016/j.jconrel.2008.02.007

Ariyaprakai, S., & Tananuwong, K. (2015). Freeze-thaw stability of edible oil-in-water emulsions stabilized by sucrose esters and Tweens. *Journal of Food Engineering*, *152*, 57–64. http://doi.org/10.1016/j.jfoodeng.2014.11.023

Artz, W.E. and Hansen, S.L. (1994). Carbohydrate Polyesters as Fat Substitutes, Editör: Akoh,C.C.,Swanson, B.G. New York:Marcel Dekker, Page: 197.

Asnin, L., & Park, S. W. (2013). Isolation and analysis of bioactive compounds in Capsicum peppers. *Critical Reviews in Food Science and Nutrition*, *8398*(August 2014), 37–41.

ASTA. (1995) American Spice Trade Association. Official communication.

Ayana, B. (2007). Production of antimicrobial edible films and characterization. Master's thesis, University of Mersin, Mersin, Turkey. Retrieved from https://tez.yok.gov.tr

Bajpai, V. K., Baek, K.-H., & Kang, S. C. (2012). Control of Salmonella in foods by using essential oils: a review. *Food Research International*, 45,722-734.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils e a review. *Food and Chemical Toxicology*, 46,446-475.

Barbaro, B., Toietta, G., Maggio, R., Arciello, M., Tarocchi, M., Galli, A., & Balsano, C. (2014). Effects of the olive-derived polyphenol oleuropein on human health. *International Journal of Molecular Sciences*, *15*(10), 18508–18524. http://doi.org/10.3390/ijms151018508

Barnadas-Rodriguez, R. & Sabes, M. (2001). Factors involved in the production of liposomes with a high-pressure homogenizer, *International Journal of Pharmaceutics*, 213, 1-2, 175-186.

Başer, K. C., & Buchbauer, G. (2010). Handbook of Essential Oils: Science, Technology, and Applications. New York: CRC Press, Taylor & Francis Group.

Bisignano, G., Tomaino, A., Lo Cascio, R., Crisafi, G., Uccella, N., & Saija, A. (1999). On the in vitro antimicrobial activity of oleuropein and hydroxytyrosol. J*ournal of Pharmacy and Pharmacology*, 51(8), 971–974.

Blanco-Padilla, A., Soto, K. M., Hernández Iturriaga, M., & Mendoza, S. (2014). Food antimicrobials nanocarriers. *Scientific World Journal*, *2014*. http://doi.org/10.1155/2014/837215

Bouchemal, K., Briançon, S., Perrier, E., & Fessi, H. (2004). Nano-emulsion formulation using spontaneous emulsification: Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. *International Journal of Pharmaceutics*, *280*(1-2), 241–251. http://doi.org/10.1016/j.ijpharm.2004.05.016

Bourne, N., Bernstein, D.I., Stanberry, L.R. (1999) Civamide (cis-capsaicin) for treatment of primary or recurrent experimental genital herpes. *Antimicrob Agents Chemother*, 43(11): 2685- 2688.

Bubonja-Sonje, M., Giacometti, J., & Abram, M. (2011). Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. *Food Chemistry*, 127(4), 1821–1827. http://doi.org/10.1016/j.foodchem.2011.02.071

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. *International Journal of Food Microbiology*, *94*(3), 223–53. http://doi.org/10.1016/j.ijfoodmicro.2004.03.022

Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., & Ferret, A. (2007). Invited review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science*, 90, 2580-2595.

Capitani, D., Segre, A. L., & Sparapani, R. (1991). Lecithin microemulsion gels: a nmr study of molecular mobility based on line widths. *Langmuir*, (7), 250–253.

Chanamai, R., & McClements, D. J. (2001). Prediction of emulsion color from droplet characteristics: Dilute monodisperse oil-in-water emulsions. *Food Hydrocolloids*, *15*, 83–91. http://doi.org/10.1016/S0268-005X(00)00055-2

Chemat, F., Grondin, I., Sing, A. S. C., & Smadja, J. (2004). Deterioration of edible oils during food processing by ultrasound. *Ultrasonics Sonochemistry*, *11*(1), 13–15. http://doi.org/10.1016/S1350-4177(03)00127-5

Chemat, F., Zill-e-Huma, & Khan, M. K. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry*, *18*(4), 813–35. http://doi.org/10.1016/j.ultsonch.2010.11.023

Chao, S. C., & Young, D. G. (2000). Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. *Journal of Essential Oil Research*, 12, 639-649.

Choi, A. J., Kim, C. J., Cho, Y. J., Hwang, J. K., & Kim, C. T. (2009). Effects of surfactants on the formation and stability of capsaicin-loaded nanoemulsions. *Food Science and Biotechnology*, *18*(5), 1161–1172.

Choi, A.-J. J., Kim, C. J. C.-T. T. C.-J., Cho, Y.-J. J., Hwang, J.-K. K., & Kim, C. J. C.-T. T. C.-J. (2011a). Characterization of Capsaicin-Loaded Nanoemulsions Stabilized with Alginate and Chitosan by Self-assembly. *Food and Bioprocess Technology*, *4*(6), 1119–1126. http://doi.org/10.1007/s11947-011-0568-9

Choi, S. J., Decker, E. A., Henson, L., Popplewell, L. M., Xiao, H., & McClements, D. J. (2011b). Formulation and properties of model beverage emulsions stabilized by sucrose monopalmitate: Influence of pH and lyso-lecithin addition. *Food Research International*, *44*(9), 3006–3012. http://doi.org/10.1016/j.foodres.2011.07.007

Choi, A. Y., Kim, C. T., Park, H. Y., Kim, H. O., Lee, N. R., Lee, K. E., & Gwak, H. S. (2013). Pharmacokinetic characteristics of capsaicin-loaded nanoemulsions fabricated with alginate and chitosan. *Journal of Agricultural and Food Chemistry*, *61*(9), 2096–2102.

Cichewicz, R. H., & Thorpe, P. A. (1996). The antimicrobial properties of chile peppers (Capsicum species) and their uses in Mayan medicine. *Journal of Etnopharmacology*, *52*, 61–70.

Clydesdale, F.M. (1975). Methods and measurements of food color, in Theory, Determination and Control of Physical Properties of Food Materials, Rha, C., Ed., D. Reidel Publishing, Boston, chap. 14.

Cimanga, K., Kambu, K., Tona, L., Apers, S., De Bruyne, T., Her- mans, N., Totte´, J., Pieters, L., Vlietinck, A.J. (2002). Correlation between chemical composition and antibacterial activity of es- sential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *Journal of Ethnopharmacology*, 79, 213–220.

Comas, D. I., Wagner, J. R., & Tomas, M. C. (2006). Creaming stability of oil in water (O/W) emulsions: Influence of pH on soybean protein-lecithin interaction. *Food Hydrocolloids*, *20*(7), 990–996. http://doi.org/10.1016/j.foodhyd.2005.11.006

Cox, S. D., Mann, C. M., Markham, J. L., Gustafson, J. E., Warmington, J. R., & Wyllie, S. G. (2001). Determining the antimicrobial actions of tea tree oil. *Molecules*, *6*(2), 87–91. http://doi.org/10.3390/60100087

Davidson, P.M. (1997). Chemical preservatives and natural antimi- crobial compounds. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food Microbiology: Fundamentals and Frontiers. ASM, Washington, pp. 520–556.

Deal, C.L. (1991). Effect of topical capsaicin: A double blind trial. *Clin. Therap.*, 13: 383−395

de Morais, J., Santos, O., Delicato, T., Goncalves, R., and Rocha, P.-Filho (2006). Physicochemical characterization of canola oil/water nano-emulsions obtained by determination of required hlb number and emulsion phase in- version methods. *Journal of Dispersion Science and Technology*, 27(1): 109–115.

Denyer, S.P., Hugo, W.B., (1991a). Biocide-induced damage to the bacterial cytoplasmic membrane. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. The Society for Applied Bacteriology, Technical Series No 27. Oxford Blackwell Scientific Publication, Ox- ford, pp. 171–188.

Denyer, S.P., Hugo, W.B., (1991b). Mechanisms of antibacterial action—A summary. In: Denyer, S.P., Hugo, W.B. (Eds.), Mechanisms of Action of Chemical Biocides. Blackwell, Ox- ford, pp. 331–334.

D'Errico, G., Ciccarelli, D., & Ortona, O. (2005). Effect of glycerol on micelle formation by ionic and nonionic surfactants at 25 degrees C. *Journal of Colloid and Interface Science*, *286*(2), 747–754. http://doi.org/10.1016/j.jcis.2005.01.030

Derry, S., Lloyd, R., Moore, R.A., and McQuay, H.J. (2009). Topical capsaicin for chronic neuropathic pain in adults (Review). Cochrane Database of Systematic Reviews 2009, Issue 4. Art. No.: CD007393

De Lourdes Reyes-Escogido, M., Gonzalez-Mondragon, E. G., & Vazquez-Tzompantzi, E. (2011). Chemical and pharmacological aspects of capsaicin. *Molecules*, *16*(2), 1253–1270. http://doi.org/10.3390/molecules16021253

Dickinson, E. (1994). Colloidal aspects of beverages, *Food Chemistry*, 51, 343.

Dima, C., Coman, G., Cotarlet, M., Alexe, P., & Dima, Ş. (2013). Antioxidant and antibacterial properties of capsaicine microemulsions. *6th International Symposium Euro-Aliment 2013*, *37*(1), 39–49.

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT - Food Science and Technology*, *44*(9), 1908–1914. http://doi.org/10.1016/j.lwt.2011.03.003

Donsì, F., Sessa, M., & Ferrari, G. (2012). Effect of emulsifier type and disruption chamber geometry on the fabrication of food nanoemulsions by high pressure homogenization. *Industrial and Engineering Chemistry Research*, *51*(22), 7606–7618. http://doi.org/10.1021/ie2017898

Ersus S, Oztop M.H., McCarthy M.J., Barrett, D.B. (2010). Disintegration efficiency of pulsed electric field (PEF) induced effects on onion (allium cepa l.) tissues as a function of pulse protocol and determination of cell membrane integrity by 1HNMR relaxation measurements. *Journal of Food Science*, 75, 7, E444-E452.

Fabri, D., Williams, M. A. K., & Halstead, T. K. (2005). Water T2 relaxation in sugar solutions. *Carbohydrate Research*, *340*(5), 889–905. http://doi.org/10.1016/j.carres.2005.01.034

Farinato, R.S. and Rowell, R.L. (1983). Optical properties of emulsions, in Encyclopedia of Emulsion Technology, Vol. 1, Becher, P., Marcel Dekker, New York, chap. 8.

Fathi, E., & Sefidkon, F. (2012). Influence of drying and extraction methods on yield and chemical composition of the essential oil of Eucalyptus sargentii. *Journal of Agricultural Science and Technology*, 14, 1035-1042.

Firouzi, R., Shekarforoush, S. S., Nazer, A. H. K., Borumand, Z., & Jooyandeh, A. R. (2007). Effects of essential oils of oregano and nutmeg on growth and survival of Yersinia enterocolitica and Listeria monocytogenes in barbecued chicken. *Journal of Food Protection*, 70, 2626-2630.

Flagan, S. F., & Leadbetter, J. R. (2006). Utilization of capsaicin and vanillylamine as growth substrates by Capsicum (hot pepper)-associated bacteria. *Environmental Microbiology*, *8*(3), 560–5. http://doi.org/10.1111/j.1462-2920.2005.00938.x

Freidman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2004). Antibacterial Activities of Plant Essential Oils and Their Components against Escherichia coli O157 : H7 and Salmonella enterica in Apple Juice. Journal of Agricultural and Food Chemistry, 52, 6042–6048.

Frischkorn, C.G., Frischkorn, H.E., Carrazzoni, E. (1978). Cercaricidal activity of some essential oils of plants from Brazil. *Naturwissenschaften*, 65, 480–483

Friedly, E. C., Crandall, P. G., Ricke, S. C., Roman, M., O'Bryan, C., & Chalova, V. I. (2009). In vitro antilisterial effects of citrus oil fractions in combination with organic acids. *Journal of Food Science*, 74,M67-M72.

Furneri, P. M., Piperno, A., Sajia, A., & Bisignano, G. (2004). Antimycoplasmal activity of hydroxytyrosol. *Antimicrobial Agents and Chemotherapy*, *48*(12), 4892–4894.

Garti, N., Clement, V., Leser, M., Aserin, a., & Fanun, M. (1999). Sucrose ester microemulsions. *Journal of Molecular Liquids*, *80*(2-3), 253–296. http://doi.org/10.1016/S0167-7322(99)80010-5

Garti, N. (2002). Food Emulsifiers. Physical Properties of Lipids. CRC Press.

Gill, A. O., Delaquis, P., Russo, P., & Holley, R. A. (2002). Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *International Journal of Food Microbiology*, *73*(1), 83–92. http://doi.org/10.1016/S0168-1605(01)00712-7

Granizo, D. P. P., Reuhs, B. L. L., Stroshine, R., & Mauer, L. J. J. (2007). Evaluating the solubility of powdered food ingredients using dynamic nuclear magnetic resonance (NMR) relaxometry. *LWT - Food Science and Technology*, *40*(1), 36–42. http://doi.org/10.1016/j.lwt.2005.08.012

Grigoriev, D. O., & Miller, R. (2009). Mono- and multilayer covered drops as carriers. *Current Opinion in Colloid & Interface Science*, *14*(1), 48–59

Hagemann, J.W. (1988). Thermal behaviour and polymorphism of acylglycerides. In: Garti, N., Sato, K. (Eds.), Crystallization and Polymorphism of Fats and Fatty Acids. Marcel Dekker, New York, pp. 9–95.

Guenther, E. (1948). The Essential Oils. New York: D. Van Nostrand Company, Inc.

Hammer, K.A., Carson, C.F. and Riley, T.V. (1999), Antimicrobial Activity of Essential Oils and Other Plant Extracts. *Journal of Applied Microbiology*, 86, 985–990.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Von Wright, A. (1998). Characterization of the action of selected essential oil compo- nents on Gram-negative bacteria. *Journal of Agricultural and Food Chemistry* 46, 3590–3595.

Henry, J. V. L., Fryer, P. J., Frith, W. J., & Norton, I. T. (2009). Emulsification mechanism and storage instabilities of hydrocarbon-in-water sub-micron emulsions stabilised with Tweens (20 and 80), Brij 96v and sucrose monoesters. *Journal of Colloid and Interface Science*, *338*(1), 201–206. http://doi.org/10.1016/j.jcis.2009.05.077

Hernandez, E. and Baker, R.A. (1991). Turbidity of beverages with citrus oil clouding agent, *Journal of Food Science*, 56, 1024.

Hernandez, E., Baker, R.A., and Crandall, P.G. (1991). Model for evaluating the turbidity in cloudy beverages, *Journal of Food Science*, 56, 747.

Hills B. (1998). Magnetic resonance imaging in food science, New York: Wiley-Interscience.

Hoeller, S., Sperger, A., & Valenta, C. (2009). Lecithin based nanoemulsions: A comparative study of the influence of non-ionic surfactants and the cationic phytosphingosine on physicochemical behaviour and skin permeation. *International Journal of Pharmaceutics*, *370*(1-2), 181–6. http://doi.org/10.1016/j.ijpharm.2008.11.014

Hornero-Méndez, D., & Mínguez-Mosquera, M. I. (2001). Rapid Spectrophotometric Determination of Red and Yellow Isochromic Carotenoid Fractions in Paprika and Red Pepper Oleoresins. *Journal of Agricultural and Food Chemistry*, *49*(8), 3584–3588. http://doi.org/10.1021/jf010400l

Hunter, R.J., Introduction to Modern Colloid Science, Oxford University Press, Oxford, 1993.

Israelachvili, J. (1992). Intermolecular and Surface Forces, Second Edition. Academic Press, London.

Jenning, V., Ma, K., & Gohla, S. H. (2000). Solid lipid nanoparticles (SLN ™) based on binary mixtures of liquid and solid lipids : a 1 H-NMR study. *International Journal of Pharmaceutics*, *205*, 15–21.

Jiang, Y., Pétrier, C., & Waite, T. D. (2002a). Effect of pH on the ultrasonic degradation of ionic aromatic compounds in aqueous solution. *Ultrasonics Sonochemistry*, *9*(3), 163–168. http://doi.org/10.1016/S1350-4177(01)00114-6

Jiang, Y., Petrier, C., & Waite, T. D. (2002b). Kinetics and mechanisms of ultrasonic degradation of volatile chlorinated aromatics in aqueous solutions. *Ultrasonics Sonochemistry*, *9*(6), 317–323. http://doi.org/10.1016/S1350-4177(02)00085-8

Jo, Y.-J., & Kwon, Y.-J. (2013). Characterization of β-carotene nanoemulsions prepared by microfluidization technique. *Food Science and Biotechnology*, *23*(1), 107–113. http://doi.org/10.1007/s10068-014-0014-7

Joe, B., and Lokesh, B.R. (1997). Prophylactic and therapeutic effects of n-3 PUFA, capsaicin & curcumin on adjuvant induced arthritis in rats. *J. Nutr. Biochem.*, 8: 397−407.

Kahlweit, M., Busse, G., & Faulhaber, B. (1995). Preparing Microemulsions with Lecithins. *Langmuir*, *11*(5), 1576–1583. http://doi.org/10.1021/la00005a027

Kanakdande, D., Bhosale, R., & Singhal, R. S. (2007). Stability of cumin oleoresin microencapsulated in different combination of gum arabic, maltodextrin and modified starch. *Carbohydrate Polymers*, *67*(4), 536–541. http://doi.org/10.1016/j.carbpol.2006.06.023

Karaca, A. C., Nickerson, M., & Low, N. H. (2013). Microcapsule production employing chickpea or lentil protein isolates and maltodextrin: Physicochemical properties and oxidative protection of encapsulated flaxseed oil. *Food Chemistry*, *139*(1-4), 448–457. http://doi.org/10.1016/j.foodchem.2013.01.040

Karlene, M. and Derick, R. (2006). Emulsions for the delivery of nutraceutical lipids. Nutraceutical and Specialty Lipids and their Co-Products. CRC Press. 281-300.

Kawada, T., Hagihara, K., and Iwai, K. (1986). Effects of capsaicin on lipid metabolism in rats fed a high fat diet. *J. Nutr.*, 116: 1272−1278.

Kentish, S., Wooster, T. J., Ashokkumar, M., Balachandran, S., Mawson, R., & Simons, L. (2008). The use of ultrasonics for nanoemulsion preparation. *Innovative Food Science & Emerging Technologies*, *9*(2), 170–175. http://doi.org/10.1016/j.ifset.2007.07.005

Kidak, R., & Ince, N. H. (2006). Ultrasonic destruction of phenol and substituted phenols: A review of current research. *Ultrasonics Sonochemistry*, *13*(3), 195–199. http://doi.org/10.1016/j.ultsonch.2005.11.004

Kim, I.-H., Oh, Y. A., Lee, H., Song, K. Bin, & Min, S. C. (2014a). Grape berry coatings of lemongrass oil-incorporating nanoemulsion. *LWT - Food Science and Technology*, *58*(1), 1–10. http://doi.org/10.1016/j.lwt.2014.03.018

Kim, J. H. J. T., Ko, J. A., Kim, J. H. J. T., Cha, D. S., Cho, J. H., Park, H. J., & Shin, G. H. (2014b). Preparation of a Capsaicin-Loaded Nanoemulsion for Improving Skin Penetration. *Journal of Agricultural and Food Chemistry*, *62*(3), 725–732. http://doi.org/10.1021/jf404220n

Kırtıl, E., & Öztop, M. H. (2014). Enkapsülasyon Maddesi Olarak Lipozom ve Gıdalarda Kullanımı : Yapısı , Karakterizasyonu , Üretimi ve Stabilitesi, *12*(4), 41–57.

Kralova, I., & Sjoblom, J. (2009). Surfactants used in food industry: a review. *Journal of Dispersion Science and Technology*, 30(9), 1363-1383.

Kulshreshtha, A.K., Singh, O.N., Wall, G.M. (2009). Pharmaceutical Suspensions: From Formulation Development to Manufacturing. 2009: Springer.

Lambert, R.J.W.,Skandamis, P.N.,Coote,P., Nychas,G.-J.E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, 91, 453–462.

Lee, S.H., Krisanapun, C., and Baek, S.J. (2010). NSAID-activated gene-1 as a molecular target for capsaicin-induced apoptosis through a novel molecular mechanism involving GSK3beta, C/EBPbeta and ATF3. *Carcinogenesis*, 31: 719−728.

Lee, L., & Norton, I. T. (2013). Comparing droplet breakup for a high-pressure valve homogeniser and a Microfluidizer for the potential production of food-grade nanoemulsions. *Journal of Food Engineering*, *114*(2), 158–163. http://doi.org/10.1016/j.jfoodeng.2012.08.009

Lee, S. Y., & Kang, D. H. (2016). Survival mechanism of Escherichia coli O157: H7 against combined treatment with acetic acid and sodium chloride. *Food Microbiology*, *55*, 95–104. http://doi.org/10.1016/j.fm.2015.10.021

Leng, T. W., Muhamad, I. I., Zaidel, D. N. A., & Khairuddin, N. (2013). Evaluation of Capsaicinoids Extracts as Bioactive Substance for Antimicrobial Films. *Jurnal Teknologi*, *64*(2), 69–74.

Leong, T. S. H., Wooster, T. J., Kentish, S. E., & Ashokkumar, M. (2009). Minimising oil droplet size using ultrasonic emulsification. *Ultrasonics Sonochemistry*, *16*(6), 721–7. http://doi.org/10.1016/j.ultsonch.2009.02.008

Liang, R., Xu, S., Shoemaker, C. F., Li, Y., Zhong, F., & Huang, Q. (2012). Physical and antimicrobial properties of peppermint oil nanoemulsions. *Journal of Agricultural and Food Chemistry*, *60*(30), 7548–55. http://doi.org/10.1021/jf301129k

Lide, D. R. (2003). CRC Handbook of Chemistry and Physics, 3485. http://doi.org/978-1466571143

Ljungdahl, A., Kristensson, K., Lundberg, J. M., Lycke, E., Svennerholm, B., Ziegler, R. (1986). Herpes simplex virus infection in capsaicin-treated mice. *J Neurol Sci*, 72, 223.

López-Carrillo, L., López-Cervantes, M., Robles-Díaz, G., Ramírez-Espitia, A., Mohar-Betancourt, A., Meneses-García, A. (2003). Capsaicin consumption, Helicobacter pylori positivity and gastric cancer in Mexico. *Int. J. Cancer*, 106: 277–282.

Maali, A. & Mosavian, M. T. H. (2013). Preparation and Application of Nanoemulsions in the Last Decade (2000–2010). *Journal of Dispersion Science and Technology*, *34*(1), 92–105. http://doi.org/10.1080/01932691.2011.648498

Mahdi Jafari, S., He, Y., & Bhandari, B. (2006). Nano-Emulsion Production by Sonication and Microfluidization—A Comparison. *International Journal of Food Properties*, *9*(3), 475–485. http://doi.org/10.1080/10942910600596464

McCarthy, G.M., and McCarthy, D.J. (1991). Effect of topical capsaicin in the therapy of painful osteoarthritis of the hand. *J.Rheumatol.*, 19: 604−607.

McClements, D. J. (1999). Food emulsions: principles, practice, and techniques. CRC series in contemporary food science. (Vol. 10). http://doi.org/10.1016/S0924-2244(99)00042-4

McClements, D. J. (2002). Theoretical prediction of emulsion color. *Advances in Colloid and Interface Science*, *97*(1-3), 63–89. http://doi.org/10.1016/S0001-8686(01)00047-1

McClements, D. J. (2012). Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, *8*(6), 1719. http://doi.org/10.1039/c2sm06903b

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrition*, *51*(4), 285–330. http://doi.org/10.1080/10408398.2011.559558

Meleson, K., Graves, S., & Mason, T. G. (2004). Formation of Concentrated Nanoemulsions by Extreme Shear. *Soft Materials*, *2*(2-3), 109–123. http://doi.org/10.1081/SMTS-200056102

Memoli, A., Palermiti, L.G., Travagli, V., Alhaique, F. (1995). Egg and soya phospholipids - sonication and dialysis - a study on liposome characterization. *International Journal of Pharmaceutics*, *117*(2): 159- 163.

Molina-torres, J., Garcı, A., & Ramı, E. (1999). Antimicrobial properties of alkamides present in flavouring plants traditionally used in Mesoamerica : affinin and capsaicin. *Journal of Ethnopharmacology*, *64*, 241–248.

Nanzai, B., Okitsu, K., Takenaka, N., Bandow, H., & Maeda, Y. (2008). Sonochemical degradation of various monocyclic aromatic compounds: Relation between hydrophobicities of organic compounds and the decomposition rates. *Ultrasonics Sonochemistry*, *15*(4), 478–483. http://doi.org/10.1016/j.ultsonch.2007.06.010

Nelson, E. K., & Dawson, L. E. (1923). The constitution of capsacicin, the pungent principle of capsicum. *Journal Of American Chemical Society*, *45*(1919), 2179–2181. http://doi.org/doi:10.1021/ja02228a011

Novak, J., Draxler, L., Gohler, I., & Franz, C. M. (2005). Essential oil composition of Vitex agnus-castus - comparison of accessions and different plant organs. *Flavor and Fragrence Journal,* 20,186-192.

O'Beirne, D., & Hamer, G. (2000). The utilisation of glucose/acetate mixtures by Escherichia coli W3110 under aerobic growth conditions. *Bioprocess Engineering*, *23*(4), 375–380. http://doi.org/10.1007/s004499900176

Okada, Y., and Okajima, H. (2001). Antioxidant effect of capsaicin on lipid peroxidation in homogeneous solution, micelle dispersions & liposomal membranes. *Redox. Rep.*, 6: 117−122.

Olawore, N. O., Ogunwande, I. A., Ekundayo, O., & Adeleke, K. A. (2005). Chemical composition of the leaf and fruit essential oils of Murraya paniculata (L.) Jack. (Syn. Murraya exotica Linn. *Flavor and Fragrance Journal,* 20,54-56.

Oztop M.H., Rosenberg M., Rosenberg, Y., McCarthy, K.L.M., McCarthy, M.J. (2010). Magnetic resonance imaging (MRI) and relaxation spectrum analysis as methods to investigate swelling in whey protein gels. *Journal of Food Science*, 75, 7, E508-E515.

Oztop, M.H., McCarthy, M.J., McCarthy, K.L., Rosenberg, M. (2012). Uptake of divalent ions (Mn+2 and Ca+2) by heat-set whey protein gels. *Journal of Food Science*, 77, 2, E68-73.

Ozturk, I., Sagdic, O., Tornuk, F., & Yetim, H. (2014). Effect of wheat sprout powder incorporation on lipid oxidation and physicochemical properties of beef patties. *International Journal of Food Science & Technology*, *49*(4), 1112–1121. http://doi.org/10.1111/ijfs.12407

Park, J.S., Choi, M.A., Kim, B.S., Han, I.S., Kurata, T., and Yu, R. (2000). Capsaicin protects against ethanol-induced oxidative injury in the gastric mucosa of rats. *Life Sci.*, 67: 3087– 3093.

Pichersky, E., Noel, J. P., & Dudareva, N. (2006). Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science*, 311, 808-811.

Piorkowski, D. T., & McClements, D. J. (2013). Beverage emulsions: Recent developments in formulation, production, and applications. *Food Hydrocolloids*. http://doi.org/10.1016/j.foodhyd.2013.07.009

Porras, M., Solans, C., González, C., & Gutiérrez, J. M. (2008). Properties of water-in-oil (W/O) nano-emulsions prepared by a low-energy emulsification method. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *324*(1-3), 181–188. http://doi.org/10.1016/j.colsurfa.2008.04.012

Prasad, N.S., Raghavendra, R., Lokesh, B.R., and Naidu, K.A. (2004). Spice phenolics inhibit human PMNL 5-lipoxygenase. *Prostagl. Leukotr. Essent. Fatty Acids*, 70: 521−528.

Qian, C., & McClements, D. J. (2011). Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: Factors affecting particle size. *Food Hydrocolloids*, *25*(5), 1000–1008. http://doi.org/10.1016/j.foodhyd.2010.09.017

Quintanilla-Carvajal, M., Camacho-Díaz, B., Meraz-Torres, L., Chanona-Pérez, J., Alamilla-Beltrán, L., Jimenéz-Aparicio, A. (2010). Nanoencapsulation: A new trend in food engineering processing, *Food Engineering Reviews*, *2*(1), 39–50.

Rao, J., & McClements, D. J. (2011a). Food-grade microemulsions, nanoemulsions and emulsions: Fabrication from sucrose monopalmitate & lemon oil. *Food Hydrocolloids*, *25*(6), 1413–1423. http://doi.org/10.1016/j.foodhyd.2011.02.004

Rao, J., & McClements, D. J. (2011b). Formation of flavor oil microemulsions, nanoemulsions and emulsions: Influence of composition and preparation method. *Journal of Agricultural and Food Chemistry*, *59*(9), 5026–5035. http://doi.org/10.1021/jf200094m

Rao, J., & McClements, D. J. (2012). Lemon oil solubilization in mixed surfactant solutions: Rationalizing microemulsion & nanoemulsion formation. *Food Hydrocolloids*, *26*(1), 268–276. http://doi.org/10.1016/j.foodhyd.2011.06.002

Rao, J., & McClements, D. J. (2013). Optimization of lipid nanoparticle formation for beverage applications: Influence of oil type, cosolvents, and cosurfactants on nanoemulsion properties. *Journal of Food Engineering*, *118*(2), 198–204. http://doi.org/10.1016/j.jfoodeng.2013.04.010

Rauha, J. P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K.,Vuorela, H., Vuorela, P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology*, *56*(1), 3–12. http://doi.org/10.1016/S0168-1605(00)00218-X

Ravichandran, M., Hettiarachchy, N. S., Ganesh, V., Ricke, S. C., & Singh, S. (2011). Enhancement of antimicrobial activities of naturally occurring phenolic compounds by nanoscale delivery against Listeria monocytogenes, Escherichia coli O157:H7 and Salmonella Typhimurium in broth and chicken meat system. *Journal of Food Safety, 31*, 462-471.

Reddy, A.C.P., and Lokesh, B.R. (1994). Studies on anti-inflammatory activity of spice principles and dietary n-3 polyunsaturated fatty acids on Carrageenan induced inflammation in rats. *Ann. Nutr. Metab.,* 38: 349−358.

Rodriguez, R.B. And Xamani M.S. (2003). Liposomes prepared by high-pressure homogenizers, Liposomes, Part A, 367: p. 28-46.

Roger, K., Cabane, B., Olsson, U. (2011). Emulsification through surfactant hydration: The PIC process revisited. *Langmuir*, 27:604-11.

Saberi, A. H., Fang, Y., & McClements, D. J. (2013). Effect of glycerol on formation, stability, and properties of vitamin-E enriched nanoemulsions produced using spontaneous emulsification. *Journal of Colloid and Interface Science*, 411, 105-113. http://doi.org/10.1016/j.jcis.2013.08.041

Salvia-Trujillo, L., Rojas-Graue, A., Soliva-Fortuny, R., & Martin-Belloso, O. (2013a). Physicochemical Characterization of Lemongrass Essential Oil-Alginate Nanoemulsions: Effect of Ultrasound Processing Parameters. *Food and Bioprocess Technology*, *6*(9), 2439–2446. http://doi.org/10.1007/s11947-012-0881-y

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2013b). Effect of processing parameters on physicochemical characteristics of microfluidized lemongrass essential oil-alginate nanoemulsions. *Food Hydrocolloids*, *30*(1), 401–407. http://doi.org/10.1016/j.foodhyd.2012.07.004

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2014). Impact of microfluidization or ultrasound processing on the antimicrobial activity against Escherichia coli of lemongrass oil-loaded nanoemulsions. *Food Control*, *37*, 292–297. http://doi.org/10.1016/j.foodcont.2013.09.015

Sari, T. P., Mann, B., Kumar, R., Singh, R. R. B., Sharma, R., Bhardwaj, M., & Athira, S. (2015). Preparation and characterization of nanoemulsion encapsulating curcumin. *Food Hydrocolloids*, *43*, 540–546. http://doi.org/10.1016/j.foodhyd.2014.07.011

Scoville,W. L. (1912). Note on capsicums. *J. Amer. Pharm. Assoc*. 1:453–454.

Shelef, L.A. (1983). Antimicrobial effects of spices. *Journal of Food Safety*, 6, 29–44.

Shchipunov, Y. A., & Schmiedel, P. (1996). Phase behavior of lecithin at the oil/water interface. *Langmuir*, *7463*(13), 6443–6445. http://doi.org/10.1021/la960082y

Shimizu, M., Iijima, S., Kobayashi, T. (1992). Production of insecticidal protein of *Bacillus thuringiensis* by cultivation of recombinant *Escherichia coli*. *J. Ferment. Bioeng, 74,* 163-168.

Shinoda, K., Friberg, S. (1986). Emulsions and Solubilization. Wiley Interscience, New York.

Sikkema, J., De Bont, J.A.M., Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews*, *59* (2), 201–222.

Silva, H. D., Cerqueira, M. Â., & Vicente, A. a. (2011). Nanoemulsions for Food Applications: Development and Characterization. *Food and Bioprocess Technology*, *5*(3), 854–867. http://doi.org/10.1007/s11947-011-0683-7

Smith-Palmer, A., Stewart, J., Fyfe, L. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Food Microbiology*, 26, 118–122.

Sokovic, M., Glamoclija, J., Marin, P. D., Brkic, D., & van Griensven, L. J. L. D. (2010). Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. *Molecules*, 15, 7532-7546.

Solans, C., & Solé, I. (2012). Nano-emulsions: Formation by low-energy methods. *Current Opinion in Colloid & Interface Science*, *17*(5), 246–254. http://doi.org/10.1016/j.cocis.2012.07.003

Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N. (2008). The antimicrobial effect of thyme essential oil, nisin, and their combination against Listeria monocytogenes in minced beef during refrigerated storage. *Food microbiology*, 25, 120-127

Srinivasan, K. (2015). Biological Activities of Red Pepper (*Capsicum annuum*) and Its Pungent Principle Capsaicin: A Review. *Critical Reviews in Food Science and Nutrition*, *8398*(November), 00–00. http://doi.org/10.1080/10408398.2013.772090

Stanberry, L.R., Bourne, N., Bravo, F. J., Bernstein, D.I. (1992). Capsaicin-sensitive peptidergic neurons are involved in the zosteriform spread of herpes simplex virus infection. *J Med Virol, 38*(2), 142- 146.

Stephen, B. J., Kumar, V. (2014). Evaluation of antimicrobial activity of capsaicin extract against *B. subtilis* species. *International Journal of Pharmaceutics and Drug Analysis*, *2*(2), 111–116.

Sudjana, A. N., D'Orazio, C., Ryan, V., Rasool, N., Ng, J., Islam, N., Riley T.V., Hammer, K. A. (2009). Antimicrobial activity of commercial Olea europaea (olive) leaf extract. International Journal of Antimicrobial Agents, 33(5), 461–463. http://doi.org/10.1016/j.ijantimicag.2008.10.026

Sugumar, S., Nirmala, J., Ghosh, V., Anjali, H., Mukherjee, a, & Chandrasekaran, N. (2013). Bio-based nanoemulsion formulation, characterization and antibacterial activity against food-borne pathogens. *Journal of Basic Microbiology*, *53*(8), 677–685. http://doi.org/10.1002/jobm.201200060

Surassmo, S., Min, S.-G., Bejrapha, P., & Choi, M.-J. (2010). Effects of surfactants on the physical properties of capsicum oleoresin-loaded nanocapsules formulated through the emulsion–diffusion method. *Food Research International*, *43*(1), 8–17. http://doi.org/10.1016/j.foodres.2009.07.008

Surh, Y.J., Kundu, J.K., Na, H.K., and Lee, J.S. (2005). Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *J. Nutr., 135* (Suppl.): 2993S−3001S.

Surh, J., Jeong, Y. G., & Vladisavljević, G. T. (2008). On the preparation of lecithin-stabilized oil-in-water emulsions by multi-stage premix membrane emulsification. *Journal of Food Engineering*, *89*(2), 164–170. http://doi.org/10.1016/j.jfoodeng.2008.04.023

Tadros, T., Izquierdo, P., Esquena, J., & Solans, C. (2004). Formation and stability of nano-emulsions. *Advances in Colloid and Interface Science*, *108-109*, 303–318. http://doi.org/10.1016/j.cis.2003.10.023

Tan, C.-T. (1990). Beverage emulsions, in Food Emulsions, 2nd ed., Larsson, K. and Friberg, S.E., Eds., Marcel Dekker, New York, chap. 10.

Terjung, N., Löffler, M., Gibis, M., Hinrichs, J., & Weiss, J. (2012). Influence of droplet size on the efficacy of oil-in-water emulsions loaded with phenolic antimicrobials. *Food & Function*, *3*(3), 290–301. http://doi.org/10.1039/c2fo10198j

Thomas, L. V, Davies, E. A., Delves-Broughton, J., & Wimpenny, J. W. T. (1998). Synergist effect of sucrose fatty acid esters on nisin inhibition of gram-positive bacteria. *Journal of Applied Microbiology*, *85*, 1013–1022.

Thoroski, J., Blank, G., Biliaderis, C., (1989). Eugenol induced in- hibition of extracellular enzyme production by Bacillus cereus. *Journal of Food Protection, 52* (6), 399–403.

Trotta, M., Cavalli, R., Ugazio, E., and Gasco, M. (1996). Phase Behaviour of Microemulsion systems containing lecithin and lysolecithin as surfactants. *International Journal of Pharmaceutics, 143*(1):67–73.

Ultee, A., Bennink, M.H.J., Moezelaar, R., (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Applied and Environmen- tal Microbiology 68 (4), 1561–1568. Ph.D. thesis, ISBN 90- 5808-219-9.

Van de Braak, S.A.A.J., Leijten, G.C.J.J. (1999). Essential Oils and Oleoresins: A Survey in the Netherlands and other Major Markets in the European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Rotterdam, p. 116

Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science, 48*(2), 333–349.

Weiss, J., Gaysinsky, S., Davidson, M., & McClements, J. (2009). Nanostructured Encapsulation Systems: Food Antimicrobials. *Global Issues in Food Science and Technology*. http://doi.org/10.1016/B978-0-12-374124-0.00024-7

Wendakoon, C.N., Sakaguchi, M., (1993). Combined effect of sodium chloride and clove on growth and biogenic amine formation of Enterobacter aerogenes in mackerel muscle extract. *Journal of Food Protection, 56* (5), 410–413.

Wichchukit, S., Oztop, M. H., McCarthy, M. J., & McCarthy, K. L. (2013). Whey protein/alginate beads as carriers of a bioactive component. *Food Hydrocolloids*, *33*(1), 66–73. http://doi.org/10.1016/j.foodhyd.2013.02.013

Woodle, M.C., Papahadjopoulos, D. (1989). Liposome preparation and size characterization, *Methods in Enzymology*, 171, Pages 193–217.

Xing, F., Cheng, G., Yi, K., & Ma, L. (2005). Nanoencapsulation of capsaicin by complex coacervation of gelatin, acacia, and tannins. *Journal of Applied Polymer Science*, *96*(6), 2225–2229. http://doi.org/10.1002/app.21698

Xue, J., & Zhong, Q. (2014). Thyme Oil Nanoemulsions Coemulsified by Sodium Caseinate and Lecithin. *Journal of Agricultural and Food Chemistry*, *62*, 9900–9907.

Yang, Y., Marshall-Breton, C., Leser, M. E., Sher, A. a., & McClements, D. J. (2012). Fabrication of ultrafine edible emulsions: Comparison of high-energy and low-energy homogenization methods. *Food Hydrocolloids*, *29*(2), 398–406. http://doi.org/10.1016/j.foodhyd.2012.04.009

Zhang, H., Shen, Y., Weng, P., Zhao, G., Feng, F., & Zheng, X. (2009). Antimicrobial activity of a food-grade fully dilutable microemulsion against Escherichia coli and Staphylococcus aureus. *International Journal of Food Microbiology*, *135*(3), 211–215. http://doi.org/10.1016/j.ijfoodmicro.2009.08.015

Zorzi, G. K., Carvalho, E. L. S., Von Poser, G. L., & Teixeira, H. F. (2015). On the use of nanotechnology-based strategies for association of complex matrices from plant extracts. *Brazilian Journal of Pharmacognosy*, *25*(4), 426–436. http://doi.org/10.1016/j.bjp.2015.07.015

APPENDIX A

HPLC RESULT

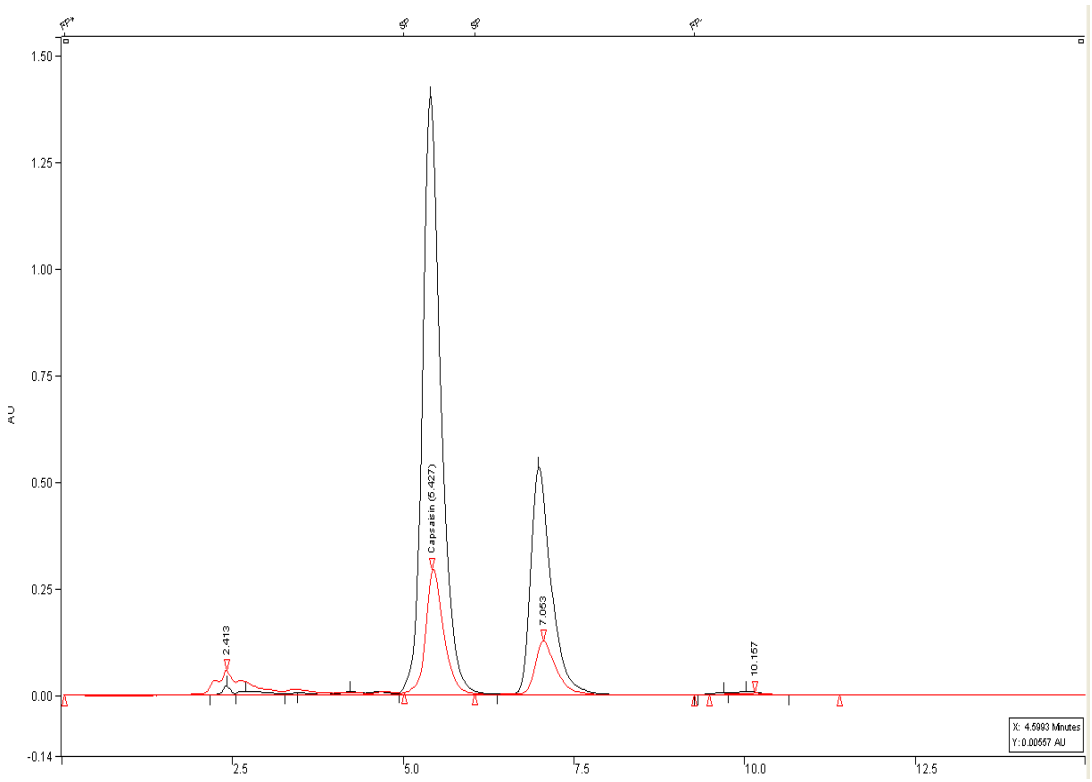

Figure A. 1 HPLC analysis result of capsaicin content in oleoresin capsicum used in this study.

APPENDIX B

ULTRASONICATION SCHEME

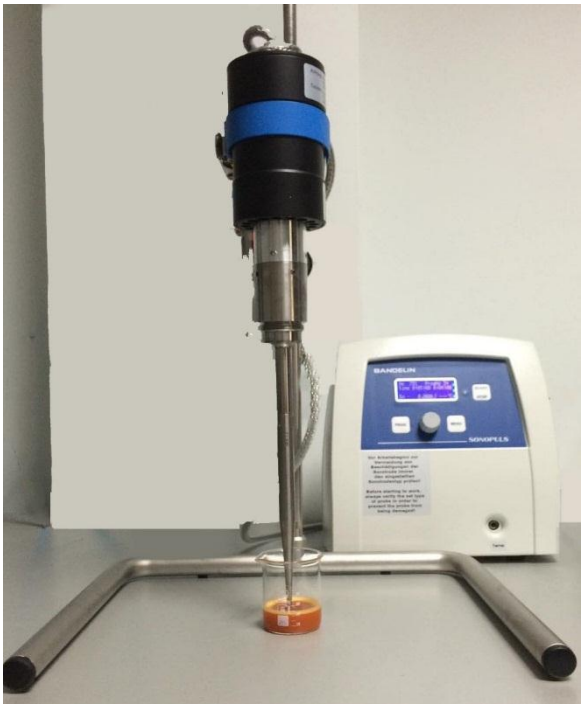

Figure B. 1 20 mL of coarse capsaicin emulsion treated with ultrasonication.

APPENDIX C

CALIBRATION CURVE

Absorbance (451 nm)=

0.045 (mg capsicum oleoresin content/mL)+ 0.012

(R^2=0.996)

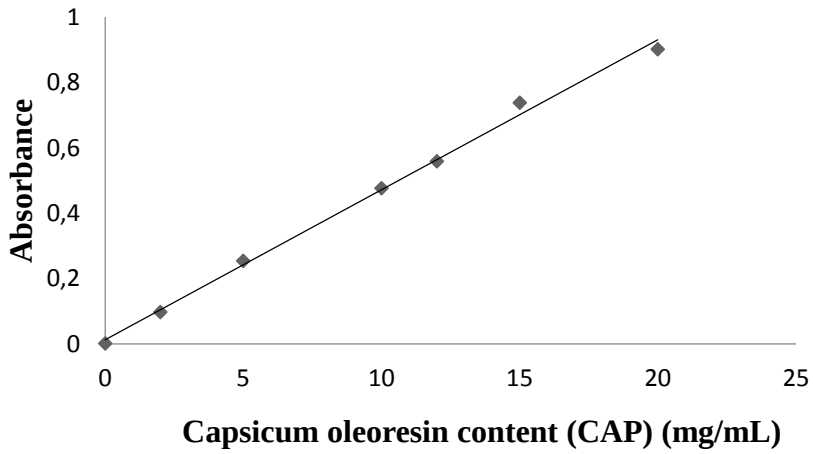

Figure C. 1 Calibration curve for encapsulation efficiency

APPENDIX D

ANOVA TABLES

Table D.1 Analysis of Variance for emulsions produced by microfluidization with the emulsifier **Tween 80**. Effect of pH and glycerol addition on particle size, turbidity, T_2 times, color-L*, a*, b* values, efficiency, *E.coli* and *S.aureus* population decrease using Adjusted SS for Tests

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	828,25	828,25	828,25	577,17	0,000
Glycerol	1	190,12	190,12	190,12	132,49	0,000
pH*Glycerol	1	34,44	34,44	34,44	24,00	0,008
Error	4	5,74	5,74	1,43		
Total	7	1058,56				

S = 1,19791 R-Sq = 99,46% R-Sq(adj) = 99,05%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	N	Mean	Grouping
7,0	4	73,5	A
3,8	4	53,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Glycerol	N	Mean	Grouping
0	4	68,1	A
1	4	58,4	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Glycerol	N	Mean	Grouping
7,0	0	2	80,4	A
7,0	1	2	66,5	B
3,8	0	2	55,9	C
3,8	1	2	50,3	D

Means that do not share a letter are significantly different.

Analysis of Variance for T2, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	369	369	369	0,29	0,619
Glycerol	1	673555	673555	673555	528,74	0,000
pH*Glycerol	1	19328	19328	19328	15,17	0,018
Error	4	5096	5096	1274		
Total	7	698346				

S = 35,6914 R-Sq = 99,27% R-Sq(adj) = 98,72%

Grouping Information Using Tukey Method and 95,0% Confidence for T2

pH	N	Mean	Grouping
7,0	4	1007,6	A
3,8	4	994,0	A

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Glycerol	N	Mean	Grouping
0	4	1291,0	A
1	4	710,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2

pH	Glycerol	N	Mean	Grouping
7,0	0	2	1346,9	A
3,8	0	2	1235,0	A
3,8	1	2	753,0	B
7,0	1	2	668,3	B

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,0059678	0,0059678	0,0059678	1435,86	0,000
Glycerol	1	0,0130815	0,0130815	0,0130815	3147,44	0,000
pH*Glycerol	1	0,0002050	0,0002050	0,0002050	49,33	0,002
Error	4	0,0000166	0,0000166	0,0000042		
Total	7	0,0192710				

S = 0,00203869 R-Sq = 99,91% R-Sq(adj) = 99,85%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	N	Mean	Grouping
7,0	4	0,4	A
3,8	4	0,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Glycerol	N	Mean	Grouping
0	4	0,4	A
1	4	0,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Glycerol	N	Mean	Grouping
7,0	0	2	0,4	A
3,8	0	2	0,3	B
7,0	1	2	0,3	C
3,8	1	2	0,3	D

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR- L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	3,9060	3,9060	3,9060	53,03	0,002
Glycerol	1	0,4371	0,4371	0,4371	5,93	0,072
pH*Glycerol	1	0,6786	0,6786	0,6786	9,21	0,039
Error	4	0,2947	0,2947	0,0737		
Total	7	5,3164				

S = 0,271408 R-Sq = 94,46% R-Sq(adj) = 90,30%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR- L

pH	N	Mean	Grouping
7,0	4	53,9	A
3,8	4	52,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR- L

Glycerol	N	Mean	Grouping
0	4	53,5	A
1	4	53,0	A

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR- L

pH	Glycerol	N	Mean	Grouping
7,0	0	2	54,4	A
7,0	1	2	53,4	A B
3,8	1	2	52,6	B
3,8	0	2	52,5	B

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,3080	0,3080	0,3080	0,56	0,470
Glycerol	1	0,1332	0,1332	0,1332	0,24	0,632
pH*Glycerol	1	0,5476	0,5476	0,5476	0,99	0,339
Error	12	6,6367	6,6367	0,5531		
Total	15	7,6256				

S = 0,743682 R-Sq = 12,97% R-Sq(adj) = 0,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	N	Mean	Grouping
3,8	8	54,4	A
7,0	8	54,1	A

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Glycerol	N	Mean	Grouping
0	8	54,4	A
1	8	54,2	A

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	Glycerol	N	Mean	Grouping
3,8	0	4	54,7	A
7,0	1	4	54,2	A
3,8	1	4	54,1	A
7,0	0	4	54,0	A

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	95,220	95,220	95,220	200,18	0,000
Glycerol	1	47,726	47,726	47,726	100,33	0,001
pH*Glycerol	1	18,911	18,911	18,911	39,76	0,003
Error	4	1,903	1,903	0,476		
Total	7	163,760				

S = 0,689692 R-Sq = 98,84% R-Sq(adj) = 97,97%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	N	Mean	Grouping
7,0	4	93,0	A
3,8	4	86,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Glycerol	N	Mean	Grouping
0	4	91,9	A
1	4	87,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Glycerol	N	Mean	Grouping
7,0	0	2	93,9	A
7,0	1	2	92,0	A B
3,8	0	2	90,0	B
3,8	1	2	82,1	C

Means that do not share a letter are significantly different.

Analysis of Variance for Efficiency, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	4,061	4,061	4,061	295,36	0,000
Glycerol	1	30,811	30,811	30,811	2240,82	0,000
pH*Glycerol	1	45,601	45,601	45,601	3316,45	0,000
Error	4	0,055	0,055	0,014		
Total	7	80,529				

S = 0,117260 R-Sq = 99,93% R-Sq(adj) = 99,88%

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	N	Mean	Grouping
3,8	4	83,8	A
7,0	4	82,4	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Glycerol	N	Mean	Grouping
0	4	85,1	A
1	4	81,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Glycerol	N	Mean	Grouping
3,8	0	2	88,2	A
7,0	1	2	82,8	B
7,0	0	2	82,0	C
3,8	1	2	79,5	D

Means that do not share a letter are significantly different.

Analysis of Variance for E.coli, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,60065	0,60065	0,60065	44,21	0,003
Glycerol	1	0,11753	0,11753	0,11753	8,65	0,042
pH*Glycerol	1	0,01083	0,01083	0,01083	0,80	0,422
Error	4	0,05434	0,05434	0,01358		
Total	7	0,78335				

S = 0,116554 R-Sq = 93,06% R-Sq(adj) = 87,86%

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	N	Mean	Grouping
7,0	4	2,6	A
3,8	4	2,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Glycerol	N	Mean	Grouping
0	4	2,5	A
1	4	2,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Glycerol	N	Mean	Grouping
7,0	0	2	2,8	A
7,0	1	2	2,5	A B
3,8	0	2	2,2	B
3,8	1	2	2,0	B

Means that do not share a letter are significantly different.

Analysis of Variance for S.aureus, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,0406	0,0406	0,0406	1,22	0,331
Glycerol	1	6,7896	6,7896	6,7896	204,74	0,000
pH*Glycerol	1	0,2701	0,2701	0,2701	8,15	0,046
Error	4	0,1326	0,1326	0,0332		
Total	7	7,2330				

S = 0,182106 R-Sq = 98,17% R-Sq(adj) = 96,79%

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	N	Mean	Grouping
7,0	4	4,8	A
3,8	4	4,6	A

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Glycerol	N	Mean	Grouping
1	4	5,6	A
0	4	3,8	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Glycerol	N	Mean	Grouping
7,0	1	2	5,9	A
3,8	1	2	5,4	A
3,8	0	2	3,9	B
7,0	0	2	3,7	B

Means that do not share a letter are significantly different.

Table D.2 Analysis of Variance for emulsions produced by microfluidization with the emulsifier **lecithin**. Effect of pH, glycerol addition, and heating before homogenization on particle size, turbidity, T2 times, color-L*, a*, b* values, efficiency, *E.coli* and *S.aureus* population decrease using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	139502	139502	139502	3720,06	0,000
Glycerol	1	47742	47742	47742	1273,13	0,000
Heat	1	8649	8649	8649	230,64	0,000
pH*Glycerol	1	52212	52212	52212	1392,33	0,000
pH*Heat	1	5929	5929	5929	158,11	0,000
Glycerol*Heat	1	3481	3481	3481	92,83	0,000
pH*Glycerol*Heat	1	12996	12996	12996	346,56	0,000
Error	8	300	300	38		
Total	15	270812				

S = 6,12372 R-Sq = 99,89% R-Sq(adj) = 99,79%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	N	Mean	Grouping
3,8	8	417,0	A
7,0	8	230,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Glycerol	N	Mean	Grouping
0	8	378,3	A
1	8	269,0	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Heat	N	Mean	Grouping
0	8	346,9	A
1	8	300,4	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Glycerol	N	Mean	Grouping
3,8	0	4	528,8	A
3,8	1	4	305,3	B
7,0	1	4	232,8	C
7,0	0	4	227,8	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Heat	N	Mean	Grouping
3,8	0	4	459,5	A
3,8	1	4	374,5	B
7,0	0	4	234,3	C
7,0	1	4	226,3	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Glycerol	Heat	N	Mean	Grouping
0	0	4	416,3	A
0	1	4	340,3	B
1	0	4	277,5	C
1	1	4	260,5	D

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Glycerol	Heat	N	Mean	Grouping
3,8	0	0	2	614,5	A
3,8	0	1	2	443,0	B
3,8	1	1	2	306,0	C
3,8	1	0	2	304,5	C
7,0	1	0	2	250,5	D
7,0	0	1	2	237,5	D E
7,0	0	0	2	218,0	E
7,0	1	1	2	215,0	E

Means that do not share a letter are significantly different.

Analysis of Variance for T2 Values, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1582	1582	1582	86,02	0,000
Glycerol	1	424288	424288	424288	23073,84	0,000
Heat	1	12412	12412	12412	675,00	0,000
pH*Glycerol	1	2766	2766	2766	150,41	0,000
pH*Heat	1	16	16	16	0,85	0,383
Glycerol*Heat	1	14204	14204	14204	772,47	0,000
pH*Glycerol*Heat	1	112	112	112	6,11	0,039
Error	8	147	147	18		
Total	15	455527				

S = 4,28815 R-Sq = 99,97% R-Sq(adj) = 99,94%

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

pH	N	Mean	Grouping
7,0	8	644,1	A
3,8	8	624,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

Glycerol	N	Mean	Grouping
0	8	797,0	A
1	8	471,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

Heat	N	Mean	Grouping
0	8	662,0	A
1	8	606,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

pH	Glycerol	N	Mean	Grouping
3,8	0	4	800,2	A
7,0	0	4	793,8	A
7,0	1	4	494,4	B
3,8	1	4	448,2	C

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

pH	Heat	N	Mean	Grouping
7,0	0	4	671,0	A
3,8	0	4	653,0	B
7,0	1	4	617,2	C
3,8	1	4	595,4	D

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

Glycerol	Heat	N	Mean	Grouping
0	0	4	854,6	A
0	1	4	739,3	B
1	1	4	473,2	C
1	0	4	469,4	C

Grouping Information Using Tukey Method and 95,0% Confidence for T2 Values

pH	Glycerol	Heat	N	Mean	Grouping
3,8	0	0	2	856,2	A
7,0	0	0	2	853,1	A
3,8	0	1	2	744,2	B
7,0	0	1	2	734,5	B
7,0	1	1	2	500,0	C
7,0	1	0	2	488,8	C
3,8	1	0	2	449,9	D
3,8	1	1	2	446,5	D

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,03340	0,03340	0,03340	7,39	0,026
Glycerol	1	0,35076	0,35076	0,35076	77,61	0,000
Heat	1	0,00026	0,00026	0,00026	0,06	0,815
pH*Glycerol	1	0,33902	0,33902	0,33902	75,01	0,000
pH*Heat	1	0,02038	0,02038	0,02038	4,51	0,066
Glycerol*Heat	1	0,04962	0,04962	0,04962	10,98	0,011
pH*Glycerol*Heat	1	0,00050	0,00050	0,00050	0,11	0,749
Error	8	0,03616	0,03616	0,00452		
Total	15	0,83008				

S = 0,0672286 R-Sq = 95,64% R-Sq(adj) = 91,83%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	N	Mean	Grouping
7,0	8	3,4	A
3,8	8	3,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Glycerol	N	Mean	Grouping
0	8	3,5	A
1	8	3,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Heat	N	Mean	Grouping
0	8	3,4	A
1	8	3,3	A

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Glycerol	N	Mean	Grouping
3,8	0	4	3,6	A
7,0	0	4	3,4	B
7,0	1	4	3,4	B
3,8	1	4	3,0	C

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Heat	N	Mean	Grouping
7,0	0	4	3,4	A
7,0	1	4	3,4	A B
3,8	1	4	3,3	A B
3,8	0	4	3,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Glycerol	Heat	N	Mean	Grouping
0	1	4	3,5	A
0	0	4	3,4	A
1	0	4	3,3	B
1	1	4	3,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Glycerol	Heat	N	Mean	Grouping
3,8	0	1	2	3,7	A
3,8	0	0	2	3,5	A B
7,0	1	0	2	3,5	A B
7,0	0	1	2	3,4	A B
7,0	0	0	2	3,4	B
7,0	1	1	2	3,3	B C
3,8	1	0	2	3,0	C D
3,8	1	1	2	3,0	D

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1,446	1,446	1,446	562,92	0,000
Glycerol	1	32,747	32,747	32,747	12748,23	0,000
Heat	1	0,014	0,014	0,014	5,37	0,049
pH*Glycerol	1	94,819	94,819	94,819	36912,47	0,000
pH*Heat	1	0,652	0,652	0,652	253,84	0,000
Glycerol*Heat	1	6,669	6,669	6,669	2596,32	0,000
pH*Glycerol*Heat	1	3,395	3,395	3,395	1321,58	0,000
Error	8	0,021	0,021	0,003		
Total	15	139,762				

S = 0,0506828 R-Sq = 99,99% R-Sq(adj) = 99,97%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	N	Mean	Grouping
7,0	8	5,3	A
3,8	8	4,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Glycerol	N	Mean	Grouping
1	8	6,4	A
0	8	3,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Heat	N	Mean	Grouping
0	8	5,0	A
1	8	5,0	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Glycerol	N	Mean	Grouping
3,8	1	4	8,6	A
7,0	0	4	6,3	B
7,0	1	4	4,3	C
3,8	0	4	0,8	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Heat	N	Mean	Grouping
7,0	0	4	5,5	A
7,0	1	4	5,1	B
3,8	1	4	4,9	C
3,8	0	4	4,5	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Glycerol	Heat	N	Mean	Grouping
1	1	4	7,1	A
1	0	4	5,8	B
0	0	4	4,3	C
0	1	4	2,9	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Glycerol	Heat	N	Mean	Grouping
3,8	1	1	2	8,9	A
3,8	1	0	2	8,2	B
7,0	0	0	2	7,7	C
7,0	1	1	2	5,2	D
7,0	0	1	2	5,0	E
7,0	1	0	2	3,4	F
3,8	0	0	2	0,9	G
3,8	0	1	2	0,8	G

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	133,69	133,69	133,69	9843,82	0,000
Glycerol	1	334,98	334,98	334,98	24665,00	0,000
Heat	1	1,22	1,22	1,22	89,50	0,000
pH*Glycerol	1	821,54	821,54	821,54	60490,67	0,000
pH*Heat	1	0,31	0,31	0,31	22,88	0,001
Glycerol*Heat	1	36,81	36,81	36,81	2710,69	0,000
pH*Glycerol*Heat	1	27,38	27,38	27,38	2015,95	0,000
Error	8	0,11	0,11	0,01		
Total	15	1356,04				

S = 0,116539 R-Sq = 99,99% R-Sq(adj) = 99,98%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	N	Mean	Grouping
7,0	8	21,5	A
3,8	8	15,8	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Glycerol	N	Mean	Grouping
1	8	23,2	A
0	8	14,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Heat	N	Mean	Grouping
1	8	18,9	A
0	8	18,4	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	Glycerol	N	Mean	Grouping
3,8	1	4	27,5	A
7,0	0	4	24,1	B
7,0	1	4	18,9	C
3,8	0	4	4,0	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	Heat	N	Mean	Grouping
7,0	1	4	22,0	A
7,0	0	4	21,1	B
3,8	1	4	15,9	C
3,8	0	4	15,6	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Glycerol	Heat	N	Mean	Grouping
1	1	4	25,0	A
1	0	4	21,4	B
0	0	4	15,3	C
0	1	4	12,8	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	Glycerol	Heat	N	Mean	Grouping
3,8	1	1	2	27,8	A
3,8	1	0	2	27,1	B
7,0	0	0	2	26,5	C
7,0	1	1	2	22,2	D
7,0	0	1	2	21,7	E
7,0	1	0	2	15,7	F
3,8	0	0	2	4,1	G
3,8	0	1	2	3,9	G

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	3,920	3,920	3,920	413,76	0,000
Glycerol	1	98,506	98,506	98,506	10396,37	0,000
Heat	1	0,058	0,058	0,058	6,08	0,039
pH*Glycerol	1	284,428	284,428	284,428	30018,81	0,000
pH*Heat	1	1,850	1,850	1,850	195,21	0,000
Glycerol*Heat	1	20,748	20,748	20,748	2189,77	0,000
pH*Glycerol*Heat	1	10,465	10,465	10,465	1104,51	0,000
Error	8	0,076	0,076	0,009		
Total	15	420,050				

S = 0,0973396 R-Sq = 99,98% R-Sq(adj) = 99,97%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	N	Mean	Grouping
7,0	8	9,1	A
3,8	8	8,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Glycerol	N	Mean	Grouping
1	8	11,0	A
0	8	6,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Heat	N	Mean	Grouping
0	8	8,6	A
1	8	8,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Glycerol	N	Mean	Grouping
3,8	1	4	14,8	A
7,0	0	4	10,8	B
7,0	1	4	7,3	C
3,8	0	4	1,4	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Heat	N	Mean	Grouping
7,0	0	4	9,5	A
7,0	1	4	8,7	B
3,8	1	4	8,3	C
3,8	0	4	7,8	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Glycerol	Heat	N	Mean	Grouping
1	1	4	12,1	A
1	0	4	10,0	B
0	0	4	7,3	C
0	1	4	4,9	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Glycerol	Heat	N	Mean	Grouping
3,8	1	1	2	15,4	A
3,8	1	0	2	14,1	B
7,0	0	0	2	13,1	C
7,0	1	1	2	8,9	D
7,0	0	1	2	8,4	E
7,0	1	0	2	5,8	F
3,8	0	0	2	1,4	G
3,8	0	1	2	1,3	G

Means that do not share a letter are significantly different.

Analysis of Variance for Efficiency, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1146,55	1146,55	1146,55	64427,47	0,000
Glycerol	1	958,71	958,71	958,71	53872,20	0,000
Heat	1	103,73	103,73	103,73	5828,67	0,000
pH*Glycerol	1	1210,92	1210,92	1210,92	68044,44	0,000
pH*Heat	1	10,77	10,77	10,77	605,00	0,000
Glycerol*Heat	1	2,76	2,76	2,76	155,20	0,000
pH*Glycerol*Heat	1	6,32	6,32	6,32	355,20	0,000
Error	8	0,14	0,14	0,02		
Total	15	3439,91				

S = 0,133402   R-Sq = 100,00%   R-Sq(adj) = 99,99%

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	N	Mean	Grouping
3,8	8	76,3	A
7,0	8	59,4	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Glycerol	N	Mean	Grouping
1	8	75,6	A
0	8	60,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Heat	N	Mean	Grouping
1	8	70,4	A
0	8	65,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Glycerol	N	Mean	Grouping
3,8	1	4	92,7	A
7,0	0	4	60,3	B
3,8	0	4	59,9	C
7,0	1	4	58,4	D

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Heat	N	Mean	Grouping
3,8	1	4	79,7	A
3,8	0	4	72,9	B
7,0	1	4	61,1	C
7,0	0	4	57,6	D

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Glycerol	Heat	N	Mean	Grouping
1	1	4	77,7	A
1	0	4	73,4	B
0	1	4	63,1	C
0	0	4	57,1	D

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Glycerol	Heat	N	Mean	Grouping
3,8	1	1	2	96,3	A
3,8	1	0	2	89,2	B
7,0	0	1	2	63,1	C
3,8	0	1	2	63,0	C
7,0	1	1	2	59,1	D
7,0	1	0	2	57,7	E
7,0	0	0	2	57,6	E
3,8	0	0	2	56,7	F

Means that do not share a letter are significantly different.

Analysis of Variance for E.coli, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1,08160	1,08160	1,08160	41,26	0,000
Glycerol	1	0,09000	0,09000	0,09000	3,43	0,101
Heat	1	0,16402	0,16402	0,16402	6,26	0,037
pH*Glycerol	1	0,19360	0,19360	0,19360	7,39	0,026
pH*Heat	1	0,57003	0,57003	0,57003	21,75	0,002
Glycerol*Heat	1	0,00062	0,00062	0,00062	0,02	0,881
pH*Glycerol*Heat	1	0,64802	0,64802	0,64802	24,72	0,001
Error	8	0,20970	0,20970	0,02621		
Total	15	2,95760				

S = 0,161903 R-Sq = 92,91% R-Sq(adj) = 86,71%

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	N	Mean	Grouping
7,0	8	3,1	A
3,8	8	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Glycerol	N	Mean	Grouping
1	8	3,0	A
0	8	2,8	A

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Heat	N	Mean	Grouping
0	8	3,0	A
1	8	2,8	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Glycerol	N	Mean	Grouping
7,0	0	4	3,2	A
7,0	1	4	3,1	A B
3,8	1	4	2,8	B
3,8	0	4	2,4	C

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Heat	N	Mean	Grouping
7,0	1	4	3,2	A
7,0	0	4	3,1	A
3,8	0	4	2,9	A
3,8	1	4	2,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Glycerol	Heat	N	Mean	Grouping
1	0	4	3,1	A
0	0	4	2,9	A
1	1	4	2,9	A
0	1	4	2,7	A

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Glycerol	Heat	N	Mean	Grouping
7,0	1	1	2	3,4	A
7,0	0	0	2	3,3	A
3,8	1	0	2	3,3	A
7,0	0	1	2	3,1	A B
7,0	1	0	2	2,8	A B C
3,8	0	0	2	2,5	B C
3,8	0	1	2	2,3	C
3,8	1	1	2	2,3	C

Means that do not share a letter are significantly different.

Analysis of Variance for S.aureus, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,22563	0,22563	0,22563	19,39	0,002
Glycerol	1	0,40322	0,40322	0,40322	34,65	0,000
Heat	1	0,59290	0,59290	0,59290	50,95	0,000
pH*Glycerol	1	0,00723	0,00723	0,00723	0,62	0,453
pH*Heat	1	0,07290	0,07290	0,07290	6,26	0,037
Glycerol*Heat	1	0,15210	0,15210	0,15210	13,07	0,007
pH*Glycerol*Heat	1	0,09610	0,09610	0,09610	8,26	0,021
Error	8	0,09310	0,09310	0,01164		
Total	15	1,64318				

S = 0,107877 R-Sq = 94,33% R-Sq(adj) = 89,38%

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	N	Mean	Grouping
7,0	8	4,1	A
3,8	8	3,9	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Glycerol	N	Mean	Grouping
1	8	4,2	A
0	8	3,9	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Heat	N	Mean	Grouping
1	8	4,2	A
0	8	3,8	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Glycerol	N	Mean	Grouping
7,0	1	4	4,3	A
3,8	1	4	4,1	A B
7,0	0	4	4,0	B
3,8	0	4	3,7	C

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Heat	N	Mean	Grouping
7,0	1	4	4,4	A
3,8	1	4	4,0	B
7,0	0	4	3,9	B C
3,8	0	4	3,8	C

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Glycerol	Heat	N	Mean	Grouping
1	1	4	4,3	A
0	1	4	4,2	A
1	0	4	4,1	A
0	0	4	3,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Glycerol	Heat	N	Mean	Grouping
7,0	0	1	2	4,4	A
7,0	1	1	2	4,4	A B
7,0	1	0	2	4,2	A B C
3,8	1	1	2	4,2	A B C
3,8	1	0	2	4,0	B C D
3,8	0	1	2	3,9	C D
3,8	0	0	2	3,6	D
7,0	0	0	2	3,6	D

Means that do not share a letter are significantly different.

Table D.3 Analysis of Variance for emulsions produced by microfluidization with the emulsifier **SMP**. Effect of pH, glycerol addition, and heating before homogenization on particle size, turbidity, T2 times, color-L*, a*, b* values, efficiency, *E.coli* and *S.aureus* population decrease using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	99,656	99,656	16,609	11,37	0,000
Error	14	20,453	20,453	1,461		
Total	20	120,110				

S = 1,20870 R-Sq = 82,97% R-Sq(adj) = 75,67%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 7.4	3	39,6	A
pH 3.8- heat	3	36,6	A B
pH 7.4 - heat	3	34,8	B C
pH 3.8- glycerol	3	33,7	B C
pH 3.8- glycerol- heat	3	33,6	B C
pH 7.4- glycerol	3	33,5	B C
pH 7.4- glycerol- heat	3	33,2	C

Means that do not share a letter are significantly different.

Analysis of Variance for T2, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	1905845	1905845	317641	368,45	0,000
Error	14	12070	12070	862		
Total	20	1917914				

S = 29,3617 R-Sq = 99,37% R-Sq(adj) = 99,10%

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	N	Mean	Grouping
pH 7.4 - heat	3	1209,0	A
pH 3.8- heat	3	1131,7	A B
pH 7.4	3	1091,5	B
pH 7.4- glycerol- heat	3	620,5	C
pH 7.4- glycerol	3	598,3	C
pH 3.8- glycerol	3	504,2	D
pH 3.8- glycerol- heat	3	464,4	D

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	2,25118	2,25118	0,37520	1584,38	0,000
Error	14	0,00332	0,00332	0,00024		
Total	20	2,25450				

S = 0,0153886   R-Sq = 99,85%   R-Sq(adj) = 99,79%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 3.8- heat	3	1,1	A
pH 7.4	3	1,0	B
pH 7.4 - heat	3	0,6	C
pH 3.8- glycerol- heat	3	0,4	D
pH 3.8- glycerol	3	0,4	D
pH 7.4- glycerol- heat	3	0,3	E
pH 7.4- glycerol	3	0,3	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	251,944	251,944	41,991	19595,64	0,000
Error	14	0,030	0,030	0,002		
Total	20	251,974				

S = 0,0462910   R-Sq = 99,99%   R-Sq(adj) = 99,98%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 7.4- glycerol	3	49,8	A
pH 7.4- glycerol- heat	3	49,7	A
pH 3.8- glycerol	3	47,6	B
pH 3.8- glycerol- heat	3	47,6	B
pH 3.8- heat	3	45,0	C
pH 7.4 - heat	3	44,4	D
pH 7.4	3	39,1	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	120,913	120,913	20,152	47,45	0,000
Error	14	5,946	5,946	0,425		
Total	20	126,859				

S = 0,651697   R-Sq = 95,31%   R-Sq(adj) = 93,30%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	3	55,2	A
pH 3.8- glycerol	3	54,8	A
pH 7.4- glycerol	3	54,8	A
pH 7.4- glycerol- heat	3	53,9	A
pH 3.8- heat	3	53,4	A
pH 7.4 - heat	3	51,5	B
pH 7.4	3	47,9	C

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	812,49	812,49	135,41	288,46	0,000
Error	14	6,57	6,57	0,47		
Total	20	819,06				

S = 0,685159 R-Sq = 99,20% R-Sq(adj) = 98,85%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	3	85,7	A
pH 7.4- glycerol	3	85,5	A
pH 3.8- glycerol	3	82,1	B
pH 3.8- glycerol- heat	3	82,1	B
pH 3.8- heat	3	77,6	C
pH 7.4 - heat	3	76,6	C
pH 7.4	3	66,4	D

Means that do not share a letter are significantly different.

Analysis of Variance for Efficiency, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	6	149,596	149,596	24,933	4,97	0,006
Error	14	70,242	70,242	5,017		
Total	20	219,838				

S = 2,23993 R-Sq = 68,05% R-Sq(adj) = 54,35%

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Treatment	N	Mean	Grouping
pH 7.4	3	70,7	A
pH 7.4 - heat	3	68,3	A
pH 7.4- glycerol- heat	3	68,2	A
pH 7.4- glycerol	3	67,8	A B
pH 3.8- glycerol	3	65,4	A B
pH 3.8- glycerol- heat	3	64,9	A B
pH 3.8- heat	3	61,9	B

Means that do not share a letter are significantly different.

Analysis of Variance for E.coli, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment_1	6	3,44311	3,44311	0,57385	78,17	0,000
Error	7	0,05139	0,05139	0,00734		
Total	13	3,49450				

S = 0,0856826 R-Sq = 98,53% R-Sq(adj) = 97,27%

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Treatment	N	Mean	Grouping
pH 3.8- glycerol	2	1,5	A
pH 3.8- glycerol- heat	2	1,4	A B
pH 3.8- heat	2	1,3	A B
pH 7.4- glycerol	2	1,1	B C
pH 7.4- glycerol- heat	2	0,9	C
pH 7.4 - heat	2	0,5	D
pH 7.4	2	0,1	E

Means that do not share a letter are significantly different.

Analysis of Variance for S.aureus, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment_1	6	0,39628	0,39628	0,06605	4,83	0,029
Error	7	0,09576	0,09576	0,01368		
Total	13	0,49204				

S = 0,116959 R-Sq = 80,54% R-Sq(adj) = 63,86%

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Treatment	N	Mean	Grouping
pH 7.4 - heat	2	2,7	A
pH 3.8- heat	2	2,7	A B
pH 3.8- glycerol- heat	2	2,6	A B
pH 3.8- glycerol	2	2,6	A B
pH 7.4	2	2,5	A B
pH 7.4- glycerol	2	2,3	A B
pH 7.4- glycerol- heat	2	2,3	B

Means that do not share a letter are significantly different.

Table D.4 Analysis of Variance for emulsions produced by microfluidization with the emulsifier **Tween 80** stored for 0 or 28 days. Effect of pH, glycerol addition, heating before homogenization and storage time on particle size, turbidity, T2 times, color-L*, a*, b* values using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	431,45	431,45	143,82	129,47	0,000
Time	1	310,32	310,32	310,32	279,36	0,000
Treatment*Time	3	119,45	119,45	39,82	35,85	0,000
Error	16	17,77	17,77	1,11		
Total	23	879,00				

S = 1,05396 R-Sq = 97,98% R-Sq(adj) = 97,09%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	43,9	A
pH 7.4	6	43,2	A
pH 7.4- glycerol	6	39,6	B
pH 3.8	6	33,2	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Time	N	Mean	Grouping
28	12	43,5	A
0	12	36,4	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	28	3	48,8	A
pH 7.4	28	3	47,3	A B
pH 7.4- glycerol	28	3	45,1	B
pH 7.4	0	3	39,1	C
pH 3.8- glycerol	0	3	38,9	C
pH 7.4- glycerol	0	3	34,1	D
pH 3.8	0	3	33,3	D
pH 3.8	28	3	33,0	D

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	1,06331	1,06331	0,35444	506,94	0,000
Time	1	0,10192	0,10192	0,10192	145,77	0,000
Treatment*Time	3	0,05609	0,05609	0,01870	26,74	0,000
Error	16	0,01119	0,01119	0,00070		
Total	23	1,23251				

S = 0,0264418 R-Sq = 99,09% R-Sq(adj) = 98,70%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 7.4	6	0,8	A
pH 3.8- glycerol	6	0,7	B
pH 3.8	6	0,4	C
pH 7.4- glycerol	6	0,3	D

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Time	N	Mean	Grouping
28	12	0,6	A
0	12	0,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	28	3	0,9	A
pH 7.4	28	3	0,8	A
pH 7.4	0	3	0,7	B
pH 3.8- glycerol	0	3	0,6	C
pH 3.8	28	3	0,4	D
pH 3.8	0	3	0,4	E
pH 7.4- glycerol	28	3	0,3	E
pH 7.4- glycerol	0	3	0,3	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	568,63	568,63	189,54	1023,42	0,000
Time	1	126,36	126,36	126,36	682,29	0,000
Treatment*Time	3	168,80	168,80	56,27	303,81	0,000
Error	16	2,96	2,96	0,19		
Total	23	866,75				

S = 0,430354 R-Sq = 99,66% R-Sq(adj) = 99,51%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 7.4	6	49,9	A
pH 3.8	6	47,8	B
pH 7.4- glycerol	6	44,3	C
pH 3.8- glycerol	6	37,1	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Time	N	Mean	Grouping
0	12	47,1	A
28	12	42,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	0	3	50,4	A
pH 7.4	28	3	50,2	A
pH 7.4	0	3	49,6	A
pH 3.8	28	3	47,9	B
pH 3.8	0	3	47,8	B
pH 3.8- glycerol	0	3	40,5	C
pH 7.4- glycerol	28	3	38,2	D
pH 3.8- glycerol	28	3	33,7	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	99,650	99,650	33,217	454,92	0,000
Time	1	35,770	35,770	35,770	489,89	0,000
Treatment*Time	3	58,967	58,967	19,656	269,19	0,000
Error	16	1,168	1,168	0,073		
Total	23	195,555				

S = 0,270216 R-Sq = 99,40% R-Sq(adj) = 99,14%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 3.8	6	54,2	A
pH 7.4	6	52,8	B
pH 7.4- glycerol	6	51,6	C
pH 3.8- glycerol	6	48,7	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Time	N	Mean	Grouping
0	12	53,1	A
28	12	50,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	0	3	55,1	A
pH 3.8	28	3	54,3	B
pH 3.8	0	3	54,1	B
pH 7.4	28	3	53,2	C
pH 7.4	0	3	52,5	D
pH 3.8- glycerol	0	3	50,6	E
pH 7.4- glycerol	28	3	48,2	F
pH 3.8- glycerol	28	3	46,8	G

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	1738,15	1738,15	579,38	1100,34	0,000
Time	1	363,64	363,64	363,64	690,60	0,000
Treatment*Time	3	500,66	500,66	166,89	316,94	0,000
Error	16	8,42	8,42	0,53		
Total	23	2610,88				

S = 0,725638 R-Sq = 99,68% R-Sq(adj) = 99,54%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 7.4	6	86,1	A
pH 3.8	6	82,5	B
pH 7.4- glycerol	6	76,5	C
pH 3.8- glycerol	6	63,7	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Time	N	Mean	Grouping
0	12	81,1	A
28	12	73,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	0	3	87,1	A
pH 7.4	28	3	86,6	A
pH 7.4	0	3	85,6	A
pH 3.8	28	3	82,6	B
pH 3.8	0	3	82,4	B
pH 3.8- glycerol	0	3	69,2	C
pH 7.4- glycerol	28	3	65,8	D
pH 3.8- glycerol	28	3	58,1	E

Means that do not share a letter are significantly different.

Analysis of Variance for T2, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	6457089	6457089	2152363	1112,52	0,000
Time	1	76862	76862	76862	39,73	0,000
Treatment*Time	3	4453	4453	1484	0,77	0,529
Error	16	30955	30955	1935		
Total	23	6569358				

S = 43,9849 R-Sq = 99,53% R-Sq(adj) = 99,32%

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	N	Mean	Grouping
pH 7.4	6	1718,4	A
pH 3.8	6	1593,8	B
pH 7.4- glycerol	6	630,5	C
pH 3.8- glycerol	6	614,5	C

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Time	N	Mean	Grouping
0	12	1195,9	A
28	12	1082,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	Time	N	Mean	Grouping
pH 7.4	0	3	1783,6	A
pH 3.8	0	3	1667,9	A B
pH 7.4	28	3	1653,3	B
pH 3.8	28	3	1519,7	C
pH 7.4- glycerol	0	3	670,9	D
pH 3.8- glycerol	0	3	661,2	D
pH 7.4- glycerol	28	3	590,2	D
pH 3.8- glycerol	28	3	567,8	D

Means that do not share a letter are significantly different.

Table D.5 Analysis of Variance for emulsions produced by microfluidization with the emulsifier **lecithin** stored for 0 or 28 days. Effect of pH, glycerol addition, heating before homogenization and storage time on particle size, turbidity, T2 times, color-L*, a*, b* values using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	0,104023	0,104023	0,020805	12,69	0,000
Time	1	0,679424	0,679424	0,679424	414,39	0,000
Treatment*Time	5	0,303789	0,303789	0,060758	37,06	0,000
Error	24	0,039350	0,039350	0,001640		
Total	35	1,126587				

S = 0,0404918 R-Sq = 96,51% R-Sq(adj) = 94,91%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 7.4- glycerol	6	2,1	A
pH 7.4	6	2,1	A
pH 3.8- glycerol- heat	6	2,1	A B
pH 7.4 - heat	6	2,0	B C
pH 3.8- glycerol	6	2,0	B C
pH 7.4- glycerol- heat	6	2,0	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Time	N	Mean	Grouping
28	18	2,2	A
0	18	1,9	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	Time	N	Mean	Grouping
pH 7.4	28	3	2,4	A
pH 7.4- glycerol	28	3	2,3	A
pH 7.4 - heat	28	3	2,2	B
pH 7.4- glycerol- heat	28	3	2,1	B
pH 3.8- glycerol- heat	28	3	2,1	B
pH 3.8- glycerol	28	3	2,1	B C
pH 3.8- glycerol- heat	0	3	2,0	B C
pH 3.8- glycerol	0	3	2,0	C D
pH 7.4- glycerol	0	3	1,9	D E
pH 7.4 - heat	0	3	1,9	D E
pH 7.4- glycerol- heat	0	3	1,9	D E
pH 7.4	0	3	1,8	E

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	2,19384	2,19384	0,43877	42,11	0,000
Time	1	0,20370	0,20370	0,20370	19,55	0,000
Treatment*Time	5	8,27747	8,27747	1,65549	158,89	0,000
Error	24	0,25007	0,25007	0,01042		
Total	35	10,92508				

S = 0,102075 R-Sq = 97,71% R-Sq(adj) = 96,66%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	2,9	A
pH 7.4 - heat	6	2,9	A
pH 3.8- glycerol- heat	6	2,6	B
pH 7.4- glycerol- heat	6	2,6	B
pH 7.4- glycerol	6	2,3	C
pH 7.4	6	2,3	C

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Time	N	Mean	Grouping
28	18	2,7	A
0	18	2,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	Time	N	Mean	Grouping
pH 7.4 - heat	0	3	3,2	A
pH 7.4	28	3	3,2	A
pH 3.8- glycerol	0	3	3,1	A
pH 3.8- glycerol- heat	0	3	3,1	A
pH 7.4- glycerol- heat	28	3	2,8	B
pH 3.8- glycerol	28	3	2,7	B C
pH 7.4- glycerol	28	3	2,7	B C
pH 7.4 - heat	28	3	2,5	B C
pH 7.4- glycerol- heat	0	3	2,4	C D
pH 3.8- glycerol- heat	28	3	2,2	D E
pH 7.4- glycerol	0	3	1,9	E
pH 7.4	0	3	1,3	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	182,553	182,553	36,511	1797,57	0,000
Time	1	796,368	796,368	796,368	39208,51	0,000
Treatment*Time	5	118,584	118,584	23,717	1167,67	0,000
Error	24	0,487	0,487	0,020		
Total	35	1097,993				

S = 0,142517 R-Sq = 99,96% R-Sq(adj) = 99,94%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	11,6	A
pH 7.4	6	9,4	B
pH 3.8- glycerol	6	8,0	C
pH 7.4- glycerol	6	7,6	D
pH 3.8- glycerol- heat	6	6,2	E
pH 7.4 - heat	6	4,5	F

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Time	N	Mean	Grouping
0	18	12,6	A
28	18	3,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	0	3	19,9	A
pH 7.4	0	3	14,1	B
pH 7.4- glycerol	0	3	12,8	C
pH 3.8- glycerol	0	3	12,0	D
pH 3.8- glycerol- heat	0	3	8,9	E
pH 7.4 - heat	0	3	7,7	F
pH 7.4	28	3	4,6	G
pH 3.8- glycerol	28	3	4,1	H
pH 3.8- glycerol- heat	28	3	3,5	I
pH 7.4- glycerol- heat	28	3	3,3	I
pH 7.4- glycerol	28	3	2,4	J
pH 7.4 - heat	28	3	1,2	K

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	443,26	443,26	88,65	286,48	0,000
Time	1	2503,00	2503,00	2503,00	8088,55	0,000
Treatment*Time	5	206,96	206,96	41,39	133,76	0,000
Error	24	7,43	7,43	0,31		
Total	35	3160,65				

S = 0,556282 R-Sq = 99,77% R-Sq(adj) = 99,66%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 7.4	6	26,6	A
pH 7.4- glycerol- heat	6	25,7	A
pH 3.8- glycerol	6	24,7	B
pH 3.8- glycerol- heat	6	21,7	C
pH 7.4- glycerol	6	21,2	C
pH 7.4 - heat	6	16,2	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Time	N	Mean	Grouping
0	18	31,0	A
28	18	14,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	0	3	37,0	A
pH 7.4	0	3	32,6	B
pH 7.4- glycerol	0	3	31,4	B C
pH 3.8- glycerol	0	3	30,6	C
pH 3.8- glycerol- heat	0	3	27,6	D
pH 7.4 - heat	0	3	26,8	D
pH 7.4	28	3	20,6	E
pH 3.8- glycerol	28	3	18,7	F
pH 3.8- glycerol- heat	28	3	15,7	G
pH 7.4- glycerol- heat	28	3	14,4	G
pH 7.4- glycerol	28	3	11,0	H
pH 7.4 - heat	28	3	5,6	I

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	549,39	549,39	109,88	2013,23	0,000
Time	1	2384,86	2384,86	2384,86	43696,49	0,000
Treatment*Time	5	355,59	355,59	71,12	1303,05	0,000
Error	24	1,31	1,31	0,05		
Total	35	3291,14				

S = 0,233619 R-Sq = 99,96% R-Sq(adj) = 99,94%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	19,8	A
pH 7.4	6	16,0	B
pH 3.8- glycerol	6	13,8	C
pH 7.4- glycerol	6	13,1	D
pH 3.8- glycerol- heat	6	10,6	E
pH 7.4 - heat	6	7,5	F

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Time	N	Mean	Grouping
0	18	21,6	A
28	18	5,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	0	3	34,2	A
pH 7.4	0	3	24,3	B
pH 7.4- glycerol	0	3	22,0	C
pH 3.8- glycerol	0	3	20,6	D
pH 3.8- glycerol- heat	0	3	15,3	E
pH 7.4 - heat	0	3	13,1	F
pH 7.4	28	3	7,8	G
pH 3.8- glycerol	28	3	6,9	H
pH 3.8- glycerol- heat	28	3	5,8	I
pH 7.4- glycerol- heat	28	3	5,4	I
pH 7.4- glycerol	28	3	4,1	J
pH 7.4 - heat	28	3	1,9	K

Means that do not share a letter are significantly different.

Analysis of Variance for T2, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	1800771	1800771	360154	10567,36	0,000
Time	1	13321	13321	13321	390,86	0,000
Treatment*Time	5	5606	5606	1121	32,90	0,000
Error	24	818	818	34		
Total	35	1820516				

S = 5,83796 R-Sq = 99,96% R-Sq(adj) = 99,93%

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	N	Mean	Grouping
pH 7.4	6	908,0	A
pH 7.4 - heat	6	766,8	B
pH 3.8- glycerol	6	395,2	C
pH 7.4- glycerol	6	391,0	C
pH 3.8- glycerol- heat	6	354,6	D
pH 7.4- glycerol- heat	6	348,6	D

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Time	N	Mean	Grouping
0	18	546,6	A
28	18	508,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	Time	N	Mean	Grouping
pH 7.4	0	3	915,9	A
pH 7.4	28	3	900,1	A
pH 7.4 - heat	0	3	800,3	B
pH 7.4 - heat	28	3	733,3	C
pH 3.8- glycerol	0	3	427,1	D
pH 7.4- glycerol	0	3	395,3	E
pH 7.4- glycerol	28	3	386,6	E
pH 3.8- glycerol- heat	0	3	383,9	E
pH 3.8- glycerol	28	3	363,2	F
pH 7.4- glycerol- heat	0	3	356,9	F G
pH 7.4- glycerol- heat	28	3	340,2	G H
pH 3.8- glycerol- heat	28	3	325,3	H

Means that do not share a letter are significantly different.

Table D.6 Analysis of Variance for emulsions produced by microfluidization with the emulsifier **SMP** stored for 0 or 28 days. Effect of pH, glycerol addition, heating before homogenization and storage time on particle size, turbidity, T2 times, color-L*, a*, b* values using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	89,255	89,255	29,752	35,40	0,000
Time	1	107,527	107,527	107,527	127,94	0,000
Treatment*Time	3	76,710	76,710	25,570	30,43	0,000
Error	16	13,447	13,447	0,840		
Total	23	286,938				

S = 0,916742 R-Sq = 95,31% R-Sq(adj) = 93,26%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	38,4	A
pH 3.8- glycerol- heat	6	36,4	B
pH 7.4- glycerol- heat	6	34,0	C
pH 7.4- glycerol	6	33,7	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Time	N	Mean	Grouping
28	12	37,7	A
0	12	33,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	28	3	43,1	A
pH 3.8- glycerol- heat	28	3	39,2	B
pH 7.4- glycerol- heat	28	3	34,7	C
pH 7.4- glycerol	28	3	33,9	C
pH 3.8- glycerol	0	3	33,7	C
pH 3.8- glycerol- heat	0	3	33,6	C
pH 7.4- glycerol	0	3	33,5	C
pH 7.4- glycerol- heat	0	3	33,2	C

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	0,138099	0,138099	0,046033	139,09	0,000
Time	1	0,073815	0,073815	0,073815	223,03	0,000
Treatment*Time	3	0,056638	0,056638	0,018879	57,04	0,000
Error	16	0,005295	0,005295	0,000331		
Total	23	0,273848				

S = 0,0181923 R-Sq = 98,07% R-Sq(adj) = 97,22%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	0,5	A
pH 3.8- glycerol- heat	6	0,4	B
pH 7.4- glycerol- heat	6	0,3	C
pH 7.4- glycerol	6	0,3	C

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Time	N	Mean	Grouping
28	12	0,5	A
0	12	0,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	28	3	0,6	A
pH 3.8- glycerol- heat	28	3	0,5	B
pH 3.8- glycerol- heat	0	3	0,4	C
pH 3.8- glycerol	0	3	0,4	C D
pH 7.4- glycerol- heat	28	3	0,4	C D
pH 7.4- glycerol	28	3	0,3	D E
pH 7.4- glycerol- heat	0	3	0,3	D E
pH 7.4- glycerol	0	3	0,3	E

Means that do not share a letter are significantly different.

Analysis of Variance for T2, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	52745	52745	17582	5,67	0,008
Time	1	52168	52168	52168	16,82	0,001
Treatment*Time	3	8278	8278	2759	0,89	0,468
Error	16	49615	49615	3101		
Total	23	162805				

S = 55,6859 R-Sq = 69,53% R-Sq(adj) = 56,19%

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	549,9	A
pH 7.4- glycerol	6	543,2	A
pH 3.8- glycerol	6	463,6	A B
pH 3.8- glycerol- heat	6	444,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Time	N	Mean	Grouping
0	12	546,8	A
28	12	453,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for T2

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	0	3	620,5	A
pH 7.4- glycerol	0	3	598,3	A
pH 3.8- glycerol	0	3	504,2	A B
pH 7.4- glycerol	28	3	488,0	A B
pH 7.4- glycerol- heat	28	3	479,3	A B
pH 3.8- glycerol- heat	0	3	464,4	A B
pH 3.8- glycerol- heat	28	3	424,0	B
pH 3.8- glycerol	28	3	423,0	B

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	9,9267	9,9267	3,3089	3931,36	0,000
Time	1	1,5914	1,5914	1,5914	1890,71	0,000
Treatment*Time	3	4,3899	4,3899	1,4633	1738,57	0,000
Error	16	0,0135	0,0135	0,0008		
Total	23	15,9214				

S = 0,0290115 R-Sq = 99,92% R-Sq(adj) = 99,88%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 7.4- glycerol	6	49,6	A
pH 7.4- glycerol- heat	6	49,5	B
pH 3.8- glycerol	6	48,4	C
pH 3.8- glycerol- heat	6	48,2	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Time	N	Mean	Grouping
28	12	49,2	A
0	12	48,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	0	3	49,8	A
pH 7.4- glycerol- heat	0	3	49,7	B
pH 7.4- glycerol	28	3	49,5	C
pH 7.4- glycerol- heat	28	3	49,3	D
pH 3.8- glycerol	28	3	49,1	E
pH 3.8- glycerol- heat	28	3	48,9	F
pH 3.8- glycerol	0	3	47,6	G
pH 3.8- glycerol- heat	0	3	47,6	G

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	0,64881	0,64881	0,21627	451,35	0,000
Time	1	2,14204	2,14204	2,14204	4470,34	0,000
Treatment*Time	3	0,48235	0,48235	0,16078	335,54	0,000
Error	16	0,00767	0,00767	0,00048		
Total	23	3,28086				

S = 0,0218899   R-Sq = 99,77%   R-Sq(adj) = 99,66%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	6	54,8	A
pH 7.4- glycerol	6	54,7	B
pH 7.4- glycerol- heat	6	54,6	C
pH 3.8- glycerol	6	54,3	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Time	N	Mean	Grouping
0	12	54,9	A
28	12	54,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol- heat	0	3	55,2	A
pH 7.4- glycerol- heat	0	3	54,8	B
pH 3.8- glycerol	0	3	54,8	B C
pH 7.4- glycerol	0	3	54,8	C
pH 7.4- glycerol	28	3	54,6	D
pH 3.8- glycerol- heat	28	3	54,4	E
pH 7.4- glycerol- heat	28	3	54,4	E
pH 3.8- glycerol	28	3	53,9	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	27,1477	27,1477	9,0492	213,40	0,000
Time	1	5,6454	5,6454	5,6454	133,13	0,000
Treatment*Time	3	11,7852	11,7852	3,9284	92,64	0,000
Error	16	0,6785	0,6785	0,0424		
Total	23	45,2568				

S = 0,205923   R-Sq = 98,50%   R-Sq(adj) = 97,84%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 7.4- glycerol	6	85,4	A
pH 7.4- glycerol- heat	6	85,4	A
pH 3.8- glycerol	6	83,4	B
pH 3.8- glycerol- heat	6	83,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Time	N	Mean	Grouping
28	12	84,8	A
0	12	83,9	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	0	3	85,7	A
pH 7.4- glycerol	0	3	85,5	A B
pH 7.4- glycerol	28	3	85,3	A B
pH 7.4- glycerol- heat	28	3	85,1	B C
pH 3.8- glycerol	28	3	84,6	C D
pH 3.8- glycerol- heat	28	3	84,3	D
pH 3.8- glycerol	0	3	82,1	E
pH 3.8- glycerol- heat	0	3	82,1	E

Means that do not share a letter are significantly different.

Table D.7 Analysis of Variance for emulsions produced by ultrasonication with the emulsifier **Tween 80**. Effect of pH and glycerol addition on particle size, turbidity, color-L*, a*, b* values, efficiency, *E.coli*, and *S.aureus* population decrease.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1,20	1,20	1,20	12,89	0,007
Glycerol	1	103,25	103,25	103,25	1106,29	0,000
pH*Glycerol	1	2442,45	2442,45	2442,45	26169,14	0,000
Error	8	0,75	0,75	0,09		
Total	11	2547,66				

S = 0,305505 R-Sq = 99,97% R-Sq(adj) = 99,96%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	N	Mean	Grouping
3,8	6	49,23	A
7,4	6	48,60	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Glycerol	N	Mean	Grouping
0	6	51,85	A
1	6	45,98	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Glycerol	N	Mean	Grouping
7,4	0	3	65,80	A
3,8	1	3	60,57	B
3,8	0	3	37,90	C
7,4	1	3	31,40	D

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,0094	0,0094	0,0094	0,79	0,399
Glycerol	1	3,1992	3,1992	3,1992	269,54	0,000
pH*Glycerol	1	1,1261	1,1261	1,1261	94,88	0,000
Error	8	0,0950	0,0950	0,0119		
Total	11	4,4296				

S = 0,108945 R-Sq = 97,86% R-Sq(adj) = 97,05%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	N	Mean	Grouping
3,8	6	1,6	A
7,4	6	1,6	A

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Glycerol	N	Mean	Grouping
0	6	2,1	A
1	6	1,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Glycerol	N	Mean	Grouping
7,4	0	3	2,4	A
3,8	0	3	1,9	B
3,8	1	3	1,4	C
7,4	1	3	0,8	D

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	47,402	47,402	47,402	5412,20	0,000
Glycerol	1	97,641	97,641	97,641	11148,36	0,000
pH*Glycerol	1	53,467	53,467	53,467	6104,75	0,000
Error	8	0,070	0,070	0,009		
Total	11	198,580				

S = 0,0935860 R-Sq = 99,96% R-Sq(adj) = 99,95%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	N	Mean	Grouping
7,4	6	71,3	A
3,8	6	67,3	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Glycerol	N	Mean	Grouping
0	6	72,2	A
1	6	66,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Glycerol	N	Mean	Grouping
3,8	0	3	72,3	A
7,4	0	3	72,0	B
7,4	1	3	70,6	C
3,8	1	3	62,4	D

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	5,6307	5,6307	5,6307	402,91	0,000
Glycerol	1	0,8965	0,8965	0,8965	64,15	0,000
pH*Glycerol	1	12,3221	12,3221	12,3221	881,73	0,000
Error	8	0,1118	0,1118	0,0140		
Total	11	18,9612				

S = 0,118216 R-Sq = 99,41% R-Sq(adj) = 99,19%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	N	Mean	Grouping
3,8	6	41,69	A
7,4	6	40,32	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Glycerol	N	Mean	Grouping
1	6	41,28	A
0	6	40,73	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH	Glycerol	N	Mean	Grouping
3,8	1	3	42,98	A
7,4	0	3	41,06	B
3,8	0	3	40,41	C
7,4	1	3	39,58	D

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	63,664	63,664	63,664	33216,07	0,000
Glycerol	1	163,541	163,541	163,541	85325,65	0,000
pH*Glycerol	1	43,548	43,548	43,548	22720,85	0,000
Error	8	0,015	0,015	0,002		
Total	11	270,769				

S = 0,0437798 R-Sq = 99,99% R-Sq(adj) = 99,99%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	N	Mean	Grouping
7,4	6	115,93	A
3,8	6	111,33	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Glycerol	N	Mean	Grouping
0	6	117,32	A
1	6	109,94	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Glycerol	N	Mean	Grouping
7,4	0	3	117,72	A
3,8	0	3	116,92	B
7,4	1	3	114,15	C
3,8	1	3	105,73	D

Means that do not share a letter are significantly different.

Analysis of Variance for Efficiency, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,0050067	0,0050067	0,0050067	129240,25	0,000
Glycerol	1	0,0000022	0,0000022	0,0000022	56,25	0,000
pH*Glycerol	1	0,0054914	0,0054914	0,0054914	141752,25	0,000
Error	8	0,0000003	0,0000003	0,0000000		
Total	11	0,0105006				

S = 0,000196824   R-Sq = 100,00%   R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	N	Mean	Grouping
3,8	6	0,75	A
7,4	6	0,71	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Glycerol	N	Mean	Grouping
1	6	0,73	A
0	6	0,73	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Glycerol	N	Mean	Grouping
3,8	0	3	0,77	A
7,4	1	3	0,73	B
3,8	1	3	0,73	C
7,4	0	3	0,69	D

Means that do not share a letter are significantly different.

Analysis of Variance for E.coli, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,38922	0,38922	0,38922	263,67	0,000
Glycerol	1	0,52456	0,52456	0,52456	355,35	0,000
pH*Glycerol	1	0,12396	0,12396	0,12396	83,98	0,001
Error	4	0,00590	0,00590	0,00148		
Total	7	1,04364				

S = 0,0384207   R-Sq = 99,43%   R-Sq(adj) = 99,01%

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	N	Mean	Grouping
7,4	4	2,1	A
3,8	4	1,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Glycerol	N	Mean	Grouping
1	4	2,1	A
0	4	1,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Glycerol	N	Mean	Grouping
7,4	1	2	2,5	A
3,8	1	2	1,8	B
7,4	0	2	1,7	B
3,8	0	2	1,5	C

Means that do not share a letter are significantly different.

Analysis of Variance for S.aureus, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,004545	0,004545	0,004545	5,31	0,082
Glycerol	1	0,074163	0,074163	0,074163	86,72	0,001
pH*Glycerol	1	0,022276	0,022276	0,022276	26,05	0,007
Error	4	0,003421	0,003421	0,000855		
Total	7	0,104405				

S = 0,0292436 R-Sq = 96,72% R-Sq(adj) = 94,27%

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	N	Mean	Grouping
3,8	4	2,9	A
7,4	4	2,8	A

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Glycerol	N	Mean	Grouping
1	4	2,9	A
0	4	2,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Glycerol	N	Mean	Grouping
3,8	1	2	3,0	A
7,4	1	2	2,9	B
7,4	0	2	2,8	B C
3,8	0	2	2,7	C

Means that do not share a letter are significantly different.

Table D.8 Analysis of Variance for emulsions produced by ultrasonication with the emulsifier **lecithin**. Effect of pH, glycerol addition, and heating before homogenization on particle size, turbidity, color-L*, a*, b* values, efficiency, *E.coli* and *S.aureus* population decrease using Adjusted SS for Tests

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,31556	0,31556	0,31556	719,30	0,000
Glycerol	1	0,21282	0,21282	0,21282	485,10	0,000
Heat	1	0,00115	0,00115	0,00115	2,62	0,125
pH*Glycerol	1	0,19548	0,19548	0,19548	445,58	0,000
pH*Heat	1	0,00224	0,00224	0,00224	5,11	0,038
Glycerol*Heat	1	0,00209	0,00209	0,00209	4,77	0,044
pH*Glycerol*Heat	1	0,00058	0,00058	0,00058	1,32	0,267
Error	16	0,00702	0,00702	0,00044		
Total	23	0,73694				

S = 0,0209454 R-Sq = 99,05% R-Sq(adj) = 98,63%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	N	Mean	Grouping
3,8	12	0,356	A
7,4	12	0,126	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Glycerol	N	Mean	Grouping
0	12	0,335	A
1	12	0,147	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Heat	N	Mean	Grouping
0	12	0,248	A
1	12	0,234	A

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Glycerol	N	Mean	Grouping
3,8	0	6	0,540	A
3,8	1	6	0,171	B
7,4	0	6	0,130	C
7,4	1	6	0,123	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Heat	N	Mean	Grouping
3,8	0	6	0,372	A
3,8	1	6	0,339	A
7,4	1	6	0,129	B
7,4	0	6	0,124	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Glycerol	Heat	N	Mean	Grouping
0	1	6	0,338	A
0	0	6	0,333	A
1	0	6	0,163	B
1	1	6	0,131	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

pH	Glycerol	Heat	N	Mean	Grouping
3,8	0	0	3	0,552	A
3,8	0	1	3	0,528	A
3,8	1	0	3	0,192	B
3,8	1	1	3	0,150	B C
7,4	0	1	3	0,147	B C
7,4	1	0	3	0,134	B C
7,4	0	0	3	0,113	C
7,4	1	1	3	0,111	C

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,02381	0,02381	0,02381	9,03	0,008
Glycerol	1	0,25544	0,25544	0,25544	96,86	0,000
Heat	1	0,04890	0,04890	0,04890	18,54	0,001
pH*Glycerol	1	0,38913	0,38913	0,38913	147,55	0,000
pH*Heat	1	0,00029	0,00029	0,00029	0,11	0,745
Glycerol*Heat	1	0,25889	0,25889	0,25889	98,17	0,000
pH*Glycerol*Heat	1	0,20400	0,20400	0,20400	77,35	0,000
Error	16	0,04220	0,04220	0,00264		
Total	23	1,22266				

S = 0,0513543 R-Sq = 96,55% R-Sq(adj) = 95,04%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	N	Mean	Grouping
3,8	12	2,7	A
7,4	12	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Glycerol	N	Mean	Grouping
0	12	2,8	A
1	12	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Heat	N	Mean	Grouping
1	12	2,7	A
0	12	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Glycerol	N	Mean	Grouping
3,8	0	6	2,9	A
7,4	1	6	2,7	B
7,4	0	6	2,6	B
3,8	1	6	2,5	C

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Heat	N	Mean	Grouping
3,8	1	6	2,8	A
7,4	1	6	2,7	A B
3,8	0	6	2,7	B
7,4	0	6	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Glycerol	Heat	N	Mean	Grouping
0	1	6	2,9	A
1	0	6	2,6	B
0	0	6	2,6	B
1	1	6	2,5	C

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

pH	Glycerol	Heat	N	Mean	Grouping
3,8	0	1	3	3,2	A
7,4	1	1	3	2,7	B
3,8	0	0	3	2,7	B
7,4	0	1	3	2,7	B
7,4	1	0	3	2,6	B
3,8	1	0	3	2,6	B
7,4	0	0	3	2,6	B
3,8	1	1	3	2,3	C

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	197,11	197,11	197,11	4274,59	0,000
Glycerol	1	1253,10	1253,10	1253,10	27174,93	0,000
Heat	1	4,90	4,90	4,90	106,18	0,000
pH*Glycerol	1	21,13	21,13	21,13	458,25	0,000
pH*Heat	1	1,14	1,14	1,14	24,62	0,000
Glycerol*Heat	1	16,57	16,57	16,57	359,27	0,000
pH*Glycerol*Heat	1	0,96	0,96	0,96	20,82	0,000
Error	16	0,74	0,74	0,05		
Total	23	1495,64				

S = 0,214738   R-Sq = 99,95%   R-Sq(adj) = 99,93%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	N	Mean	Grouping
7,4	12	41,804	A
3,8	12	36,072	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Glycerol	N	Mean	Grouping
1	12	46,164	A
0	12	31,712	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Heat	N	Mean	Grouping
1	12	39,390	A
0	12	38,487	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Glycerol	N	Mean	Grouping
7,4	1	6	48,092	A
3,8	1	6	44,237	B
7,4	0	6	35,517	C
3,8	0	6	27,908	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Heat	N	Mean	Grouping
7,4	1	6	42,038	A
7,4	0	6	41,570	B
3,8	1	6	36,742	C
3,8	0	6	35,403	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Glycerol	Heat	N	Mean	Grouping
1	1	6	47,447	A
1	0	6	44,882	B
0	0	6	32,092	C
0	1	6	31,333	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

pH	Glycerol	Heat	N	Mean	Grouping
7,4	1	1	3	48,957	A
7,4	1	0	3	47,227	B
3,8	1	1	3	45,937	C
3,8	1	0	3	42,537	D
7,4	0	0	3	35,913	E
7,4	0	1	3	35,120	F
3,8	0	0	3	28,270	G
3,8	0	1	3	27,547	H

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	12,557	12,557	12,557	19074,03	0,000
Glycerol	1	100,942	100,942	100,942	153329,65	0,000
Heat	1	0,411	0,411	0,411	624,03	0,000
pH*Glycerol	1	14,789	14,789	14,789	22464,91	0,000
pH*Heat	1	1,162	1,162	1,162	1764,46	0,000
Glycerol*Heat	1	0,742	0,742	0,742	1127,11	0,000
pH*Glycerol*Heat	1	0,437	0,437	0,437	664,41	0,000

```
Error             16    0,011    0,011    0,001
Total             23  131,051

S = 0,0256580   R-Sq = 99,99%   R-Sq(adj) = 99,99%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH    N    Mean  Grouping
7,4  12  42,126  A
3,8  12  40,679    B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Glycerol   N    Mean  Grouping
1         12  43,453  A
0         12  39,352    B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Heat   N    Mean  Grouping
1     12  41,533  A
0     12  41,272    B


Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH   Glycerol  N    Mean  Grouping
3,8  1         6  43,515  A
7,4  1         6  43,392    B
7,4  0         6  40,860      C
3,8  0         6  37,843        D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH   Heat  N    Mean  Grouping
7,4  0     6  42,215  A
7,4  1     6  42,037    B
3,8  1     6  41,030      C
3,8  0     6  40,328        D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Glycerol  Heat  N    Mean  Grouping
1         1     6  43,760  A
1         0     6  43,147    B
0         0     6  39,397      C
0         1     6  39,307        D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

pH   Glycerol  Heat  N    Mean  Grouping
3,8  1         1     3  44,177  A
7,4  1         0     3  43,440    B
7,4  1         1     3  43,343      C
3,8  1         0     3  42,853        D
7,4  0         0     3  40,990          E
7,4  0         1     3  40,730            F
3,8  0         1     3  37,883              G
3,8  0         0     3  37,803                H

Means that do not share a letter are significantly different.
```

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	577,51	577,51	577,51	13734,00	0,000
Glycerol	1	3771,78	3771,78	3771,78	89697,54	0,000
Heat	1	11,08	11,08	11,08	263,59	0,000
pH*Glycerol	1	69,94	69,94	69,94	1663,24	0,000
pH*Heat	1	3,00	3,00	3,00	71,42	0,000
Glycerol*Heat	1	40,38	40,38	40,38	960,24	0,000
pH*Glycerol*Heat	1	2,85	2,85	2,85	67,77	0,000
Error	16	0,67	0,67	0,04		
Total	23	4477,22				

S = 0,205061 R-Sq = 99,98% R-Sq(adj) = 99,98%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	N	Mean	Grouping
7,4	12	72,046	A
3,8	12	62,235	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Glycerol	N	Mean	Grouping
1	12	79,677	A
0	12	54,604	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Heat	N	Mean	Grouping
1	12	67,820	A
0	12	66,461	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Glycerol	N	Mean	Grouping
7,4	1	6	82,875	A
3,8	1	6	76,478	B
7,4	0	6	61,217	C
3,8	0	6	47,992	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Heat	N	Mean	Grouping
7,4	1	6	72,372	A
7,4	0	6	71,720	B
3,8	1	6	63,268	C
3,8	0	6	61,202	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Glycerol	Heat	N	Mean	Grouping
1	1	6	81,653	A
1	0	6	77,700	B
0	0	6	55,222	C
0	1	6	53,987	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

pH	Glycerol	Heat	N	Mean	Grouping
7,4	1	1	3	84,153	A
7,4	1	0	3	81,597	B
3,8	1	1	3	79,153	C
3,8	1	0	3	73,803	D
7,4	0	0	3	61,843	E
7,4	0	1	3	60,590	F
3,8	0	0	3	48,600	G
3,8	0	1	3	47,383	H

Means that do not share a letter are significantly different.

Analysis of Variance for Efficiency, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,0046983	0,0046983	0,0046983	46,52	0,000
Glycerol	1	0,0046034	0,0046034	0,0046034	45,58	0,000
Heat	1	0,0027603	0,0027603	0,0027603	27,33	0,000
pH*Glycerol	1	0,0164839	0,0164839	0,0164839	163,22	0,000
pH*Heat	1	0,0021415	0,0021415	0,0021415	21,20	0,000
Glycerol*Heat	1	0,0142428	0,0142428	0,0142428	141,03	0,000
pH*Glycerol*Heat	1	0,0109683	0,0109683	0,0109683	108,60	0,000
Error	16	0,0016159	0,0016159	0,0001010		
Total	23	0,0575142				

S = 0,0100496 R-Sq = 97,19% R-Sq(adj) = 95,96%

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	N	Mean	Grouping
3,8	12	0,680	A
7,4	12	0,652	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Glycerol	N	Mean	Grouping
1	12	0,680	A
0	12	0,653	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Heat	N	Mean	Grouping
1	12	0,677	A
0	12	0,656	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Glycerol	N	Mean	Grouping
3,8	1	6	0,720	A
7,4	0	6	0,665	B
3,8	0	6	0,640	C
7,4	1	6	0,640	C

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Heat	N	Mean	Grouping
3,8	1	6	0,701	A
3,8	0	6	0,660	B
7,4	1	6	0,654	B
7,4	0	6	0,651	B

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Glycerol	Heat	N	Mean	Grouping
1	1	6	0,715	A
0	0	6	0,666	B
1	0	6	0,645	C
0	1	6	0,639	C

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

pH	Glycerol	Heat	N	Mean	Grouping
3,8	1	1	3	0,786	A
7,4	0	0	3	0,666	B
3,8	0	0	3	0,666	B
7,4	0	1	3	0,663	B C
3,8	1	0	3	0,655	B C
7,4	1	1	3	0,644	B C
7,4	1	0	3	0,636	C D
3,8	0	1	3	0,615	D

Means that do not share a letter are significantly different.

Analysis of Variance for E.coli, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,53889	0,53889	0,53889	253,47	0,000
Glycerol	1	0,01740	0,01740	0,01740	8,19	0,021
Heat	1	0,00165	0,00165	0,00165	0,78	0,404
pH*Glycerol	1	0,21215	0,21215	0,21215	99,79	0,000
pH*Heat	1	0,00770	0,00770	0,00770	3,62	0,093
Glycerol*Heat	1	0,00486	0,00486	0,00486	2,29	0,169
pH*Glycerol*Heat	1	0,05250	0,05250	0,05250	24,70	0,001
Error	8	0,01701	0,01701	0,00213		
Total	15	0,85217				

S = 0,0461088 R-Sq = 98,00% R-Sq(adj) = 96,26%

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	N	Mean	Grouping
7,4	8	2,1	A
3,8	8	1,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Glycerol	N	Mean	Grouping
0	8	1,9	A
1	8	1,9	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Heat	N	Mean	Grouping
0	8	1,9	A
1	8	1,9	A

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Glycerol	N	Mean	Grouping
7,4	1	4	2,2	A
7,4	0	4	2,0	B
3,8	0	4	1,9	C
3,8	1	4	1,6	D

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Heat	N	Mean	Grouping
7,4	0	4	2,1	A
7,4	1	4	2,1	A
3,8	1	4	1,7	B
3,8	0	4	1,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Glycerol	Heat	N	Mean	Grouping
0	1	4	2,0	A
0	0	4	1,9	A
1	0	4	1,9	A
1	1	4	1,9	A

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

pH	Glycerol	Heat	N	Mean	Grouping
7,4	1	0	2	2,3	A
7,4	1	1	2	2,1	B
7,4	0	1	2	2,1	B
7,4	0	0	2	2,0	B C
3,8	0	0	2	1,9	B C
3,8	0	1	2	1,8	C
3,8	1	1	2	1,6	D
3,8	1	0	2	1,5	D

Means that do not share a letter are significantly different.

Analysis of Variance for S.aureus, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0,095697	0,095697	0,095697	205,97	0,000
Glycerol	1	0,000122	0,000122	0,000122	0,26	0,622
Heat	1	0,012531	0,012531	0,012531	26,97	0,001
pH*Glycerol	1	0,005733	0,005733	0,005733	12,34	0,008
pH*Heat	1	0,004525	0,004525	0,004525	9,74	0,014
Glycerol*Heat	1	0,000065	0,000065	0,000065	0,14	0,717
pH*Glycerol*Heat	1	0,045407	0,045407	0,045407	97,73	0,000
Error	8	0,003717	0,003717	0,000465		
Total	15	0,167797				

S = 0,0215547   R-Sq = 97,78%   R-Sq(adj) = 95,85%

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	N	Mean	Grouping
7,4	8	2,7	A
3,8	8	2,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Glycerol	N	Mean	Grouping
1	8	2,6	A
0	8	2,6	A

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Heat	N	Mean	Grouping
1	8	2,7	A
0	8	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Glycerol	N	Mean	Grouping
7,4	0	4	2,7	A
7,4	1	4	2,7	A
3,8	1	4	2,6	B
3,8	0	4	2,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Heat	N	Mean	Grouping
7,4	1	4	2,7	A
7,4	0	4	2,7	A
3,8	1	4	2,6	B
3,8	0	4	2,5	C

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Glycerol	Heat	N	Mean	Grouping
1	1	4	2,7	A
0	1	4	2,6	A
1	0	4	2,6	B
0	0	4	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

pH	Glycerol	Heat	N	Mean	Grouping
7,4	0	1	2	2,8	A
7,4	1	0	2	2,7	A B
3,8	1	1	2	2,7	B
7,4	0	0	2	2,7	B
7,4	1	1	2	2,6	B
3,8	0	0	2	2,5	C
3,8	0	1	2	2,5	C
3,8	1	0	2	2,5	C

Means that do not share a letter are significantly different.

Table D.9 Analysis of Variance for emulsions produced by ultrasonication with the emulsifier **SMP**. Effect of pH, glycerol addition, and heating before homogenization on particle size, turbidity, color-L*, a*, b* values, efficiency, *E.coli* and *S.aureus* population decrease, using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	5895,0	5895,0	1179,0	104,90	0,000
Error	12	134,9	134,9	11,2		
Total	17	6029,9				

S = 3,35253 R-Sq = 97,76% R-Sq(adj) = 96,83%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 7.4 - heat	3	84,7	A
pH 7.4	3	64,9	B
pH 3.8- glycerol	3	62,6	B
pH 7.4- glycerol	3	39,9	C
pH 7.4- glycerol- heat	3	37,1	C
pH 3.8- glycerol- heat	3	35,4	C

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	6,1056	6,1056	1,2211	72007,49	0,000
Error	12	0,0002	0,0002	0,0000		
Total	17	6,1058				

S = 0,00411805 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 3.8- glycerol	3	1,8	A
pH 7.4	3	1,8	B
pH 7.4 - heat	3	1,1	C
pH 7.4- glycerol	3	0,6	D
pH 7.4- glycerol- heat	3	0,5	E
pH 3.8- glycerol- heat	3	0,3	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	9410,1	9410,1	1882,0	2117283,16	0,000
Error	12	0,0	0,0	0,0		
Total	17	9410,2				

S = 0,0298142 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	3	72,9	A
pH 3.8- glycerol	3	69,2	B
pH 7.4- glycerol- heat	3	44,6	C
pH 7.4- glycerol	3	43,5	D
pH 7.4	3	16,4	E
pH 7.4 - heat	3	13,9	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	1269,73	1269,73	253,95	408128,37	0,000
Error	12	0,01	0,01	0,00		
Total	17	1269,74				

S = 0,0249444 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	3	52,8	A
pH 7.4- glycerol	3	52,1	B
pH 3.8- glycerol	3	41,7	C
pH 3.8- glycerol- heat	3	39,9	D
pH 7.4	3	33,1	E
pH 7.4 - heat	3	31,0	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	24678,1	24678,1	4935,6	1147819,42	0,000
Error	12	0,1	0,1	0,0		
Total	17	24678,2				

S = 0,0655744 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	3	117,6	A
pH 3.8- glycerol	3	115,3	B
pH 7.4- glycerol- heat	3	76,9	C
pH 7.4- glycerol	3	75,0	D
pH 7.4	3	28,1	E
pH 7.4 - heat	3	23,9	F

Means that do not share a letter are significantly different.

Analysis of Variance for Efficiency, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	277,486	277,486	55,497	20,21	0,000
Error	12	32,948	32,948	2,746		
Total	17	310,434				

S = 1,65701   R-Sq = 89,39%   R-Sq(adj) = 84,96%

Grouping Information Using Tukey Method and 95,0% Confidence for Efficiency

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	3	70,2	A
pH 3.8- glycerol	3	63,0	B
pH 7.4- glycerol- heat	3	61,8	B C
pH 7.4 - heat	3	59,8	B C
pH 7.4- glycerol	3	59,3	B C
pH 7.4	3	58,5	C

Means that do not share a letter are significantly different.

Analysis of Variance for E.coli, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment_1	5	3,03634	3,03634	0,60727	61,32	0,000
Error	6	0,05942	0,05942	0,00990		
Total	11	3,09576				

S = 0,0995152   R-Sq = 98,08%   R-Sq(adj) = 96,48%

Grouping Information Using Tukey Method and 95,0% Confidence for E.coli

Treatment	N	Mean	Grouping
pH 7.4- glycerol	2	2,7	A
pH 7.4- glycerol- heat	2	2,6	A
pH 7.4	2	2,5	A B
pH 7.4 - heat	2	2,5	A B
pH 3.8- glycerol- heat	2	2,2	B
pH 3.8- glycerol	2	1,2	C

Means that do not share a letter are significantly different.

Analysis of Variance for S.aureus, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment_1	5	0,10625	0,10625	0,02125	1,45	0,328
Error	6	0,08763	0,08763	0,01461		
Total	11	0,19388				

S = 0,120854   R-Sq = 54,80%   R-Sq(adj) = 17,13%

Grouping Information Using Tukey Method and 95,0% Confidence for S.aureus

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	2	2,9	A
pH 3.8- glycerol	2	2,8	A
pH 7.4 - heat	2	2,8	A
pH 7.4- glycerol- heat	2	2,7	A
pH 7.4- glycerol	2	2,7	A
pH 7.4	2	2,6	A

Means that do not share a letter are significantly different.

Table D.10 Analysis of Variance for emulsions produced by ultrasonication with the emulsifier **Tween 80** stored for 0 or 28 days. Effect of pH, glycerol addition, heating before homogenization and storage time on particle size, turbidity, color-L*, a*, b* values using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	4373,5	4373,5	1457,8	5716,96	0,000
Time	1	1235,5	1235,5	1235,5	4845,24	0,000
Treatment*Time	3	4706,9	4706,9	1569,0	6152,87	0,000
Error	16	4,1	4,1	0,3		
Total	23	10320,0				

S = 0,504975 R-Sq = 99,96% R-Sq(adj) = 99,94%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	76,2	A
pH 7.4- glycerol	6	56,0	B
pH 7.4	6	53,9	C
pH 3.8	6	38,3	D

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Time	N	Mean	Grouping
28	12	63,3	A
0	12	48,9	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	28	3	91,9	A
pH 7.4- glycerol	28	3	80,5	B
pH 7.4	0	3	65,8	C
pH 3.8- glycerol	0	3	60,6	D
pH 7.4	28	3	42,0	E
pH 3.8	28	3	38,6	F
pH 3.8	0	3	37,9	F
pH 7.4- glycerol	0	3	31,4	G

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	1,6676	1,6676	0,5559	86,22	0,000
Time	1	0,0639	0,0639	0,0639	9,90	0,006
Treatment*Time	3	6,0953	6,0953	2,0318	315,13	0,000
Error	16	0,1032	0,1032	0,0064		
Total	23	7,9300				

S = 0,0802958 R-Sq = 98,70% R-Sq(adj) = 98,13%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	1,9	A
pH 7.4	6	1,7	B
pH 3.8	6	1,5	C
pH 7.4- glycerol	6	1,2	D

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Time	N	Mean	Grouping
0	12	1,6	A
28	12	1,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	Time	N	Mean	Grouping
pH 7.4	0	3	2,4	A
pH 3.8- glycerol	28	3	2,3	A
pH 3.8	0	3	1,9	B
pH 7.4- glycerol	28	3	1,6	C
pH 3.8- glycerol	0	3	1,4	C
pH 3.8	28	3	1,1	D
pH 7.4	28	3	1,0	D E
pH 7.4- glycerol	0	3	0,8	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	572,702	572,702	190,901	1215,35	0,000
Time	1	77,042	77,042	77,042	490,48	0,000
Treatment*Time	3	42,471	42,471	14,157	90,13	0,000
Error	16	2,513	2,513	0,157		
Total	23	694,728				

S = 0,396327 R-Sq = 99,64% R-Sq(adj) = 99,48%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 3.8	6	72,6	A
pH 7.4	6	70,4	B
pH 7.4- glycerol	6	67,4	C
pH 3.8- glycerol	6	59,7	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Time	N	Mean	Grouping
0	12	69,3	A
28	12	65,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	Time	N	Mean	Grouping
pH 3.8	28	3	72,9	A
pH 3.8	0	3	72,3	A
pH 7.4	0	3	72,0	A
pH 7.4- glycerol	0	3	70,6	B
pH 7.4	28	3	68,7	C
pH 7.4- glycerol	28	3	64,3	D
pH 3.8- glycerol	0	3	62,4	E
pH 3.8- glycerol	28	3	57,0	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	37,3851	37,3851	12,4617	94,62	0,000
Time	1	0,7526	0,7526	0,7526	5,71	0,029
Treatment*Time	3	17,3144	17,3144	5,7715	43,82	0,000
Error	16	2,1072	2,1072	0,1317		
Total	23	57,5593				

S = 0,362905 R-Sq = 96,34% R-Sq(adj) = 94,74%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	42,7	A
pH 7.4- glycerol	6	40,7	B
pH 7.4	6	40,7	B
pH 3.8	6	39,2	C

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Time	N	Mean	Grouping
0	12	41,0	A
28	12	40,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	0	3	43,0	A
pH 3.8- glycerol	28	3	42,4	A B
pH 7.4- glycerol	28	3	41,9	B C
pH 7.4	0	3	41,1	C D
pH 3.8	0	3	40,4	D E
pH 7.4	28	3	40,4	D E
pH 7.4- glycerol	0	3	39,6	E
pH 3.8	28	3	38,0	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	755,33	755,33	251,78	770,77	0,000
Time	1	177,89	177,89	177,89	544,57	0,000
Treatment*Time	3	22,51	22,51	7,50	22,97	0,000
Error	16	5,23	5,23	0,33		
Total	23	960,95				

S = 0,571540   R-Sq = 99,46%   R-Sq(adj) = 99,22%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 7.4	6	115,7	A
pH 3.8	6	115,4	A
pH 7.4- glycerol	6	110,8	B
pH 3.8- glycerol	6	101,8	C

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Time	N	Mean	Grouping
0	12	113,6	A
28	12	108,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	Time	N	Mean	Grouping
pH 7.4	0	3	117,7	A
pH 3.8	0	3	116,9	A
pH 7.4- glycerol	0	3	114,1	B
pH 3.8	28	3	113,9	B
pH 7.4	28	3	113,6	B
pH 7.4- glycerol	28	3	107,4	C
pH 3.8- glycerol	0	3	105,7	D
pH 3.8- glycerol	28	3	97,9	E

Means that do not share a letter are significantly different.

Table D.11 Analysis of Variance for emulsions produced by ultrasonication with the emulsifier **lecithin** stored for 0 or 28 days. Effect of pH, glycerol addition, heating before homogenization and storage time on particle size, turbidity, color-L*, a*, b* values using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	0,26759	0,26759	0,08920	45,62	0,000
Time	1	0,34994	0,34994	0,34994	178,99	0,000
Treatment*Time	3	0,39108	0,39108	0,13036	66,68	0,000
Error	16	0,03128	0,03128	0,00196		
Total	23	1,03989				

S = 0,0442162 R-Sq = 96,99% R-Sq(adj) = 95,68%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	2,4	A
pH 7.4- glycerol- heat	6	2,4	A
pH 3.8- glycerol- heat	6	2,2	B
pH 7.4- glycerol	6	2,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Time	N	Mean	Grouping
28	12	2,4	A
0	12	2,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	28	3	2,7	A
pH 3.8- glycerol	28	3	2,5	B
pH 3.8- glycerol	0	3	2,3	C
pH 3.8- glycerol- heat	28	3	2,2	C D
pH 3.8- glycerol- heat	0	3	2,2	C D
pH 7.4- glycerol	28	3	2,2	C D
pH 7.4- glycerol	0	3	2,1	D E
pH 7.4- glycerol- heat	0	3	2,0	E

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	0,319700	0,319700	0,106567	39,86	0,000
Time	1	0,275418	0,275418	0,275418	103,03	0,000
Treatment*Time	3	0,038189	0,038189	0,012730	4,76	0,015
Error	16	0,042771	0,042771	0,002673		
Total	23	0,676078				

S = 0,0517029 R-Sq = 93,67% R-Sq(adj) = 90,91%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 7.4- glycerol	6	2,8	A
pH 7.4- glycerol- heat	6	2,8	A
pH 3.8- glycerol	6	2,7	A
pH 3.8- glycerol- heat	6	2,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Time	N	Mean	Grouping
28	12	2,8	A
0	12	2,6	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	28	3	2,9	A
pH 7.4- glycerol- heat	28	3	2,8	A B
pH 3.8- glycerol	28	3	2,8	A B C
pH 7.4- glycerol- heat	0	3	2,7	B C D
pH 3.8- glycerol- heat	28	3	2,6	C D
pH 7.4- glycerol	0	3	2,6	C D
pH 3.8- glycerol	0	3	2,6	D
pH 3.8- glycerol- heat	0	3	2,3	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	4042,4	4042,4	1347,5	19826,86	0,000
Time	1	2633,6	2633,6	2633,6	38751,14	0,000
Treatment*Time	3	2936,6	2936,6	978,9	14402,96	0,000
Error	16	1,1	1,1	0,1		
Total	23	9613,7				

S = 0,260696 R-Sq = 99,99% R-Sq(adj) = 99,98%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	48,9	A
pH 7.4- glycerol	6	48,4	B
pH 3.8- glycerol- heat	6	23,5	C
pH 3.8- glycerol	6	21,9	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Time	N	Mean	Grouping
0	12	46,2	A
28	12	25,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	28	3	49,5	A
pH 7.4- glycerol- heat	0	3	49,0	A
pH 7.4- glycerol- heat	28	3	48,9	A
pH 7.4- glycerol	0	3	47,2	B
pH 3.8- glycerol- heat	0	3	45,9	C
pH 3.8- glycerol	0	3	42,5	D

pH 3.8- glycerol	28	3	1,3	E
pH 3.8- glycerol- heat	28	3	1,2	E

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	1940,23	1940,23	646,74	40495,21	0,000
Time	1	2330,71	2330,71	2330,71	145935,25	0,000
Treatment*Time	3	1968,44	1968,44	656,15	41084,07	0,000
Error	16	0,26	0,26	0,02		
Total	23	6239,63				

S = 0,126376 R-Sq = 100,00% R-Sq(adj) = 99,99%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	42,6	A
pH 7.4- glycerol	6	42,5	A
pH 3.8- glycerol- heat	6	24,9	B
pH 3.8- glycerol	6	24,4	C

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Time	N	Mean	Grouping
0	12	43,5	A
28	12	23,7	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol- heat	0	3	44,2	A
pH 7.4- glycerol	0	3	43,4	B
pH 7.4- glycerol- heat	0	3	43,3	B
pH 3.8- glycerol	0	3	42,9	C
pH 7.4- glycerol- heat	28	3	41,9	D
pH 7.4- glycerol	28	3	41,6	D
pH 3.8- glycerol	28	3	5,9	E
pH 3.8- glycerol- heat	28	3	5,5	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	11821,9	11821,9	3940,6	63776,94	0,000
Time	1	8057,9	8057,9	8057,9	130412,61	0,000
Treatment*Time	3	8670,6	8670,6	2890,2	46776,26	0,000
Error	16	1,0	1,0	0,1		
Total	23	28551,3				

S = 0,248571 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	84,1	A
pH 7.4- glycerol	6	83,0	B
pH 3.8- glycerol- heat	6	40,5	C
pH 3.8- glycerol	6	37,9	D

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Time	N	Mean	Grouping
0	12	79,7	A
28	12	43,0	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol	28	3	84,4	A
pH 7.4- glycerol- heat	0	3	84,2	A
pH 7.4- glycerol- heat	28	3	84,0	A
pH 7.4- glycerol	0	3	81,6	B
pH 3.8- glycerol- heat	0	3	79,2	C
pH 3.8- glycerol	0	3	73,8	D
pH 3.8- glycerol	28	3	2,0	E
pH 3.8- glycerol- heat	28	3	1,8	E

Means that do not share a letter are significantly different.

Table D.12 Analysis of Variance for emulsions produced by ultrasonication with the emulsifier **SMP** stored for 0 or 28 days. Effect of pH, glycerol addition, heating before homogenization and storage time on particle size, turbidity, color-L*, a*, b* values using Adjusted SS for Tests.

Analysis of Variance for D[3,2], using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	11361,8	11361,8	2272,4	270,70	0,000
Time	1	396,0	396,0	396,0	47,18	0,000
Treatment*Time	5	1900,2	1900,2	380,0	45,27	0,000
Error	24	201,5	201,5	8,4		
Total	35	13859,4				

S = 2,89732 R-Sq = 98,55% R-Sq(adj) = 97,88%

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	N	Mean	Grouping
pH 3.8- glycerol	6	80,2	A
pH 7.4 - heat	6	78,4	A
pH 7.4	6	64,9	B
pH 7.4- glycerol	6	41,5	C
pH 3.8- glycerol- heat	6	40,9	C
pH 7.4- glycerol- heat	6	38,6	C

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Time	N	Mean	Grouping
28	18	60,7	A
0	18	54,1	B

Grouping Information Using Tukey Method and 95,0% Confidence for D[3,2]

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol	28	3	97,7	A
pH 7.4 - heat	0	3	84,7	B
pH 7.4 - heat	28	3	72,2	C
pH 7.4	0	3	64,9	C D
pH 7.4	28	3	64,9	C D
pH 3.8- glycerol	0	3	62,6	D
pH 3.8- glycerol- heat	28	3	46,5	E
pH 7.4- glycerol	28	3	43,1	E F
pH 7.4- glycerol- heat	28	3	40,1	E F
pH 7.4- glycerol	0	3	39,9	E F
pH 7.4- glycerol- heat	0	3	37,1	F
pH 3.8- glycerol- heat	0	3	35,4	F

Means that do not share a letter are significantly different.

Analysis of Variance for Turbidity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	15,0250	15,0250	3,0050	472,09	0,000
Time	1	2,1714	2,1714	2,1714	341,14	0,000
Treatment*Time	5	1,0060	1,0060	0,2012	31,61	0,000

Error	24	0,1528	0,1528	0,0064
Total	35	18,3553		

S = 0,0797826 R-Sq = 99,17% R-Sq(adj) = 98,79%

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	N	Mean	Grouping
pH 7.4	6	2,2	A
pH 3.8- glycerol	6	2,1	A
pH 7.4 - heat	6	1,2	B
pH 3.8- glycerol- heat	6	0,8	C
pH 7.4- glycerol	6	0,6	D
pH 7.4- glycerol- heat	6	0,6	D

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Time	N	Mean	Grouping
28	18	1,5	A
0	18	1,0	B

Grouping Information Using Tukey Method and 95,0% Confidence for Turbidity

Treatment	Time	N	Mean	Grouping
pH 7.4	28	3	2,6	A
pH 3.8- glycerol	28	3	2,4	A
pH 3.8- glycerol	0	3	1,8	B
pH 7.4	0	3	1,8	B
pH 7.4 - heat	28	3	1,4	C
pH 3.8- glycerol- heat	28	3	1,3	C
pH 7.4 - heat	0	3	1,1	D
pH 7.4- glycerol	28	3	0,7	E
pH 7.4- glycerol- heat	28	3	0,7	E
pH 7.4- glycerol	0	3	0,6	E
pH 7.4- glycerol- heat	0	3	0,5	E F
pH 3.8- glycerol- heat	0	3	0,3	F

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	19397,4	19397,4	3879,5	236714,10	0,000
Time	1	0,5	0,5	0,5	32,22	0,000
Treatment*Time	5	6,9	6,9	1,4	84,71	0,000
Error	24	0,4	0,4	0,0		
Total	35	19405,3				

S = 0,128019 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	6	73,6	A
pH 3.8- glycerol	6	69,3	B
pH 7.4- glycerol- heat	6	44,5	C
pH 7.4- glycerol	6	43,0	D
pH 7.4	6	15,9	E
pH 7.4 - heat	6	13,5	F

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Time	N	Mean	Grouping
0	18	43,4	A
28	18	43,2	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-L

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol- heat	28	3	74,4	A
pH 3.8- glycerol- heat	0	3	72,9	B
pH 3.8- glycerol	28	3	69,3	C
pH 3.8- glycerol	0	3	69,2	C
pH 7.4- glycerol- heat	0	3	44,6	D
pH 7.4- glycerol- heat	28	3	44,3	D
pH 7.4- glycerol	0	3	43,5	E
pH 7.4- glycerol	28	3	42,4	F
pH 7.4	0	3	16,4	G
pH 7.4	28	3	15,5	H
pH 7.4 - heat	0	3	13,9	I
pH 7.4 - heat	28	3	13,1	J

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-a, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	2591,68	2591,68	518,34	116844,62	0,000
Time	1	0,43	0,43	0,43	96,71	0,000
Treatment*Time	5	0,60	0,60	0,12	27,07	0,000
Error	24	0,11	0,11	0,00		
Total	35	2592,81				

S = 0,0666041 R-Sq = 100,00% R-Sq(adj) = 99,99%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	N	Mean	Grouping
pH 7.4- glycerol- heat	6	52,9	A
pH 7.4- glycerol	6	52,0	B
pH 3.8- glycerol	6	41,8	C
pH 3.8- glycerol- heat	6	39,6	D
pH 7.4	6	32,9	E
pH 7.4 - heat	6	30,7	F

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Time	N	Mean	Grouping
0	18	41,8	A
28	18	41,5	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-a

Treatment	Time	N	Mean	Grouping
pH 7.4- glycerol- heat	28	3	53,0	A
pH 7.4- glycerol- heat	0	3	52,8	A
pH 7.4- glycerol	0	3	52,1	B
pH 7.4- glycerol	28	3	51,9	C
pH 3.8- glycerol	28	3	41,8	D
pH 3.8- glycerol	0	3	41,7	D
pH 3.8- glycerol- heat	0	3	39,9	E

pH 3.8- glycerol- heat	28	3	39,4			F			
pH 7.4	0	3	33,1				G		
pH 7.4	28	3	32,7					H	
pH 7.4 - heat	0	3	31,0						I
pH 7.4 - heat	28	3	30,5							J

Means that do not share a letter are significantly different.

Analysis of Variance for COLOR-b, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	49898,7	49898,7	9979,7	487279,18	0,000
Time	1	10,1	10,1	10,1	493,24	0,000
Treatment*Time	5	3,3	3,3	0,7	32,31	0,000
Error	24	0,5	0,5	0,0		
Total	35	49912,6				

S = 0,143110 R-Sq = 100,00% R-Sq(adj) = 100,00%

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	N	Mean	Grouping
pH 3.8- glycerol- heat	6	117,5	A
pH 3.8- glycerol	6	115,0	B
pH 7.4- glycerol- heat	6	76,7	C
pH 7.4- glycerol	6	74,1	D
pH 7.4	6	27,4	E
pH 7.4 - heat	6	23,2	F

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Time	N	Mean	Grouping
0	18	72,8	A
28	18	71,8	B

Grouping Information Using Tukey Method and 95,0% Confidence for COLOR-b

Treatment	Time	N	Mean	Grouping
pH 3.8- glycerol- heat	0	3	117,6	A
pH 3.8- glycerol- heat	28	3	117,4	A
pH 3.8- glycerol	0	3	115,3	B
pH 3.8- glycerol	28	3	114,6	C
pH 7.4- glycerol- heat	0	3	76,9	D
pH 7.4- glycerol- heat	28	3	76,4	E
pH 7.4- glycerol	0	3	75,0	F
pH 7.4- glycerol	28	3	73,1	G
pH 7.4	0	3	28,1	H
pH 7.4	28	3	26,6	I
pH 7.4 - heat	0	3	23,9	J
pH 7.4 - heat	28	3	22,5	K

Means that do not share a letter are significantly different.