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Optimization of the fabrication, stability, and performance of food grade nanoemulsions with low and high energy methods

Item Type	Dissertation (Open Access)
Authors	Komaiko, Jennifer
DOI	10.7275/7909334.0
Download date	2025-07-04 00:11:45
Link to Item	https://hdl.handle.net/20.500.14394/19811

Optimization of the fabrication, stability, and performance
of food grade nanoemulsions with low and high energy
methods

A Dissertation Presented

By

JENNIFER KOMAIKO

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

DOCTOR OF PHILOSOPHY

February 2016

Department of Food Science

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my advisor and mentor, Dr. Julian McClements, who supported and guided me through my graduate studies. His knowledge and encouragement has helped shape me into the young scientist I am today. I have learned how to design experiments that have a purpose and hopefully made an impact on the area of low-energy nanoemulsion formation. I am appreciative for all of the discussions I entered his office in a panic and left feeling relief throughout the last three years. I would also like to thank my dissertation committee members: Dr. Eric Decker and Dr. Richard Ludescher. Dr. Decker has been supportive in both my research and extracurricular pursuits, and I am thankful for all of the assistance throughout my time at the University of Massachusetts. Dr. Ludescher has served as a mentor since I first met him and entered his lab in the spring of 2009. I can say confidently I do not think I would be here if it was not for Dr. Ludescher and thus I am so grateful for having entered his lab all those years ago.

I would also like to thank all of my past and current lab mates in the Biopolymers and Colloids Research Laboratory for their support, especially Cansu Gumus, Rebecca Walker, Bicheng Wu, Cynthia Lopez-Pena, Anna Joos, Inge Liberloo, Iris Joye, and Gabriel Davidov. It was an amazing experience to be able to meet so many people from around the world who helped make working on a Saturday night enjoyable. My special thanks go to Jean Alamed for her support and friendship. I am also thankful for all of the times I entered Jean's office and vented about my latest concern. Jean always helped make my day better and am so appreciative for all of our talks.

Lastly, I need to give thanks to my family who despite not always understanding my stress were always supportive of my endeavors. I have enjoyed being able to live in the same state as my older brother Ryan and for the many visits from my younger sister Heather. Having two incredibly intelligent and driven siblings has helped push me towards my success. Finally, to my mom and dad thank you for giving me the strength to chase my dreams. I do not know if more supportive parents exist but I am so happy to call you both mine.

ABSTRACT

OPTIMIZATION OF THE FABRICATION, STABILITY, AND PERFORMANCE OF
FOOD GRADE NANOEMULSIONS WITH LOW AND HIGH ENERGY METHODS

FEBRUARY 2016

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There is interest in the production of emulsions by low-energy methods because no expensive equipment is required thus making emulsion formation inexpensive and simple to implement. The goal of this research is to establish the major factors that affect emulsion formation using low-energy methods and possible applications of the emulsions and nanoemulsions formed by this method. Lastly, the use of natural emulsifiers with low- and high-energy methods was investigated.

Initially, formation of nanoemulsions using isothermal low energy methods was investigated with a model system (hexadecane, Brij 30). Preliminary experiments showed that nanoemulsions could only be formed when the surfactant was initially mixed in with the oil phase. The major factors that affected particle size included order of addition, surfactant concentration, and storage temperature, while addition rate and stirring speed had minimal effects. The optimal formulation conditions were determined to be at a surfactant-to-oil ratio (SOR) of 0.375, an addition time of 5 minutes, and a stir speed of 700 rpm for both spontaneous emulsification and emulsion phase inversion methods. Additionally, emulsions could be stored for up to a month at temperatures less than 25°C

without showing any instability. Experiments were then carried out to establish which factors affect nanoemulsion formation when using food grade ingredients and the spontaneous emulsification method. Droplet size decreased with increasing SOR and was smallest when the non-ionic surfactant Tween 80 was utilized. In order for spontaneous emulsification to occur, the surfactant had to be initially dissolved in the organic phase rather than the aqueous phase. Oil composition affected particle size with medium chain triglycerides (MCT) forming the smallest droplets followed by flavor oils and then long chain triglycerides forming the largest droplets. However, no physiochemical correlation could be made between oil characteristics and particle size. The results obtained using spontaneous emulsification were then compared to those obtained using emulsion phase inversion and similarities were found, implying a common underlying mechanism for the two methods.

Next, the formation of nanoemulsions using the spontaneous emulsification method was demonstrated in a model food system: a gelatin-based dessert. The influence of preparation and storage conditions on nanoemulsion formation and stability were investigated. Droplet size decreased with increasing preparation temperature. Translucent filled hydrogels could be formed by incorporating nanoemulsions into the gelatin system. Optical and rheological properties remained unchanged with emulsion incorporation into a model gelatin gel and commercial gelatin dessert. The use of spontaneous emulsification to produce nanoemulsions may be helpful in the production of functional food gels.

Finally, sunflower phospholipids were investigated as an emulsifier using spontaneous emulsification. Initial particle diameter was influenced by phospholipid

composition, phospholipid concentration, initial phospholipid location, and storage time. Relatively large emulsion droplets ($d > 10 \mu\text{m}$) could be formed which means it is possible to form emulsions using natural emulsifiers when fine droplets are not essential. However, often fine droplets are more desirable so the use of sunflower phospholipids with the high energy method of microfluidization was also investigated to see if an ω -3 fatty acid nanoemulsion delivery system could be formed. Relatively small droplets ($d < 150 \text{ nm}$) could be formed by optimizing the phospholipid type and concentration. These results suggest that sunflower phospholipids are a viable emulsifier choice to form nanoemulsions and have added benefits due to their low allergenicity and non-genetically modified sources.

Keywords: Emulsions; Nanoemulsions; Low-energy Methods; Spontaneous Emulsification; Emulsion Phase Inversion; Hydrogels; Phospholipids

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CHAPTER 1

INTRODUCTION

Emulsion-based delivery systems are important for the incorporation of lipophilic components, such as oils, flavors, colors, vitamins, or nutraceuticals, into aqueous based food products. Nanoemulsions are defined as emulsions that have a diameter between 20-200 nm [1]. This small size leads to optical clarity [2], enhanced stability against gravitational separation [3], and high bioavailability of encapsulated components. Therefore, recently there has been increased interest in the production of nanoemulsions.

High- or low-energy methods can be utilized to produce nanoemulsions. Low-energy methods are of interest because there is no requirement for expensive equipment. Rather, the physiochemical properties of the surfactant-oil-water system are utilized to produce fine emulsion droplets at the oil-water interface. Low-energy methods can be broadly broken into isothermal or thermal methods, with isothermal methods relying on a change in composition and thermal methods relying on a change in temperature. The use of isothermal methods may bring greater cost savings because there is no requirement for a rapid temperature change.

The goal of this research was to better understand the factors that influence the low-energy production of nanoemulsions and explore potential applications of these nanoemulsions in food products. Initially, the factors affecting the two main isothermal low-energy methods, spontaneous emulsification and emulsion phase inversion, were studied in a model system. The influence of system composition and preparation method on the efficiency of nanoemulsion formation by spontaneous emulsification with food-grade ingredients was then examined. Next, the practical utility of nanoemulsions

produced by spontaneous emulsification was demonstrated by incorporating them into a filled hydrogel system. Lastly, the use of sunflower phospholipids using both low- and high-energy methods was investigated. Consumers are demanding clean labels and therefore there is a demand to find natural emulsifier choices that could be utilized to form delivery systems from a variety of methods.

CHAPTER 2

LITERATURE REVIEW: FORMATION OF FOOD-GRADE NANOEMULSIONS USING LOW-ENERGY PREPARATION METHODS, A REVIEW OF AVAILABLE METHODS

2.1. Abstract

There is considerable interest in the production of emulsions and nanoemulsions using low-energy methods due to the fact they are simple to implement and no expensive equipment is required. In this chapter, the principles of isothermal (spontaneous emulsification and emulsion phase inversion) and thermal (phase inversion temperature) low-energy methods for nanoemulsion production are presented. The major factors influencing nanoemulsion formation using low-energy methods and food grade components are reviewed: preparation conditions, oil type, surfactant type, surfactant-to-oil ratio, cosolvent, or cosurfactant addition. The advantages and disadvantages of different low-energy and high-energy methods for fabricating nanoemulsions are highlighted, and potential applications for these techniques are discussed.

2.2. Introduction

Emulsions are generally defined as two immiscible liquids with one of the liquids being dispersed as spherical droplets within the other [4]. The two most common liquids used to form emulsions in the food industry are oil and water. When the oil phase is dispersed in the water phase the system is called an oil-in-water emulsion, but when the water phase is dispersed in the oil phase it is called a water-in-oil emulsion. As most food

emulsions are predominantly aqueous based (such as beverages, milks, creams, dressings, sauces, soups, and dips) this review will mainly focus on the formation of oil-in-water emulsions. Emulsions are categorized based on their particle diameter and thermodynamic stability into conventional emulsions, nanoemulsions, or microemulsions (Table 1).

Table 1. Classification of emulsion type based on diameter and thermodynamic stability.

Emulsion Type	Diameter Range	Thermodynamic Stability
(Conventional) Emulsion	> 200 nm	Metastable
Nanoemulsion	< 200 nm	Metastable
Microemulsion	<100 nm	Stable

Both conventional emulsions and nanoemulsions are metastable systems meaning they have a tendency to breakdown over time due to a variety of destabilization mechanisms, such as gravitational separate, coalescence, flocculation, and Ostwald ripening [5]. The smaller size of the droplets in nanoemulsions typically gives them better stability to gravitational separation and droplet aggregation than conventional emulsions [6]. For instance, the rate of gravitational separation can be described by Stokes' Law which states the velocity that a droplet moves upward is related to gravity (g), particle radius (r), the difference in density of the continuous and dispersed phase ($\Delta\rho$) and shear viscosity of the continuous phase (η):

$$V_{\text{stokes}} = - \frac{2gr^2(\Delta\rho)}{9\eta} \quad (1.1)$$

Therefore, the smaller diameter of nanoemulsions corresponds to greater stability against gravitational separation [7]. In addition, the small size of the droplets in nanoemulsions means that Brownian motion effects may oppose gravitational forces,

which can also inhibit droplet movement [6]. Microemulsions share a similar small size to nanoemulsions thereby giving them good stability to gravitational separation and leading to systems that are optically clear or only slightly turbid due to weak light scattering [2], which is advantageous for incorporation into some food and beverage systems. In contrast to nanoemulsions however, microemulsions are thermodynamically stable [8-10]. Because the small droplet size of nanoemulsions can lead to good kinetic stability [11], there is often confusion about whether a nanoemulsion or microemulsion was formed. Practical ways to distinguish between the two include measurements of long term stability, the shape of the particle size distribution, and the morphology of the individual particles (**Table 2**) [8]. Additionally, nanoemulsions typically require less surfactant and are thus of interest for the food industry.

Table 2. Practical methods to distinguish between nanoemulsions and microemulsions. Adapted from [8].

Method	Microemulsion	Nanoemulsion
Particle size distribution	Single narrow peak	Single peak that may be narrow or broad
Particle shape analysis	Spherical or non-spherical due to ultralow interfacial tension	Spherical due to Laplace pressure
Stability analysis	Properties do not change over time	Properties may change over time

Preparation of all food grade emulsions requires oil, water, emulsifier, and energy input (mechanical or physiochemical). The free energy required (ΔG) to form a nanoemulsion is given by:

$$\Delta G = \Delta A\gamma - T\Delta S \quad (1.2)$$

Here, $\Delta A\gamma$ is the free energy needed to increase the oil-water interface (where A is the interfacial area and γ is the interfacial tension) and $T\Delta S$ is the free energy associated with increasing the number of possible arrangements of droplets in a nanoemulsion (where T is the temperature and S is the entropy) compared to the separated phases. In both emulsions and nanoemulsions, the change in entropy is not great enough to overcome the free energy required to expand the interface, and thus the process of emulsion or nanoemulsion formation requires some free energy input [11]. This free energy can be provided by mechanical devices or by the chemical potential of the system [1]. In high-energy methods, this free energy comes from mechanical forces applied to the system (such as shear, turbulence, or cavitation), although most of this energy is actually lost as heat due to friction. In low-energy methods, the majority of the free energy associated with emulsion formation comes from physiochemical processes rather than the application of mechanical forces.

Recently, there has been growing interest in producing nanoemulsions using low-energy means due to the fact that expensive specialized equipment (such as homogenizers) is not required [3, 12], and therefore there is a need to understand what the optimal conditions for low-energy production of nanoemulsions are. In particular, there is a need for a better understanding of the types and amounts of ingredients required to form nanoemulsions by low-energy methods, and to establish the most appropriate preparation methods to use for particular applications.

2.2.1. Surfactant classification schemes

Emulsifiers play a major role in facilitating the formation of nanoemulsions by reducing the interfacial tension, and thereby lowering the free energy penalty associated

with droplet formation [11]. Emulsifiers are surface-active agents capable of adsorbing to the oil-water interface and forming a protective coating around droplets [5]. This protective coating helps prevent droplet aggregation during and after emulsion formation. Examples of food-grade emulsifiers include small molecule surfactants, phospholipids, amphiphilic proteins, and amphiphilic polysaccharides (**Table 3**) [13]

Table 3. Types of surfactants used in food grade emulsion formation. Adapted from [6].

Surfactant Type	Example/Source
Small molecule surfactants	Tweens, Spans
Phospholipids	Egg, soy, sunflower, or dairy lecithin
Amphiphilic proteins	Whey protein isolate, caseinate
Amphiphilic polysaccharides	Gum Arabic, modified starches

Previous studies suggest that small molecule surfactants and phospholipids are the most effective emulsifiers for fabricating nanoemulsions using low-energy approaches due to the specific structures and properties [14-16]. As a result, only these types of emulsifiers will be considered in detail in this article. Nevertheless, it should be noted that there is considerable interest in developing effective means of forming nanoemulsions from biopolymers, since they have advantages from a labeling perspective [17].

Surfactants and phospholipids can be classified based on their molecular geometry, hydrophilic lipophilic balance (HLB) number, or hydrophilic-lipophilic deviation (HLD) number [18-20]. The molecular geometry of a surfactant molecule can be characterized by a packing parameter (p), which is equal to the ratio of the tail group to head group cross-sectional areas: $p = a_T/a_H$. The packing parameter determines the optimum packing of surfactants when they assemble into monolayers, which in turn determines the optimum curvature that tends to be adopted by a given surfactant [20]. When the tail

group is appreciably larger than the head group ($p > 1$), then the monolayer adopts a curvature where the tail groups point outwards, which favors the formation of reverse micelles and W/O emulsions. Conversely, when the head group is appreciably larger than the tail group ($p < 1$), then the monolayer adopts a curvature where the head groups points outward, which favors the formation of micelles and O/W emulsions. Finally, if the head group and tail group cross-sectional areas are similar ($p = 1$), then the monolayer tends to be planar, which favors the formation of bilayers and vesicles. An understanding of the factors that influence the packing parameter of a surfactant is often extremely useful for optimizing the formation of nanoemulsions by low energy methods.

The HLB system was developed more than 50 years ago [21, 22] in an attempt to identify the optimum surfactant required to formulate emulsions with certain properties *e.g.*, oil-in-water or water-in-oil. In this system, hydrophilic surfactants have high HLB values (above 10) while lipophilic surfactants have low HLB values (1-10) [23]. This classification can be further broken down into 5 categories according to surfactant functionality (**Table 4**) [24]. While the HLB system is valuable and convenient it does have some shortcomings. For example, it says nothing about the amount of surfactant that must be utilized to form a stable emulsion [23], which is critical from a manufacturing and cost standpoint. It also provides limited information about how a surfactant will perform under different environmental conditions or in systems with different compositions [5].

Table 4. Classification of surfactants based on HLB values. Adapted from [24]

Range of HLB Values	Application
3.5-6	Water-in-oil emulsifier
7-9	Wetting agent
8-18	Oil-in-water emulsifier
13-15	Detergent
15-18	Solubilization

The HLD number is a dimensionless parameter that describes the relative affinity of a surfactant for either the aqueous (hydrophilic) phase or organic (lipophilic) phase [25]. This classification scheme explicitly takes into consideration the nature of the system and is dependent on surfactant type, oil type, aqueous phase composition (pH, ionic strength, salinity, cosolvent, etc.) and environmental conditions (such as temperature) [26]. This classification scheme may also be referred to as the surfactant affinity difference (SAD) or the hydrophilic-lipophilic difference [27], but SAD is simply related to HLD by taking the thermal energy into consideration: $SAD \times RT = HLD$, where R is the gas constant and T is the absolute temperature. The HLD numbers can be divided into three categories depending on the relative affinity for the surfactant for the oil or water phases: $HLD < 0$, $HLD = 0$, or $HLD > 0$ (**Table 5**) [7].

Table 5. Classification of surfactants based on HLD values. Adapted from [7].

Range of HLD values	Surfactant Affinity	Microstructure formed	Emulsion type stabilized
$HLD < 0$	Higher affinity for water than oil	Micelles in water	Oil-in-water emulsions
$HLD = 0$	Equal affinity for water and oil	Bicontinuous microemulsions or liquid crystalline phases	Neither oil-in water or water-in-oil emulsions
$HLD > 0$	Higher affinity for oil than water	Reverse micelles in oil	Water-in-oil emulsions

The HLD classification scheme is particularly helpful in understanding the formation of nanoemulsions by low-energy approaches since it categorizes conditions where phase inversions may occur. Typically, a two-dimensional map of surfactant affinity (HLD) *versus* system composition (water-to-oil-ratio or WOR) is constructed, which contains different regions that describe where stable nanoemulsions or emulsions can exist (**Figure 1**). Based on how system conditions are changed, phase inversion can either occur through a transitional or catastrophic mechanism. If one moves downwards in a vertical direction, from a region where a W/O emulsion is stable to one where an O/W emulsion is stable (i.e. a change in HLD number), then a transitional phase inversion occurs. This can be achieved by a change in environmental conditions (such as temperature) or product formulation (such as surfactant type, pH, or salt concentration), with the most appropriate method depending on the nature of the surfactant present. The phase inversion temperature (PIT) method of producing nanoemulsions is based on this principle. In contrast, if one moves rightwards by adding increasing amounts of water to an oil phase (i.e., increasing the WOR), then a catastrophic phase inversion may occur from a W/O emulsion to an O/W emulsion [27]. The spontaneous emulsification and emulsion phase inversion methods are partly based on this principle, and partly based on a change in HLD number.

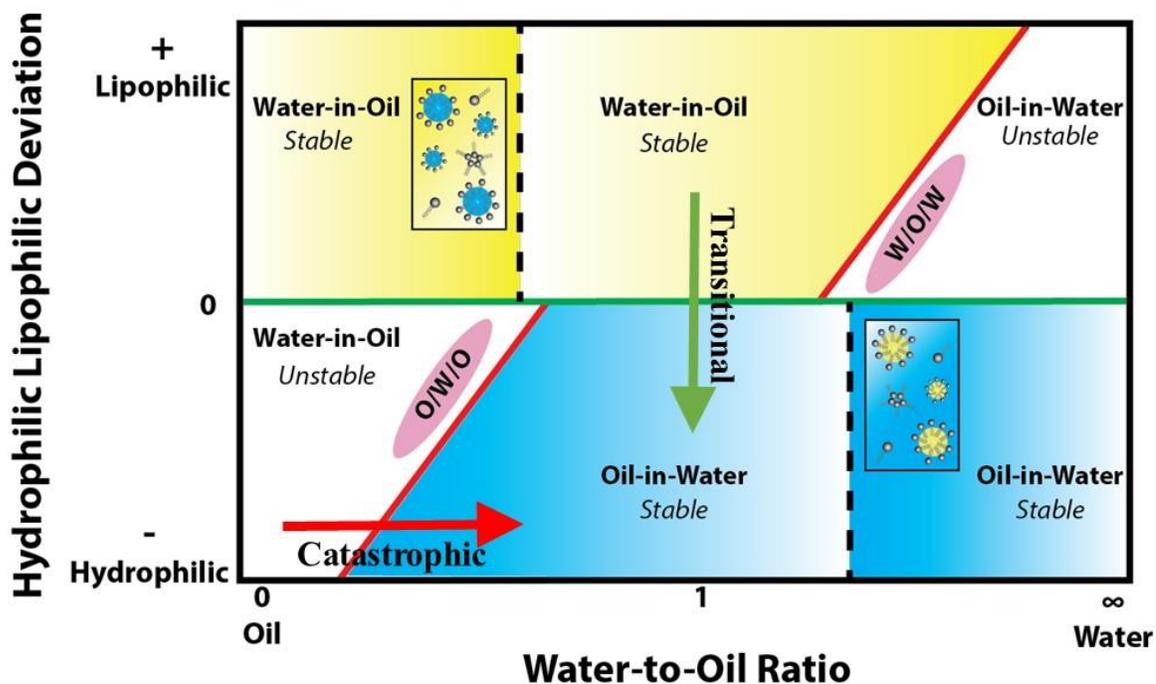


Figure 1. Hydrophilic lipophilic deviation (HLD) versus water-to-oil ratio (WOR) map. Adapted from [4, 27]. Nanoemulsions can be formed through transitional phase inversions where the HLD of a surfactant is changed, or through catastrophic phase inversions where the WOR is changed.

In this review the low-energy methods have been divided into isothermal and thermal approaches for the sake of convenience, with isothermal approaches requiring a change in composition and thermal approaches requiring a change in temperature to produce fine droplets. Changing the temperature of large volumes of liquid is likely to be energy intensive and therefore the isothermal low energy methods may be more appropriate for nanoemulsion formation in the food industry.

Many authors have reviewed low-energy formation of nanoemulsions as it applies to other fields of study such as pharmaceuticals [3, 28]. As recent as 2007, authors have stated that spontaneous emulsification is being investigated but not in the field of food science [29]. The goal of this review is to demonstrate the potential applications of the

low-energy approach in the food industry by showing that it can be used to form nanoemulsions with novel physicochemical and functional properties using all food-grade ingredients.

2.3. Isothermal low energy methods for nanoemulsion formation

Isothermal low energy methods are those that do not utilize any specialized equipment or require a change in temperature in order to produce fine droplets. There are a number of advantages to use isothermal versus thermal methods including the ability to prepare nanoemulsions over a wide range of temperatures rather than fixed at a temperature close to the phase inversion temperature, no requirement for temperature quenching after preparation which could correspond to energy savings, and the capacity to encapsulate heat sensitive compounds. Many bioactive compounds may demonstrate temperature degradation and therefore heating during emulsion formation could be unfavorable. The two main isothermal low energy methods that been utilized in food science are spontaneous emulsification and emulsion phase inversion methods.

2.3.1. Spontaneous emulsification

Spontaneous emulsification (SE) can take place through numerous physicochemical mechanisms True spontaneous emulsification occurs when two immiscible liquids are placed in contact and then emulsify without any external aid, be it thermal or mechanical. Solvents can be utilized to facilitate this process in either the presence [30] or the absence [31] of surfactants. When SE takes place using only oil, water, and a water-miscible solvent without a surfactant it is called the Ouzo effect, after the well-known aperitif [31]. With food grade systems, where the use of a solvent is often

not ideal due to cost, flavor, and safety concerns, SE generally involves the addition of an organic phase (containing oil and hydrophilic surfactant) into an aqueous phase (containing water and possibly a co-surfactant) [28, 32, 33]. In this section, the main focus will be on the isothermal SE method where the temperature is kept constant throughout the process.

Practically, the SE method is usually implemented by titrating an organic phase (oil + hydrophilic surfactant) into a container containing an aqueous phase (initially only water or buffer solution). Fine oil droplets (< 100 nm) can be formed if both the system composition (surfactant and oil type and level) and preparation conditions (temperature, stirring rate, addition rate) are optimized. A proposed mechanism for spontaneous emulsification is the formation of a bicontinuous microemulsion at the boundary where the organic and aqueous phases come into contact, which leads to the spontaneous generation of fine oil droplets when the bicontinuous microemulsion phase breaks up (**Figure 2**). A bicontinuous microemulsion will only form over a certain range of surfactant-oil-water (SOW) ratios that depend on the system. These particular SOW ratios may be reached when surfactant, oil, and water molecules diffuse across the boundary between the organic and aqueous phases. The bicontinuous microemulsion then breaks down and forms small oil droplets with dimensions similar to the hydrophobic domains in the microemulsion [34]. Mild stirring may facilitate the breakdown of the bicontinuous microemulsion, as well as the movement of the surfactant, oil, and water molecules.

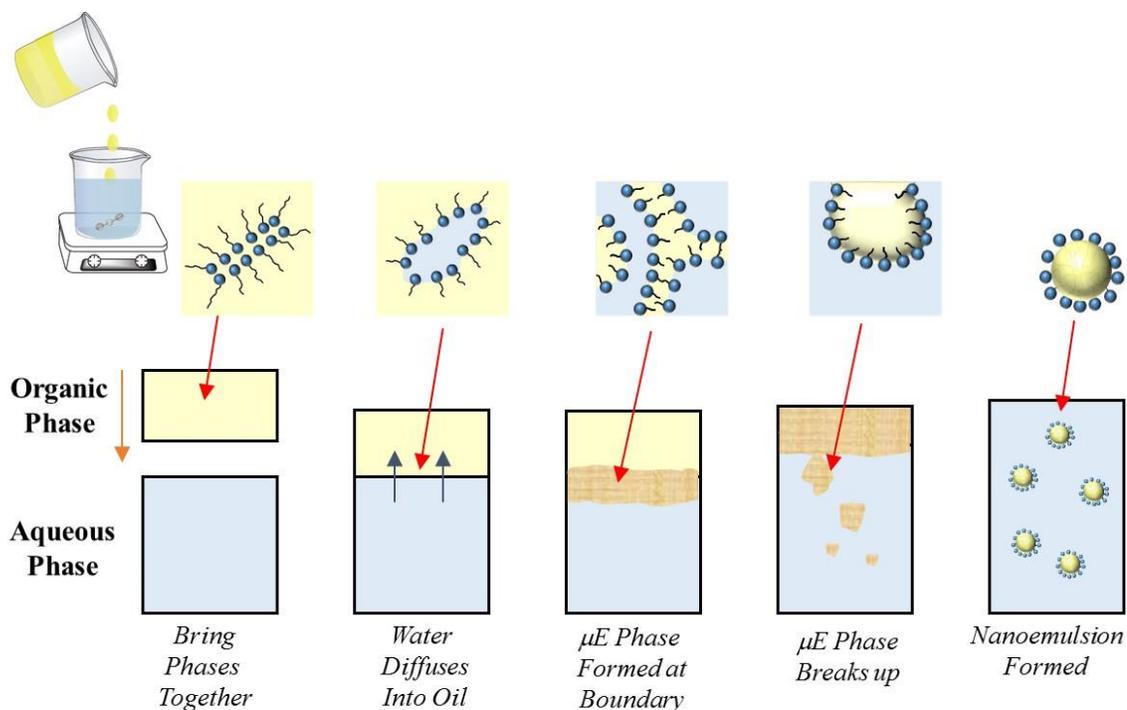


Figure 2. Schematic diagram of potential mechanism for formation of nanoemulsions by the spontaneous emulsification method. When the organic phase (oil + hydrophilic surfactant) and aqueous phase (water) are brought into contact a bicontinuous microemulsion (mE) is formed at the boundary, which breaks up and forms tiny oil droplets.

An overview of recent research articles on emulsions formed using spontaneous emulsification with food grade ingredients can be found in **Table 6**. This process can be affected by a variety of factors including preparation conditions, oil composition, surfactant type, surfactant concentration, cosolvents, cosurfactants, and system composition. In addition, factors affecting the thermal and isothermal stability of emulsions prepared using SE will also be discussed.

Table 6. An overview of recent research articles on O/W nanoemulsion formation using spontaneous emulsification with food grade ingredients

Ingredients				Preparation Conditions		Results	Ref.
% Oil	Oil/Bioactive Component	% Surfactant	Surfactant / Co-Surfactant or Co-Solvent	Preparation Temp.	Stir Speed (rpm)	Particle Dimensions	
20	MCT	0-20	Tween 20, Tween 80 or Tween 85	Room Temperature ($\approx 25^{\circ}\text{C}$)	500	$r < 100$ nm-comparison of low and high energy methods	[35]
10	MCT/Carvacrol	5-20	Tween 20, Tween 40, Tween 60, Tween 80 or Tween 85	Room Temperature ($\approx 25^{\circ}\text{C}$)	500	$d \approx 55$ nm	[36]
10	MCT/Vitamin E	5-10	Tween 80/Glycerol	Room Temperature ($\approx 25^{\circ}\text{C}$)	600	$d < 50$ nm	[37]
10	Vitamin E	10	Tween 80/Propylene glycol or ethanol	Room Temperature ($\approx 25^{\circ}\text{C}$)	600	$d < 50$ nm	[38]
10	MCT/Vitamin E	2.5-10	Tween 20, Tween 40, Tween 60, Tween 80 or Tween 85	$25-90^{\circ}\text{C}$	200, 500, or 800	$d < 50$ nm	[39]
10	MCT/Vitamin E	10	Tween 80	25°C	600	$d \approx 50$ nm-looked at effect of salt	[40]
10	MCT or Corn Oil or Lemon Oil/Vitamin E	10	Tween 80/Ethanol	Room Temperature ($\approx 25^{\circ}\text{C}$)	600	$d \approx 25-40$ nm	[41]
10	Vitamin E	10	Tween 80/Ethanol and Tween 20 or SDS or Lauric Arginate	Room Temperature ($\approx 25^{\circ}\text{C}$)	600	$d \approx 25$ nm-looked at effect of cosurfactant addition	[42]
10	MCT/Vitamin E	10	Tween 80/glycerol	Room Temperature ($\approx 25^{\circ}\text{C}$)	600	$d \approx 45$ nm-looked at effect of temperature	[43]
5	MCT/Capsanthin	10	Tween 80 and Span 20	50°C	400	$d \approx 30-150$ nm	[44]

10	MCT/orange oil	2.5-10	Tween 20, Tween 40, Tween 60, Tween 80, Tween 85, or Span 20	Room Temperature ($\approx 25^{\circ}\text{C}$)	500	$d \approx 25$ nm	[45]
10	Fish Oil with Lemon Oil or MCT as a carrier oil/polyunsaturated (ω -3) oils	2.5-10	Tween 80/glycerol, ethanol, or propylene glycol	Room Temperature ($\approx 25^{\circ}\text{C}$)	500	Smallest size formed was $d \approx 51$ nm when prepared with 40% glycerol and 50% fish oil/50% lemon oil	[12]
1	MCT	2	Tween 80	60°C	750	$d \approx 43$ nm	[46]
10	MCT, Lemon Oil, Orange Oil, Fish Oil, Grapeseed Oil, Sesame Oil, Mineral Oil, Canola Oil, Peanut Oil, or Olive Oil	SOR 0.05-2	Tween 20, Tween 40, Tween 60, Tween 80, Tween 85 or Span 20	Room Temperature ($\approx 20^{\circ}\text{C}$)	750	Smallest size formed was $d \approx 0.1$ μm when prepared with MCT and Tween 80	[15]
10	Grapeseed & Orange Oil/Resveratrol	10	Tween 80	Room Temperature ($\approx 25^{\circ}\text{C}$)	700	$d \approx 100$ nm	[47]
10	MCT/Vitamin D	5-17.5	Tween 20, Tween 40, Tween 60, Tween 80 or Tween 85/SDS	Room Temperature ($\approx 25^{\circ}\text{C}$)	200, 500, or 800	$d \approx 100$ nm	[33]
10	Lemon Oil/Fish Oil	2.5-20	Tween 80	Room Temperature ($\approx 25^{\circ}\text{C}$)	500	$d \approx 100$ nm	[48]

2.3.1.1. Influence of preparation conditions

There are a number of important factors related to preparation conditions that must be taken into account when preparing emulsions or nanoemulsions from food grade ingredients using spontaneous emulsification [30, 49, 50]. Prior to addition of the organic phase to the aqueous phase, it is necessary to ensure that the organic phase is

homogenous. Typically, oil and a slightly hydrophilic surfactant are mixed together to ensure they are thoroughly mixed. The resulting organic phase is then titrated into the aqueous phase at a controlled rate leading to the formation of small oil droplets. Finally, some additional mixing may be required to ensure that the system is homogeneous and any residual bicontinuous microemulsion phases are fully broken up. The entire process can be broadly broken down into 3 steps:

1. Mixing of organic phase (oil + surfactant)
2. Addition of organic phase into aqueous phase
3. Additional mixing time

Preparation conditions that have been investigated include holding temperature, stirring speed, addition rate, and surfactant location.

2.3.1.1.1. Influence of preparation of temperature

Preparation temperature can be controlled by holding the organic phase at specified temperatures prior to preparing at ambient temperature [39] or actually preparing the emulsions at a specified temperature [44, 46]. For emulsions made with MCT and Vitamin E, it was found that there is a moderate decrease in particle size with an increase in holding temperature. When comparing emulsions whose organic phase was held at 25°C versus those whose organic phase was held at 90°C, the mean particle diameter decreased from 55 to 48 nm at a surfactant-to-emulsion ratio (SER) of 10% and from 107 to 89 nm at an SER of 5% [39]. This difference in particle size may be due to a decrease in viscosity (which facilitates the rapid movement of surfactant, oil, and water molecules), a change in molecular geometry of the non-ionic surfactants used, an increase in oil-solubility of the non-ionic surfactant, and/or a decrease in interfacial tension as the

phase inversion temperature (PIT) is reached [39, 46]. Similar observations were reported when controlling temperature during the SE process [44, 46]. Particle size decreased when moving from 25 to 50°C in a system containing MCT and capsanthin and utilizing a mix of Tween 80 and Span 20 as the surfactants. However, when moving from 50 to 75°C the effect of temperature was greatly reduced and actually increased the particle size at high surfactant levels; This effect is likely due to an increase in droplet coalescence rate as the PIT of the system is approached [44]. In general, it seems that SE is initially facilitated when the preparation temperature is increased, but is then adversely impacted at temperatures close to the PIT of the system due to rapid droplet coalescence.

2.3.1.1.2. Influence of stir speed

Agitation conditions during emulsion formation by spontaneous emulsification also influence the size of the particles produced, with the particle diameter typically decreasing with increasing stirring speed. In a system with 8% Vitamin E/2% MCT and either 5 or 10wt% Tween 80, it was found that increasing the stir speed from 200 to 500 to 800 decreased the particle diameter at both surfactant concentrations [39]. A similar result was observed in a system with MCT/2.5wt% Vitamin D and either 10 or 17.5wt% Tween 80 [33]. Stirring is likely necessary to facilitate the transport of surfactant, oil, and water molecules, as well as to facilitate the disruption of the bicontinuous microemulsion formed at the boundary between the organic and aqueous phases [39]. Other studies have shown that the effect of stir speed may be dependent on the surfactant concentration. In a system consisting of MCT/capsanthin, with Tween 80 and Span 20 as surfactants, different results were seen at low (5 wt%) and high (10wt%) surfactant levels. At lower surfactant concentrations, increasing the stir speed decreased the particle size due to the reasons cited above. In contrast, at higher surfactant concentrations, the particle size was

independent of stir speed [44]. Emulsions made at the higher surfactant concentration were smaller than those made at the lower surfactant concentration at all stir speeds. It is possible that at the higher surfactant concentrations used in this study the bicontinuous microemulsion rapidly dispersed into the aqueous phase without the need for stirring. It is therefore critical to understand and optimize all system components for each preparation factor.

2.3.1.1.3. Influence of addition time

Some studies have shown that it is important to control the addition rate of the organic phase into the aqueous phase when using the SE method. If the addition rate of the organic phase (oil + surfactant) into the aqueous phase is carried out too quickly, then large viscous SOW clumps may form that are difficult to breakup and disperse [35]. In a model system, it was found that if the organic phase is added too quickly significantly larger droplets may be formed. In the case of the model system, this cut off was 0.25 minutes. As the addition time was increased from 0.75-20 minutes, there was no significant difference in particle size [51]. Each surfactant-oil-water system would have to be investigated to determine what the maximum addition rate was to achieve small oil droplets on a reasonable timescale. In general, most researchers are using addition times between about 5 and 15 minutes, which is likely to be appreciably longer than actually required to form small oil droplets.

2.3.1.1.4. Influence of surfactant location

The influence of initial surfactant location (organic versus aqueous phase) has also been investigated. When surfactant (Tween 80) was dissolved 100% in the aqueous phase, particle diameter was significantly larger than when dissolved 100% in the organic phase (MCT) [15]. This result suggests that the movement of the hydrophilic surfactant

from the oil phase into the aqueous phase may be important in the formation of nanoemulsions by this method.

2.3.1.2. Influence of oil composition

Only certain types of oil phases can be used to successfully form nanoemulsions using the SE technique. The choice of oil phase will impact both the formation and stability of low-energy nanoemulsions. Nanoemulsion formation can be impacted by differences in viscosity, interfacial tension, interfacial flexibility, and phase behavior, while stability is affected by differences in polarity and water-solubility of the oil molecules [36, 47]. When preparing emulsions using Tween 80, it was found that MCT formed the smallest droplets, followed by the flavor oils (lemon and orange) and long chain triglycerides (fish, grapeseed, sesame, mineral, canola, peanut and olive) [15]. However, no physiochemical correlation could be made between particle size and refractive index, density, interfacial tension or viscosity [15]. This lack of physiochemical correlation has been reported in other works as well [41]. These results suggest that it may be the phase behavior of the specific surfactant-oil-water system rather than the physicochemical properties of the oil phase that are more important in nanoemulsion formation.

The influence of mixed oils on nanoemulsion formation by SE has also been investigated. When investigating different ratios of orange oil and grapeseed oil, an optimum oil composition to achieve minimum particle size was 50% grapeseed oil and 50% orange oil. The triglyceride oils alone were unsuitable for formation of nanoemulsions, presumably because they did not exhibit the appropriate phase behavior, *i.e.*, they did not form bicontinuous microemulsions that could easily break up.

Consequently, they are unable to form small oil droplets during the SE process. In contrast, polar oils (such as orange oil) are highly susceptible to Ostwald ripening due to their relatively high water-solubility, which leads to rapid droplet growth during storage [45, 47]. Therefore different ratios of orange to grapeseed oil were tested in order to optimize emulsion formation and stability [47]. The addition of the long chain triglycerides (low water-solubility) to the polar oils (high water-solubility) inhibits droplet growth due to Ostwald ripening through an entropy of mixing effect, while still allowing small droplets to be formed. Similar results were also reported for mixtures of orange oil with MCT [45].

Medium chain triglycerides (MCT) are commonly used as the oil phase in the SE process. In a system of MCT and carvacrol, the best ratio for stability was found when 75% MCT and 25% carvacrol was used [36]. When encapsulating capsanthin in MCT, particle size decreased when low amounts (≤ 1.5 wt%) were incorporated but increased rapidly when higher amounts (1.5-2.5 wt%) were incorporated [44]. Therefore, a critical balance between oil blends is required to optimize the system during formation and storage. Additionally, the carrier oil chosen cannot be at random. When long chain triglycerides, such as corn or canola oil, were substituted for MCT in the encapsulation of carvacrol or orange oil, no stable nanoemulsions could be formed [36, 45].

In some cases, it has been reported that stable nanoemulsions can be formed using relatively low ratios of MCT. For emulsions (10% oil, 10% Tween 80) made with vitamin E acetate (VE) and medium chain triglycerides (MCT), nanoemulsions ($d < 200$ nm) could be made at all compositions tested but the smallest particle diameter was produced when the oil phase was composed mostly of VE. As the composition was

altered from all MCT to all VE, the particle diameter decreased until 80% of the oil phase was made up of VE; at higher concentrations of VE the particle diameter increased. To further understand what was occurring, interfacial tension and shear viscosity were measured for the different VE/MCT compositions; no simple relationship between droplet size and viscosity or interfacial tension was observed [39]. Adding long chain triglycerides (LCT) to the VE system increased initial particle size but also increased the thermal stability of the emulsions formed [40]. Each system therefore appears to be unique in what percentage of carrier oil should be used.

In certain applications the use of a flavor oil, such as lemon oil, as a carrier may be advantageous. The encapsulation of polyunsaturated oils (ω -3) can be achieved by incorporating fish oil into an emulsion or nanoemulsion system. However, fish oil alone is incapable of producing fine nanoemulsion droplets utilizing the SE process and therefore requires a carrier oil. While MCT has shown success with a variety of systems, lemon oil is of interest to help mask any off-flavors that may develop. Mixing intermediate concentrations of fish oil in with the carrier oils ($\leq 40\%$ for MCT and $\leq 50\%$ for lemon oil) led to droplet diameters less than 200 nm [12]. Therefore, in certain systems, flavor oils may act as an appropriate carrier oil during the SE process.

In some systems, when only low amounts of bioactive compound are required to be encapsulated, the inclusion may have no effect on the overall emulsion formed. When comparing pure MCT with MCT that had 2.5 wt% vitamin D dissolved, there was little difference on the size of the droplets produced [33].

In summary, it is important to identify an appropriate oil phase to prepare stable nanoemulsions from the SE method. At present there is no simple method of selecting an

appropriate oil for a particular surfactant-oil-water (SOW) system based purely on its physiochemical properties. It is more likely, that a ternary phase diagram will have to be prepared for the surfactant-oil-water system used, and regions where a bicontinuous microemulsion can form be established for the particular oil types utilized.

2.3.1.3. Influence of surfactant type

As with oils, only certain types of surfactants are suitable for forming nanoemulsions using the SE method. Most of the previous work using food-grade ingredients has focused on synthetic small molecule non-ionic surfactants, such as Tweens and Spans. There is likely an optimum surfactant geometry that works best to promote the spontaneous formation of fine droplets at the oil-water boundary [33]. Tween 80, which has an optimized packing parameter due to its single unsaturated tail, generally forms smaller droplets than emulsions produced by Tween 20, 40, 60 or 85. Tween 20, 40, and 60 all have saturated linear chains while Tween 85 has three unsaturated tails so Tween 80 has a higher packing parameter than Tween 20, 40, and 60 and a smaller packing parameter than Tween 85 [33, 36, 39]. Tween 80 stabilized emulsions also had a smaller particle diameter than those made with Span 20 [15].

In addition to packing parameter, the hydrophilic lipophilic balance (HLB) may also play a role in promoting fine droplet formation. In a system of 10 wt% MCT, it was found that the smallest emulsion diameter was formed when emulsions were prepared with Tween 40, Tween 80 or an equal mixture of Tween 20, 80 and 85 [15]. The HLB values for the two surfactants and surfactant mixture were 15.6, 15.0, and 14.2 respectively. When using orange oil and MCT, intermediate HLB values of 15.6, 14.9, and 15.0, belonging to Tween 40, 60, and 80, produced the most stable nanoemulsions

[45]. These HLB values are significant because they are high enough that the surfactant is hydrophilic but not so high that the surfactant is unable to be soluble in the organic phase. The requirement for the HLB value to be at this critical level supports the notion that it is the movement of the surfactant from the oil phase to the aqueous phase that drives the formation of fine oil droplets at the oil-water interface.

Other studies have shown that an HLB value of 13.4, achieved by varying Tween 80 and Span 20 ratios, produced the smallest particle size in a system consisting of 5 wt% MCT and 10 wt% surfactant [44]. Additionally, when studying a system consisting of MCT and Vitamin E, no strong correlation could be found between particle size and HLB numbers [39]. Therefore, HLB value can be used as a rough guide but each system must be optimized based on its specific components. Just because an HLB value works for pure MCT at a specific surfactant concentration does not mean it will be best for blended compositions, such as MCT and Vitamin E, or at a different surfactant concentration. Therefore both HLB values and packing parameter should be considered when choosing the appropriate surfactant for each system [36].

2.3.1.4. Influence of surfactant concentration

Surfactant concentration is one of the most important factors to control when using the SE method. One of the major drawbacks of this method is the requirement for a high amount of synthetic surfactant which can lead to cost, taste, and safety concerns [12, 15, 33, 36, 45, 48]. Therefore, when optimizing a system it is important to ensure there is enough surfactant present to stabilize the emulsion system [12] but not so much that there is excess non-functional surfactant. Many researchers have found that increasing the surfactant concentration decreases the particle size [15, 33, 36, 39, 44, 45, 47, 48].

However, in other studies it has been found that there is a point where increasing the surfactant concentration further no longer decreases the particle size, and may actually lead to an increase [12, 15, 33, 44, 47]. With excess surfactant, gel-like clumps can be observed which may be attributed to the formation of cubic phases that are not easy to dissolve or that coexist with the nanoemulsions [33, 47, 48]. The high level of surfactant may also increase droplet size due to the high viscosity of the liquid crystalline phase making it harder for the spontaneous formation of fine emulsion droplets [37, 39, 44]. In summary, it is important to use enough surfactant to form small stable droplets, but not so much that there is excess surfactant or large clumps formed.

2.3.1.5. Influence of cosolvents

Cosolvents are used because they can alter the bulk properties of aqueous solutions (such as viscosity, density, refractive index, solubility, and interfacial tension) and/or alter structural properties of the surfactant solutions (such as optimum curvature, critical micelle concentration, and phase behavior) [38]. Common cosolvents that may be used are short-chain alcohols such as ethanol, propylene glycol (PG) and glycerol. Initial droplet size in emulsions produced with Vitamin E acetate (VE) as the oil phase depended strongly on the cosolvent type, concentration, and location [38]. When using PG, particle diameter decreased slightly from 0-20% PG, steeply from 20-30% and remained low between 30-40% PG so the optimized concentration of PG was set at 30% [38]. For ethanol, which could be dissolved in the aqueous or organic phase, drastically different results were observed depending on location. When dissolved in the aqueous phase, particle diameter decreased steeply from 0-20% ethanol before increasing steeply from 20-30% ethanol and remaining high between 30-50% ethanol. When ethanol was dissolved in the organic phase, particle diameter decreased steeply from 0-5% then

increased steeply from 5-20%; ethanol was not soluble in the organic phase at higher concentrations. Smaller droplets could be formed when ethanol was dissolved in the aqueous phase than the organic phase [38]. The reason PG and ethanol help decrease the particle diameter is likely linked to their ability to alter the solubility and optimum curvature of the surfactants [38]. While small initial particle diameters can be achieved using cosolvents, the resulting nanoemulsions are often highly unstable to droplet growth during storage, which may be due to increased coalescence or Ostwald ripening. This can often be overcome by the use of dilution of the nanoemulsions after formation as to reduce the cosolvent concentration [38]. When using glycerol, the oil phase composition can impact the distribution of particles. Utilizing glycerol in a system with a mixed oil phase (80% VE & 20% MCT) produced a particle size distribution that was monomodal; in contrast, emulsions formed with pure VE had distributions that were much wider and varied more [37].

In a mixture of lemon oil and fish oil with Tween 80, it was found that the use of ethanol (10-50%) led to an increase in particle size and emulsion instability and thus was not a good cosolvent choice [12]. In the same system, propylene glycol (PG) had a complex response: increasing from 0-35% PG led to an increase in mean particle diameter, increasing from 35-40% PG led to a decrease in mean particle diameter, and increasing from 35-45% PG led to a steep increase in mean particle diameter [12]. Thus, there is a critical level of PG that must be determined in order to ensure the smallest particle diameter can be formed. Lastly, when using glycerol as a cosolvent with the same fish/lemon oil system, there was little change from 0-20% glycerol but a significant decrease in particle size moving from 20-40% glycerol with the smallest droplet size

being formed ($d \approx 51$ nm) at 40% glycerol [12]. Additionally, the emulsions made with glycerol were optically transparent which may be a result of their small size or the reduced refractive index achieved with the incorporation of glycerol.

The amount of surfactant required to produce fine emulsion droplets may be reduced by the use of cosolvents such as glycerol [37]. The benefits from a reduction in surfactant would have to be weighed against the costs of an increase in cosolvent in order to determine what the optimum composition would be. Additionally, the complexities of incorporating cosolvents would have to be individually investigated for each system. In some foods, the use of cosolvents may be undesirable for labeling purposes, and therefore this approach of producing nanoemulsions may not be viable.

2.3.1.6. Influence of cosurfactants

Cosurfactants can be added before, during, or after emulsion fabrication to facilitate the formation of small droplets, or to improve their subsequent stability or functionality. The ability of cosurfactants to modulate emulsion properties depends on their molecular characteristics, such as head and tail group properties. The head groups of cosurfactants may vary in charge (anionic, cationic, zwitterionic, or nonionic), size, and shape. The tail groups may vary in the number of chains involved, as well as their length, degree of unsaturation, and flexibility. In addition, the head and tail group characteristics can change in response to environmental conditions (such as temperature, pH, or ionic strength). In this section, a number of applications of co-surfactants within emulsions and nanoemulsions are given.

In a system consisting of 10% oil phase (Vitamin E), 10% surfactant (Tween 80), 20% cosolvent (ethanol), and 60% buffer solution (pH 3), the use of a 0.5% cosurfactant

(anionic/SDS, cationic/lauric arginate, or nonionic/Tween 20) was investigated for their ability to improve the thermal stability of the nanoemulsions [42]. Nanoemulsions stabilized by certain types of nonionic surfactants (such as Tween 80) are prone to instability are prone to instability when they are heated near to their PIT. The incorporation of cosurfactants into these systems may be able to alter the PIT by changing the optimum curvature of the surfactant monolayer and/or by altering the colloidal interactions between the droplets. In this study, use of a nonionic cosurfactant had no effect on the PIT while the use of ionic cosurfactants significantly increased it. However, this did not lead to an increase in storage stability at lower temperatures [42]. The authors suggest further research would have to be conducted to understand why the increase in PIT did not correspond to an increase in storage stability. One possible explanation was that the droplet growth that occurred at low temperatures was due to Ostwald ripening, rather than coalescence.

In the case of MCT/Vitamin D emulsions prepared using Tween 80 as the main surfactant, there was some improvement of isothermal storage stability when they were diluted in a non-ionic surfactant solution compared to when they were diluted in water [33]. It is likely the tendency of droplet coalescence was reduced due to a change in the surfactant optimum curvature and increase in repulsive interactions between emulsion droplets [33]. This study showed that dilution into a cosurfactant may be more effective at increasing isothermal stability than dilution simply into water. Each system should be investigated to see if the use of a cosurfactant could also lead to an improved isothermal storage stability.

2.3.1.7. Influence of system composition

Food systems are usually much more compositionally complex than the simple model systems tested in research laboratories. In addition to oil, surfactant, and aqueous phases they can also contain other components, such as sugars, minerals or biopolymers. Therefore, it is important to understand how emulsions made by SE act in these more complex systems. Currently not much research has been conducted in this area. In this section, a brief review of previous work on the effect of salts on the formation and stability of food-grade nanoemulsions produced by low energy methods is presented.

To investigate the effect of salt on emulsion formation, 0-1 N NaCl or 0-0.5 N CaCl₂ was added to the aqueous phase of emulsions prepared with 8% Vitamin E acetate, 2% MCT and 10% Tween 80 [40]. It was found that the mean droplet diameter ($d \approx 50$ nm) was unchanged as the salt concentration was increased. It is likely the salts did not influence the surfactant solubility or optimum curvature at the levels tested [40]. However, each system is unique and just because the tested salt concentration had no effect on initial particle size with this system does not mean it would have the same effect if oil or surfactant composition or concentration were altered. Therefore, as with most aspects related to the low-energy methods, it is important to not make assumptions and test out each set of components and conditions to determine how salt will impact initial particle size.

2.3.1.8. Thermal stability

An issue with emulsions prepared by the spontaneous emulsification technique is they are often not thermally stable due to the use of non-ionic surfactants. When non-ionic surfactants, such as Tweens, are exposed to high temperatures they can often experience dehydration of the head groups which vastly decreases the interfacial tension,

changes the optimum curvature of the surfactant, and can lead to coalescence of the emulsion droplets [12, 33, 40-43]. The degree of thermal instability is related to what temperature the emulsion is exposed to. Emulsions can be heated to temperatures well below the PIT, just below the PIT, or around or above the PIT. When exposed to these three temperature ranges and then quench cooled with an emulsion system consisting of Vitamin E, Tween 80, pH 3 buffer and glycerol, drastically different results were observed [43]. Heating to temperatures well below the PIT and around or above the PIT caused only a slight increase in the particle diameter whereas heating to temperatures just below the PIT caused significant increase in the particle diameter [43]. Rapid growth around the PIT is due to surfactant head dehydration. Commercially it is important to keep emulsions away from this rapid growth area, also called the *droplet coalescence zone*. Each surfactant-oil-water system will have its own unique *droplet coalescence zone* that must be investigated [43]. The relationship of stability to temperature is complex and also depends on factors such as the nature of the surfactant and oil phase that are used to produce the emulsion. It is likely very important to understand coalescence stability near the PIT in order to determine droplet growth after heating [45].

Thermal instability can be very complex and broken down into a number of stages. A system consisting of 10% oil phase (50% fish oil/50% lemon oil), 10% surfactant (Tween 80), and 80% aqueous phase (40% glycerol in pH 3 citrate buffer) observed thermal instability, represented by an increase in turbidity, when the emulsion was exposed to temperatures of 50-68°C. This was due to droplet coalescence. At higher temperatures, 68-70°C, the turbidity rapidly decreased before leveling off at a low turbidity between 70-74°C. As the temperature was increased past 74°C there was another

rapid increase in turbidity [12]. The decrease observed between 70-74°C may be attributed to a single isotropic microemulsion being formed. The impact on particle size is dependent on how high of a temperature the emulsion was exposed to.

The incorporation of the cosolvent glycerol in Vitamin E emulsions made with Tween 80 actually decreases the thermal stability by lowering the phase inversion temperature (PIT) [37]. While the presence of glycerol is advantageous during the production of nanoemulsions, it is disadvantageous during storage as it dehydrates the polar head groups of Tween 80 thus altering the optimum curvature and decreasing the PIT [37]. The effect of this is that emulsions made without glycerol are stable until 70°C while those made with glycerol are only stable until 55°C due to the decrease in the PIT [37]. Similarly, the addition of salt also decreases the PIT and thus decreases thermal stability [40]. The cloud point was also decreased from 78°C (no salt) to 65°C (1 M NaCl) or 70°C (0.5 N CaCl₂) [40]. These decreases in both the PIT and cloud point are likely due to the dehydration of the surfactant head group which negatively modifies the optimum curvature of the surfactant monolayer formed at the interface [40]. In contrast, cosurfactants may be helpful in increasing the thermal stability [33]. The use of ionic surfactants (SDS or lauric arginate) have been found to increase the cloud point at low levels ($\leq 0.5\%$) due to their ability to positively modify the optimum curvature of the surfactant monolayer as well as provide an electrostatic repulsion between emulsion droplets [42].

Another method that can be utilized to help with thermal stability is dilution. Many commercial based products (such as functional beverages or foods) only require emulsion-based delivery systems to be used in a highly diluted form so simple dilution

may be a practical solution for improving isothermal stability. The thermal stability of emulsions fabricated from Vitamin E and cosolvents was enhanced by dilution (100x) in water [37, 38]. Dilution reduces the amount of cosolvents present in the system thereby reducing their effect on the optimum curvature and solubility of surfactants and thus their impact on cloud points and PITs [41].

In summary, thermal instability is a major concern for nanoemulsions prepared by SE when non-ionic surfactants are utilized in their formation, but there are strategies available to increase their thermal stability.

2.3.1.9. Isothermal stability

Emulsions and nanoemulsions are not typically used immediately after production and therefore there is a need for them to stay physically stable throughout storage. The isothermal stability of these systems depends on how close the storage temperature is to the *droplet coalescence zone*. At temperatures well below the PIT, emulsions stay stable to droplet coalescence, but may still be susceptible to Ostwald ripening [43]. However, if the storage temperature is close to the PIT, in the *droplet coalescence zone*, then it is likely the emulsion will not be stable to droplet growth. The *droplet coalescence zone* will be different for every system as it is affected by emulsion composition.

Some researchers found that their systems were highly unstable during storage. A significant increase in particle diameter over the span of 2 months at room temperature was observed for emulsions prepared with 8% Vitamin E and 2% MCT. Emulsions formed with the highest initial surfactant concentration, and thus smallest initial droplet size, were surprisingly the least stable to droplet growth [39]. The instability of these droplets may be due to droplet flocculation, coalescence and/or Ostwald ripening [39]. It

was also found that stability of nanoemulsions made with Vitamin E was decreased by the addition of 40% glycerol [37]. It is likely that the addition of the glycerol decreased the temperature range where the *droplet coalescence zone* occurred.

In contrast, other researchers found their systems to be mostly stable throughout storage. In emulsions prepared with Tween 80 and fish oil/lemon oil, emulsions were stable over 30 days at temperatures of 5 and 20°C but unstable at 37°C [12]. Additionally, emulsions prepared with 5 wt% MCT/capsanthin and a surfactant mixture of Tween 80 and Span 20 were stable over a month at 25°C [44]. Instability at higher storage temperatures may be due to droplet coalescence when held at temperatures close to the PIT, in the *droplet coalescence zone*. Many systems observed an increase in storage instability with an increase in isothermal storage temperature [40]. The real world outcome of this is that emulsions formed by spontaneous emulsification using non-ionic surfactants that have a PIT of approximately 30-40°C may be best stored at refrigeration or room temperatures to avoid being in the *droplet coalescence zone*.

Interestingly, the presence of salts decreased the rate of droplet growth throughout isothermal storage due to the reduction of attractive van der Waals interactions (thus reducing coalescence) and reduction of the solubility of oil molecules in water (thus reducing Ostwald ripening) [40]. However, the use of cosurfactants (ionic or nonionic) did not improve the storage stability of emulsions made with Vitamin E, Tween 80 and ethanol [42]. Each system should be investigated individually to determine how system composition alters the *droplet coalescence zone* and subsequently affects stability.

Isothermal stability, similar to thermal stability, was also affected by the use of dilution. In nanoemulsions prepared using Vitamin E acetate (VE) and cosolvents

(propylene glycol and ethanol), enhanced isothermal stability was achieved by dilution (100x) because the cosolvent concentration was significantly reduced and thus had less effect on surfactant properties [38]. Additionally, emulsions formed with VE with and without glycerol also saw improved stability at all temperatures tested (5, 20, and 37°C) when diluted [37]. Dilution (5x) in water of emulsions made with MCT and carvacrol significantly improved the isothermal stability [36] and those made with MCT and orange oil saw improved storage stability over 40 days when diluted 10x [45]. Dilution of emulsions that have high surfactant levels may increase isothermal storage stability by reducing the amount of free surfactant micelles that are capable of transferring oil molecules between droplets during Ostwald ripening in the undiluted emulsions [45]. However, in a study comparing undiluted emulsions containing Vitamin D to those diluted with water (2- or 6-fold), there was little improvement in isothermal storage stability after storage at 25°C for one month [33]. It is possible the level of dilution tested by Guttoff et al (2 or 6x) was not significant enough for that particular system to observe beneficial effects. The other dilution levels tested (5, 10 or 100x) were appropriate for those particular systems while (2 or 6x) may not have been for the Vitamin D emulsions. These results emphasize how important it is to individually investigate each system as no overall trend has been observed between varying system compositions.

2.3.2. Emulsion phase inversion

Emulsion phase inversion (EPI) involves the addition of an aqueous phase into a stirring organic phase, which usually consists of oil and surfactant. The experimental set-up is reversed from spontaneous emulsification, which involves the addition of the aqueous phase into the stirring organic phase. The EPI method may also be referred to as catastrophic phase inversion (CPI) as this method also involves adding water into oil [52-

54]. For the sake of this review, all methods involving addition of the aqueous phase into the organic phase will be referred to as the emulsion phase inversion method. An overview of recent research articles on emulsions formed using EPI/CPI with food grade ingredients can be found in **Table 7**.

Table 7. An overview of recent research articles on O/W nanoemulsion formation using emulsion phase inversion with food grade ingredients

Method Name Used in Paper	Ingredients				Preparation Conditions		Results	Ref.
	% Oil	Oil/Bioactive Component	% Surfactant	Surfactant/Co-Surfactant or Co-Solvent	Prep. Temp.	Stir Speed (rpm)	Particle Dimensions	
Catastrophic Phase Inversion	≈ 20	Acetem/Oregano and Cinnamon Oil	≈ 10-20	Tween 60	Room Temp. (≈25°C)	700-1300	$d \approx 100-200$ nm	[52]
Emulsion Phase Inversion	10	MCT, Canola Oil, Grapeseed Oil, Limonene, Olive Oil, Orange Oil, Peanut Oil or Sesame Oil	6.5-25	Tween 20, Tween 80 and Tween 85	Room Temp. (≈25°C)	500	$d \approx 150$ nm	[7]
Emulsion Phase Inversion	10	MCT/Vitamin E	1-10	Tween 20, Tween 40, Tween 60, Tween 80, Tween 85, Q-natural, whey protein isolate, casein, or sucrose monopalmitate	Room Temp. (≈25°C)	500	$d \approx 40$ nm	[16]
Catastrophic Phase Inversion	4	D-Limonene, Olive Oil, Corn Oil, Sunflower Oil, Soybean Oil	2-6	Tween 80/Propylene Glycol	Not specified, assumed Room Temp. (≈25°C)	1300	$d \approx 40$ nm	[53]
Catastrophic Phase Inversion	4	D-Limonene/Nisin	6	Tween 80/Propylene Glycol	Not specified, assumed Room Temp. (≈25°C)	Gentle agitation (no rpm specified)	$d \approx 20$ nm	[54]
Emulsion Phase Inversion	10	Tributylin/Vitamin E	10	Tween 80	Not specified, assumed Room Temp. (≈25°C)	500 rpm	$d \approx 110$ nm	[55]

The proposed mechanism of the EPI method (**Figure 10**) has some similarities to the proposed mechanism for spontaneous emulsification. When the aqueous phase is initially titrated into the organic phase, a water-in-oil (W/O) emulsion is formed. As more water is added, a liquid crystalline phase may be formed that can be so viscous that it prevents the stir bar from continuing to rotate. The formation of this liquid crystalline phase may be an important intermediate step in nanoemulsion production, as it may be related to the formation of the bicontinuous microemulsion that eventually breaks down and forms small droplets [16, 55]. As more water is added, a multiple emulsion (oil-in-water-in-oil, O/W/O) is formed and the viscosity of the system decreases. This multiple emulsion may result from the W/O emulsion by a mechanism that is closely related to spontaneous emulsification. The inner oil droplets are spontaneously formed at the boundary between the organic and aqueous phases, which may exist as a bicontinuous microemulsion at a certain SOW ratio that breaks down. Thus, the formation of this multiple emulsion is believed to be another important intermediate step in the creation of the final O/W emulsion or nanoemulsion [56]. As more water is added, a catastrophic phase inversion takes place (O/W/O to O/W) and the small oil droplets present within the water phase of the O/W/O emulsions are released. The critical water content where this phase inversion occurs depends on factors such as stirring speed, rate of water addition, or surfactant concentration [52].

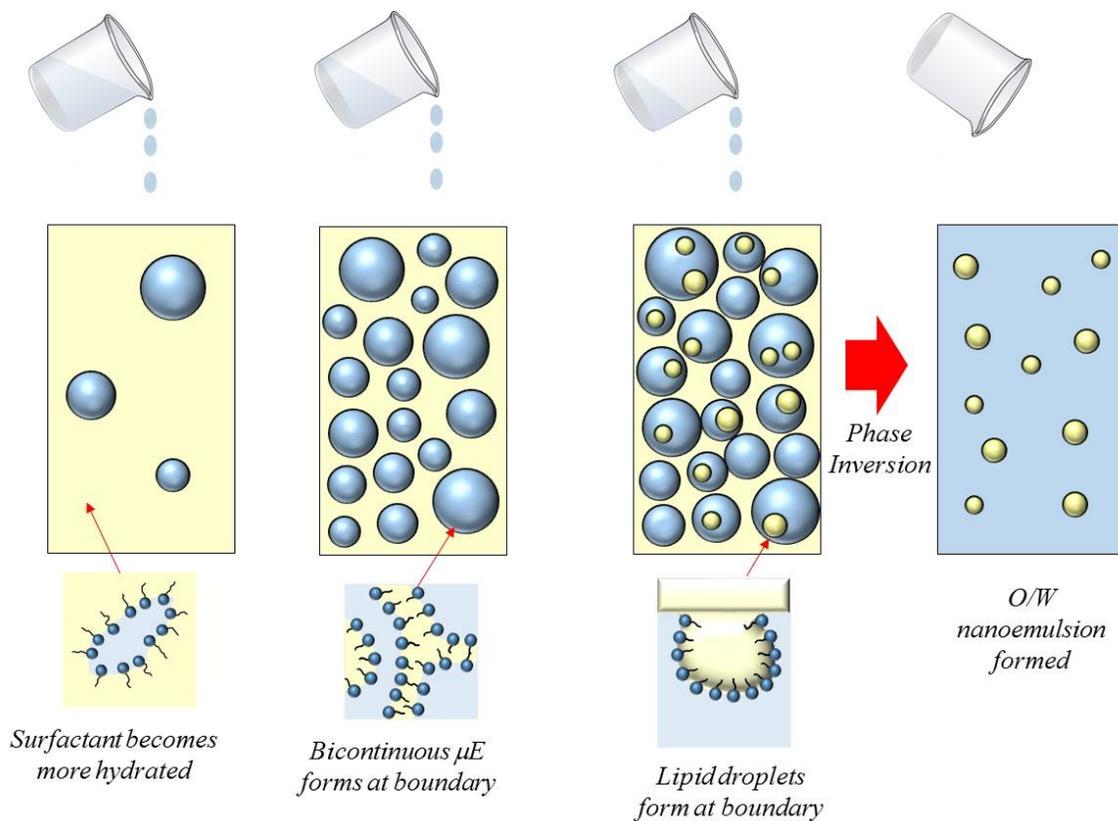


Figure 3. Schematic diagram of proposed mechanism for formation of nanoemulsions by the emulsion phase inversion method. In this case, water is titrated into a surfactant-oil mixture with constant stirring.

2.3.2.1. Influence of preparation conditions

The preparation of the components and set-up for the EPI method is quite similar to the SE method. Initially, the organic phase is mixed together until homogenous; this process can range from 10-30 minutes. Then the aqueous phase is added into the organic phase over time (5-60 minutes). Lastly, additional mixing time is allowed (5-360 minutes). The entire emulsion phase inversion process can broadly be broken down into three steps:

1. Mixing of organic phase (oil + surfactant)
2. Addition of aqueous phase into organic phase

3. Additional mixing time

Preparation factors that have been investigated when optimizing the emulsion phase inversion method with food grade ingredients include surfactant location, order of addition, stir speed, and addition time.

2.3.2.1.1. Influence of surfactant location

Surfactant location has been investigated to test the hypothesis that ultrafine droplets are spontaneously formed by the movement of surfactant from the oil phase into the aqueous phase during the EPI method. The location of the surfactant was varied from being 100% in the organic phase (where it is typically found) to 100% in the aqueous phase at 25% intervals. When the surfactant was placed in the aqueous phase larger droplets were formed compared to when the surfactant was mixed with the oil phase [7]. These results support the notion that surfactant must be dissolved in the organic phase in order to drive the formation of fine emulsion droplets. When the surfactant is initially dissolved in the oil phase, the formation of an O/W/O emulsion is promoted and thus fine droplets can be produced [7].

2.3.2.1.2. Influence of order of addition

Order of addition was investigated to determine if there is a critical action that takes place when the aqueous phase is added into the organic phase or if the same results could be achieved by simply mixing all components together. In a system using Tween 80 as the surfactant (1-10%) and MCT as the oil (10%), order of addition was found to be significant. Emulsions formed at all surfactant concentrations by simply mixing were quite large ($d > 40 \mu\text{m}$) whereas nanoemulsions ($d < 200 \text{ nm}$) could be formed at higher surfactant concentrations utilizing the emulsion phase inversion method [16]. This agrees with the assumption that something critical must happen when the aqueous phase is

added into the organic phase, likely the movement of surfactant from the organic phase to aqueous phase, in order to form fine droplets. Alternatively, having the surfactant initially in the organic phase may lead to a particular SOW ratio at the boundary between the two phases, which results in the formation of a microemulsion that can breakdown into small oil droplets.

2.3.2.1.3. Influence of stir speed

In a system consisting of Acetem 90-50K and Tween 60 it was found that increasing the stir speed (700 to 1,300 rpm) decreased the particle size (190 to 120 nm) at a high surfactant level (surfactant-to-oil ratio of 1). This is related to the greater amount of mechanical energy provided by the higher stir speed thus promoting droplet breakage. Additionally, the critical value of water required to induce a phase inversion was also dependent on stir speed when using a high surfactant concentration. A higher amount of water is required to offset the droplet disruption caused by an increase in stir speed [52].

2.3.2.1.4. Influence of addition time

Some researchers use shorter mixing times (5-15 minutes) while others use longer mixer times (up to 6 hours) when utilizing the emulsion phase inversion method to produce nanoemulsions. The effect of mixing time has not been thoroughly investigated, other than an increased mixing time leads to greater stability for emulsions made with low surfactant concentration [52]. This may be an area of interest for future research. Ideally for scale up of the EPI method it would be best to form the smallest droplets in the shortest amount of time, least amount of energy, and lowest amount of surfactant. Therefore it may be interesting to investigate the optimum amount of mixing time to form the smallest droplets.

2.3.2.2. Influence of oil composition

Like other low energy methods, only certain types of oil are appropriate to be used in the emulsion phase inversion (EPI) method. In a study using Tween 80 as the surfactant, it was found that emulsions with the smallest particle diameter could be formed with medium chain triglycerides (MCT) followed by the flavor oils (orange oil and limonene) and the largest particles were formed with long chain triglyceride oils (olive, grapeseed, sesame, peanut and canola). However, no physiochemical correlation was found between particle size and density, viscosity, or interfacial tensions of the oils [7]. No explanation has been found for why MCT works so well and other oils are incapable of forming fine nanoemulsions using the EPI method. Further work on characterizing the phase diagrams of different surfactant-oil-water systems may provide valuable insights into this phenomenon. In particular, it is important that the combination of surfactant and oil components used is able to form a microemulsion at an appropriate SOW ratio that will breakdown and produce fine oil droplets.

In another study, the impact of mixing plant oils (olive oil, corn oil, sunflower oil, and soybean oil) with D-limonene using Tween 80 was investigated. It was found that the oil phase could consist up to 10% plant oil without having a significant increase in particle size. However, once 15% plant oil was incorporated the particle size greatly increased. All plant oils tested had considerably higher viscosities than D-Limonene and it is hypothesized that for this system the viscosity impacted the size of the droplets by promoting droplet coalescence. Additionally, the influence of all the plant oils was similar which was attributed to the similarities of the viscosities. Nevertheless, as discussed earlier, a conclusive relationship between oil phase viscosity and particle size has not been established and further work is needed to prove this hypothesis. Although

smaller droplets could be achieved without the use of plant oils (<40 nm without versus \approx 40-60 nm with 10% plant oils), oil blending may be advantageous for storage stability. Ostwald ripening was observed to be the main instability mechanism because D-Limonene is a non-polar molecule with a relatively high water-solubility. In contrast, olive oil is composed of mostly long chain triglycerides with low water-solubility. Therefore, the addition of olive oil was very effective at reducing the amount of Ostwald ripening and thus preventing droplet growth [53]. When optimizing oil composition it is important to understand what factors may affect droplet formation (such as oil viscosity) and which factors affect droplet stability (such as water-solubility).

Often nanoemulsions are utilized to encapsulate lipophilic bioactive compounds, such as the fat soluble vitamins, ω -3 oils, or nutraceuticals, into aqueous based products. Therefore, it is critical to understand how the presence of these compounds affects the formation and stability of the nanoemulsions. In a study with 10% oil (MCT + Vitamin E) and 10% surfactant, it was found that oil composition affected particle size. Adding up to 8% Vitamin E acetate (VE) decreased particle size; particle size increased, however, when the entire oil phase (10%) was composed of VE [16]. This was the same result that was observed when using spontaneous emulsification [39] suggesting a similar underlying mechanism for the two methods.

As mentioned earlier, oils with a relatively high water-solubility (such as flavor and essential oils) cannot form stable nanoemulsions due to their tendency to undergo rapid Ostwald ripening without the use of a carrier oil. Examples of these oils include oregano and cinnamon which had to be mixed in with a carrier oil (Acetem) in order to form nanoemulsions [52]. Additionally, the presence of some bioactive compounds may have

minimal effect on particle size. A small increase in particle diameter was observed when nisin was added to a nanoemulsion made with 4% oil (D-limonene) and 6% surfactant (Tween 80) [54]. The small increase in particle size observed may be related to the relatively high molecular weight of nisin.

2.3.2.3. Influence of surfactant type

As mentioned earlier, the movement of a hydrophilic surfactant from the aqueous phase to the organic phase is an important factor in the formation of nanoemulsions by the EPI method. Consequently, it is only possible to use a hydrophilic surfactant that has an appreciable oil solubility using this method. Surfactants that exhibit poor oil solubility, such as label-friendly options like Q-natural (powder or liquid form), sucrose monopalmitate, casein, or whey protein isolate, form opaque colloidal suspensions when mixed with oil rather than the desirable transparent solution [16]. Food-grade non-ionic surfactants, such as Tweens, are generally considered to be the best option for low-energy methods, such as EPI [7]. However, there is a strong tendency in the food industry to move away from using synthetic surfactants, which is one of the major drawbacks of low-energy methods.

Some researchers have found success in correlating the HLB of a surfactant with its ability to produce nanoemulsions using the EPI method. In a study with 10% oil (MCT) and 25% surfactant, it was found that emulsions formed with surfactants that had intermediate hydrophilic lipophilic balance (HLB) values (≈ 15) produced the smallest particle size. Additionally, emulsions made with Tween 85, which is a relatively hydrophobic surfactant with three 3 non-polar tail groups, produced the largest particle

diameter [7]. The success of Tween 80 as a surfactant was likely due to an optimized balance of solubility, molecular geometry and HLB values.

In other studies, molecular geometry was found to be more important than HLB numbers. Molecular geometry can affect factors such as the packing parameter, which in part influences interfacial properties such as mobility and surface tension. Tween 60, which has an HLB value of 14.9, produced significantly larger droplets than Tween 80, which has an HLB of 15, in a system consisting of 10% oil (MCT + VE) and 10% surfactant. As the HLB values are so similar it was likely the molecular packing that caused the difference in particle size. Tween 60 has a single saturated chain (C18:0) while Tween 80 has a single unsaturated chain (C18:1) thus Tween 80 has a higher packing parameter making it more optimal for small particle formation [16]. Therefore, it is important to consider both HLB values and molecular packing when choosing a surfactant for the emulsion phase inversion method.

2.3.2.4. Influence of surfactant concentration

Controlling surfactant concentration is important for financial, quality and health concerns. Therefore it is important to optimize the surfactant concentration so that the desired droplet size can be formed without any negative consequences. . Researchers have found that increasing the surfactant concentration typically leads to a decrease in particle size using the EPI method [7, 16, 52, 53]. The decrease in droplet diameter with increasing surfactant concentration may be due to the need to form O/W/O emulsions throughout the process [16]. The final emulsion diameter is related to the size of the inner oil droplet in the intermediate O/W/O emulsion [7]. Smaller droplets are going to have a larger surface area and thus require a higher surfactant concentration in order to be

stabilized. Increasing surfactant concentration leads to an increase in interfacial area as well as a decrease in interfacial tension [52]. In addition, the structures required to spontaneously form nanoemulsions may only occur at a certain SOW composition.

Another theory on why a higher surfactant concentration leads to smaller particle size is related to the equilibrium phases of the system. For emulsions made with D-Limonene (4%) and Tween 80 (2-6%), a higher surfactant concentration lead to the oil phase being completely dissolved in the water at the emulsion inversion point. A lamellar liquid crystalline phase was able to coexist with an excess oil phase. As water was added into the system with a high surfactant concentration, a large increase in system viscosity was observed which shifted the flow from turbulent to laminar. Therefore, catastrophic phase inversion occurred close to the mixer and the excess oil was incorporated into the liquid crystalline phase which was further diluted with water. At lower surfactant concentrations, only a single phase of lamellar liquid crystals was observed which thus resulted in larger droplets being formed [53].

Surfactant concentration can also affect the shape of the particle size distribution. In a system consisting of 20% oil (Acetem) and varying surfactant concentrations, a bimodal particle size distribution was observed at low surfactant levels (surfactant-to-oil ratio of 0.5) compared to a monomodal particle size distribution at a high surfactant levels (surfactant-to-oil ratio of 1) [52]. This difference in particle size distribution suggests there may be alternate mechanisms of droplet formation depending on surfactant concentration. At lower surfactant levels it is likely that small particles were initially formed but may become trapped between coalescing water droplets in the phase inversion process. With a higher surfactant concentration this is likely not observed. In the same

study it was also found that the critical amount of water required for phase inversion is less when a higher amount of surfactant is used. The presence of more surfactant in the aqueous phase may cause a larger volume of oil droplets to be formed within the aqueous phase thus increasing the total aqueous phase volume and encouraging phase inversion to occur [52].

A large amount of surfactant may not always be the solution for forming fine droplets, as was observed when testing the effect of increased mixing time. Although initially smaller droplets were formed at a higher surfactant concentration, with time this trend was reversed and those made at a lower surfactant concentration were the smallest after mixing for 360 minutes. The increase in particle size at a higher surfactant concentration observed with increased mixing time is likely due to the destabilization of the droplets by Ostwald ripening. The higher surfactant concentration promotes the transfer of oil between droplets and allows the larger droplets to grow at the expense of the smaller ones [52]. Therefore, it is critical to consider preparation conditions such as mixing time when optimizing the surfactant concentration for each system.

2.3.2.5. Thermal stability

Many foods will be exposed to thermal treatments throughout processing (such as pasteurization, sterilization, and/or cooking). Therefore, it is important to understand how emulsions made by the emulsions phase inversion method behave when exposed to high temperatures. Emulsions made with 10% oil (tributylin and Vitamin E) and 10% surfactant (Tween 80) using the EPI method were found to be stable when exposed to temperatures less than 75°C but were unstable at temperatures greater than or equal to 75°C. Exposure time was limited to 30 minutes in order to simulate heat treatment. At the

lower temperatures emulsion droplets were stabilized by electrostatic or steric repulsion great enough to prevent droplet aggregation. With increasing temperatures, however, the water solubility of tributyrin increased which lead to Ostwald ripening and droplet coalescence [55]. Additionally, the non-ionic surfactant (Tween 80) used is to have undergone head-group dehydration at elevated temperatures, which changed the surfactant monolayer curvature and thus promoted droplet growth through coalescence. The thermal instability of emulsions produced by the EPI method may therefore limit their use for some applications.

2.3.2.6. Isothermal stability

Storage conditions, such as temperature and light exposure, will greatly impact the storage stability of emulsions formed by the EPI method. Emulsions produced with tributyrin, Vitamin E and Tween 80 saw greater instability over the span of 30 days when exposed to light and relatively high (40°C) temperatures [55]. In contrast, emulsions made with D-Limonene and Tween 80 displayed greater instability when stored at 28°C versus 4°C over the span of 12 days. This can be explained by Lifshitz–Slezov–Wagner (LSW) theory which states that the Ostwald ripening rate, the main instability mechanism observed in the emulsions, is reciprocally proportional to temperature and indirectly affected by temperature for this system [53]. Other systems exhibited storage stability throughout the tested conditions. In emulsions containing propylene glycol in the aqueous phase and using 4% oil (D-Limonene) with 6% surfactant (Tween 80), good stability (no stratification or turbidity change in samples) was observed when stored at 28°C for 3 months [54]. It is critical when storing emulsions made by the emulsion phase inversion method to consider temperature and light exposure in order to ensure emulsions are stable.

2.3.2.7. Influence of cosolvents/cosurfactants/system composition

The influence of cosolvents, cosurfactants, and system composition has not been thoroughly investigated for the EPI method when using food grade ingredients, and this is therefore an important area for further research. The influence of propylene glycol on the formation of emulsions by the EPI method is one of the few exceptions [53, 54]. Future work could investigate the effect of different types and concentrations of cosolvents on the formation and stability of emulsions made by the EPI method. Additionally, the use of cosurfactants, which has found success in stabilizing emulsions made by spontaneous emulsification, may be suitable for stabilizing emulsions made by EPI. Lastly, as food systems are rarely as simple as those tested in laboratory experiments, it would be interesting to look at the effect of system composition (salt, sugar, biopolymers, pH, etc.) on the formation and stability of emulsions made by this method.

2.3.3. Other isothermal methods

The spontaneous emulsification and emulsion phase inversion methods have been the most commonly used isothermal methods of producing nanoemulsions. Nevertheless, there are other isothermal methods that can also be used. The emulsion inversion point (EIP) method, easily confused with emulsion phase inversion (EPI) method based on their acronyms, involves the progressive addition of water or oil to a preformed microemulsion (water-in-oil or oil-in water) [57, 58]. The EIP method has been used to form allyl isothiocyanate containing nanoemulsions ($d = 137\text{-}215\text{ nm}$) using 6% surfactant (Tween 80 + Span 80) and 19% oil (mineral oil) [57]. The EPI method is really similar to the latter stages of the SE method, which involve the breakdown of a microemulsion into small oil droplets. This method has also be referred to as the phase

inversion composition (PIC) technique by some researchers [59-61]. In addition to the titration of water to the preformed microemulsions, phase inversion may also be promoted by the addition of electrolytes (such as salt), cosurfactants, or cosolvents. For example, an O/W emulsion containing oil droplets coated by an ionic surfactant may be converted to a W/O emulsion by adding salt to reduce the electrostatic repulsion between the surfactant head groups (thereby altering their optimum curvature).

Adding to the confusion of the EIP method, some researchers have defined it as the addition of the aqueous phase into a stirring organic phase [62], which is the same definition used for the EPI method. There is certainly discrepancy over the naming of the low energy methods for nanoemulsion formation. Additionally, some methods combine factors of low energy methods (phase inversion) with elements of high energy methods (use of equipment capable of providing high amounts of mechanical energy like high shear mixers). An example of this is the direct emulsification inversion (DEI) technique which relies on catastrophic phase inversion while applying high shear [63]. This may be viewed as an intermediate low-high method. The EIP/PIC and the DEI methods could be further explored in the realm of food science as no articles could be found on these topics.

2.4. Thermal low energy methods for nanoemulsion formation

Thermal methods, in contrast to isothermal methods, require a change in temperature to induce the formation of a nanoemulsion. The main thermal low energy method is the phase inversion temperature (PIT) method. Nanoemulsions produced by the PIT method were initially thought to be novel because no solvent usage was required

[58]. However, since isothermal methods (such as SE and emulsion phase inversion) can also be used to fabricate nanoemulsions without the use of cosolvents [7, 12, 15, 33, 46, 64], this is no longer a real advantage for the PIT method. Like other low energy methods, the PIT method requires no specialized equipment which is advantageous for lowering capital, operation and maintenance costs [65].

2.4.1. Phase inversion temperature

The phase inversion temperature (PIT) method is typically used to form nanoemulsions from a mixture of a relatively hydrophilic non-ionic surfactant, oil, and water using three main steps (**Figure 4**) [28]:

1. Oil, water, and nonionic surfactant are slightly stirred at room temperature to form a coarse emulsion
2. The mixture is gradually heated up to around or above the PIT
3. The solution is either rapidly cooled or diluted into cold water to form an O/W nanoemulsion

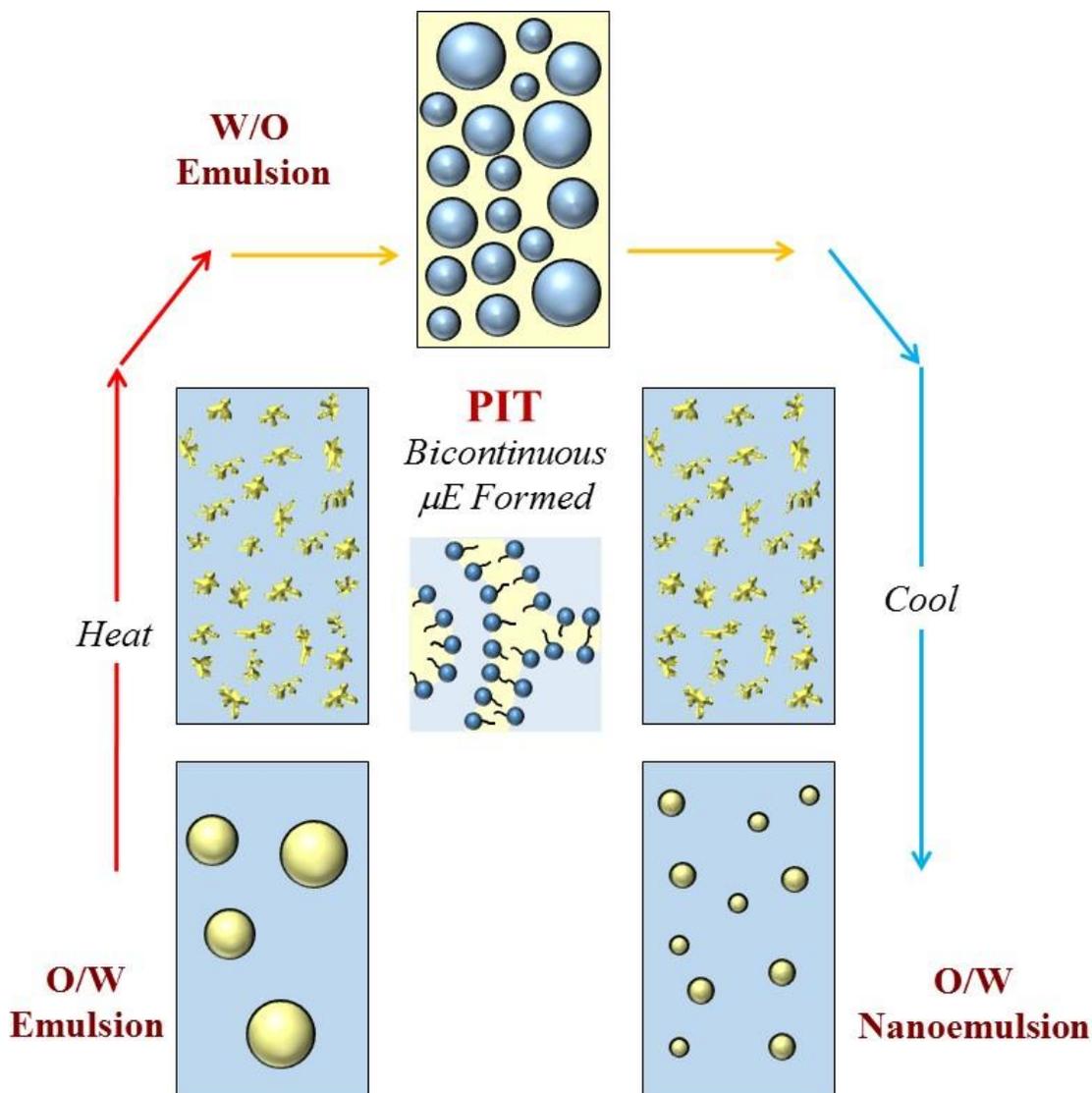


Figure 4. Schematic diagram of proposed mechanism for formation of nanoemulsions by the phase inversion temperature (PIT) method. Typically, a surfactant-oil-water mixture is heated above the phase inversion temperature, and then rapidly cooled with stirring to spontaneously form small oil droplets.

The origin of the formation of small lipid droplets in the SOW system during this process can be related to changes in the structural and physicochemical characteristics of the surfactants during heating. At low temperature, the surfactant head groups are highly hydrated, which means that the surfactant is predominantly hydrophilic and tends to be located in the aqueous phase. At high temperature, the surfactant head groups are largely dehydrated, and so the surfactant is predominately lipophilic and tends to be located in

the organic phase. At a certain intermediate temperature, which is believed to be around the PIT, the surfactant is evenly distributed between the organic phase and the aqueous phase. Thus, the mechanism of nanoemulsion formation by the PIT method has been proposed to be similar to that of the SE method [28]. When an SOW mixture is cooled from above to below the PIT, the surfactant molecules change from lipophilic to hydrophilic and therefore have a tendency to move from the organic phase to the aqueous phase. This process leads to the formation of a bicontinuous microemulsion at the boundary between the two phases, which can break down into small lipid droplets [43]. Therefore, the main driving force for the production of small nanoemulsion droplets using PIT methods is the movement of surfactant from the organic phase into the aqueous phase, similar to the isothermal low energy methods [9, 28].

An alternative or complementary explanation for nanoemulsion formation by the PIT method is based on changes in the optimum curvature of the surfactant molecules with temperature [66]. At relatively low temperatures, the surfactant head groups are highly hydrated and have a molecular geometry ($p < 1$) that favors the formation of O/W emulsions. At intermediate temperatures (near the PIT), the surfactant head groups are partially dehydrated and have a molecular geometry ($p \approx 1$) that favors the formation of planar monolayers. Under these conditions the interfacial tension is extremely low and the formation of bicontinuous microemulsions or other liquid crystalline structures is favored. At high temperature, the head groups are highly dehydrated and have a molecular geometry ($p > 1$) that favors the formation of W/O emulsions. Consequently, when a SOW mixture is cooled from above to below the PIT it moves from a W/O emulsion to a bicontinuous microemulsion to a O/W emulsion. To form small droplets,

the cooling process must typically be carried out rapidly with continuous stirring. Nanoemulsions containing small droplets were formed when SOW systems were rapidly cooled from above to below the PIT, but emulsions containing large droplets were formed when they were cooled slowly [43]. The reason for this effect can be attributed to the fact that extensive droplet coalescence occurs when the systems spend more time in the *droplet coalescence zone* during slow cooling.

Most previous studies have focused on the formation of O/W nanoemulsions using the PIT method, with very limited studies investigating the formation of W/O nanoemulsions [67]. The phase inversion temperature (PIT) method has only successfully been implemented with food grade ingredients in one study, to the best of the author's knowledge: using anhydrous milk fat and Tween 80 transparent nanostructured lipid carriers (NLCs) were produced [65]. The PIT method is specifically suited for the production of NLCs or solid lipid nanoparticles (SLNs) versus the isothermal methods because heating is already required to dissolve lipophilic compounds. Optimizing surfactant concentration is necessary because at higher surfactant concentrations gels may be formed and at lower surfactant concentrations phase separation occurs, just like in the isothermal methods. In contrast, however, NLCs were stable to dilution and more stable against instability mechanisms (such as coalescence and Ostwald ripening); this is likely due to the solid lipid core being more rigid and thus less fluidic [65].

Another way nanoemulsions produced by the PIT method could be stabilized is by surfactant displacement. Researchers found that in a non-food-grade model system of Brij 30 and tetradecane higher temperatures lead to droplet growth by coalescence and

lower temperatures lead to gelation. Nanoemulsions formed by PIT could be stabilized by the addition of a different surfactant, such as Tween 80 or SDS. The proposed explanation for this phenomenon is that the optimum curvature of the interfacial layer is altered as well as an increase in repulsive interactions between droplets thus leading to stability [66]. The use of surfactant displacement could be easily applied when using food grade ingredients.

2.5. Low energy methods for microemulsion formation

There is certainly confusion over the difference between nanoemulsions and microemulsions made by low energy methods [8, 9]. Both types of emulsions require much higher amounts of surfactant than conventional emulsions and may appear to be quite similar in structural and visual aspects. Additionally the formulation of microemulsions and low energy production of nanoemulsions can be analogous also making the two hard to differentiate. The main difference is the thermal instability but it is often confused by the kinetic stability of the nanoemulsions formed. Some researchers claim they have prepared microemulsions but actually formed nanoemulsions, or vice versa [8, 9]. Therefore, having methods to distinguish the two methods (**Table 2**) is incredibly important. Microemulsion production may prove difficult for use in food industry because dilution, which is often necessary in food and beverage systems, can cause instability [9].

The formation of microemulsions using food grade ingredients has been proven in a variety of systems (**Table 8**). Microemulsions can be further distinguished from nanoemulsions by the order of addition: in order to form nanoemulsions the surfactant

must be dissolved in the oil phase. In contrast, microemulsion formation is independent of the order of addition after an equilibration time [9]. Therefore, if order of addition affects particle size it is likely that nanoemulsions, not microemulsions, are formed.

Table 8. An overview of some recent research articles on O/W microemulsion formation with food grade ingredients. A more comprehensive list can be found at [68].

Ingredients				Results	
% Oil	Oil/Bioactive Component	% Surfactant	Surfactant/ Co-Surfactant or Co-Solvent	Particle Dimensions	Reference
10	Lemon Oil	10+	Sucrose monopalmitate	$r < 10 \text{ nm}$	[69]
10	Lemon Oil	20+	Tween 80/Propylene glycol	$r < 10 \text{ nm}$	[70]
10	Clove bud oil	1	Whey protein concentrate, gum arabic, and/or lecithin	$d \approx 150\text{-}550 \text{ nm}$	[71]
3-20	Peppermint Oil	20-26	Tween 20 and sunflower lecithin	$d < 12 \text{ nm}$	[72]
3	Peppermint Oil/ β -carotene	20-23	Tween 20 and sunflower lecithin	$d < 10 \text{ nm}$	[73]

2.6. High energy methods for emulsion formation

In contrast to the low energy methods, high energy methods require the use of devices to form small droplets. These devices often entail a large initial cost as well as expenses to maintain throughout use. The purpose of the devices in high energy methods is to provide intense mechanical energy that helps break up macroscopic phases or turn larger droplets into smaller droplets [11, 74]. In addition, the high energy methods for

nanoemulsion formation are not limited by the types of oil and emulsifiers that can be used like the low energy methods are [75, 76]. Currently high energy methods are more frequently utilized in the food industry than low energy methods with high pressure valve homogenization, microfluidization, and sonication being the most common [77]. All of the high energy methods are impacted by emulsion component characteristics (i.e. oil, type, surfactant type, surfactant concentration, viscosity, etc.) and equipment characteristics (i.e. size of the equipment, pressure used, number of passes/time in equipment, design, etc.) [78]. These parameters should be optimized for each system and high energy method.

2.6.1. High pressure valve homogenizer

Homogenization can be achieved using a high pressure valve homogenizer (HPVH). The use of HPVH is common in applications from ketchup processing to milk homogenization [79-82]. When using a HPVH, a coarse emulsion is initially made using a high-speed mixer, fed into the input valve of the HPVH, and then flowed between the valve seat and valve at a high velocity [83]. With an increase in velocity, the pressure decreases causing an instantaneous pressure drop and encouraging the coarse emulsion to impinge on the impact ring [81]. Some HPVH will pass through two valves and thus emulsion production will be broken up into two stages: in the first stage the droplets are broken up while in the second stage a lower pressure is utilized to disrupt any 'flocs' formed by the initial valve [4, 68].

2.6.2. Sonication

Emulsions produced by sonication use ultrasonic homogenizers (UH) to provide high intensity ultrasonic waves to the sample. The frequency of the waves is higher than the maximum frequency audible to the human ear (16-18 kHz). These waves provide

disruptive forces to breakup oil and water phases thus forming small droplets on the principle of cavitation [84]. Energy input comes from a sonicator probe which can be directly placed in the sample [77]. Intense mechanical vibrations provided by the probe cause pressure gradients to be formed and thus the deformation of droplets that lead to cavitation effects, either the formation, growth or collapse of small bubbles [76]. Currently sonication has been well established for the laboratory scale but may be difficult to implement on a production scale because of issues such as low throughput [80, 85]. Additionally, the high local intensity provided by sonication could lead to detrimental quality effects by way of protein denaturation, polysaccharide polymerization or lipid oxidation of the emulsion components [76].

2.6.3. Microfluidization

Microfluidizers (MF) are utilized when microfluidization is the preferred choice for nanoemulsion formation. Microfluidization is gaining popularity as a novel technique within the food industry having already been proven in the pharmaceutical and cosmetic industries [76, 79, 86]. Initially a coarse emulsion is made using a high speed mixer which is then fed into the hood and accelerated at high velocities within the channels using a pumping device. The channels are made to collide into each other within the interaction chamber [77, 81, 83, 87]. The main parts of a MF include a fluid inlet (where the coarse emulsion is fed), a pumping device (to help move the emulsion through), and the interaction chamber (where the particle collision occurs) [4].

2.7. General comments about low energy methods

In the food industry, it is often important to choose the most appropriate nanoemulsion formation method for a particular application. It is therefore useful to compare different low-energy methods with each other, and to compare low-energy methods with high-energy methods, so that their advantages and disadvantages can be critically assessed.

2.7.1. Comparison between low energy methods

As stated before, low energy methods can be broken down into isothermal and thermal methods. Isothermal methods, like spontaneous emulsification (SE), have many advantages over the thermal low energy method, phase inversion temperature (PIT): 1) SE is easier to implement in that it just requires the addition of a surfactant/oil mixture into an aqueous phase with constant mixing at room temperature, 2) there is no requirement for a temperature sensitive surfactant, 3) there is no requirement for high temperatures which could lead to thermal degradation of sensitive components, and 4) it is capable of producing smaller droplets [43]. In many situations, isothermal methods would be preferred versus thermal methods. However, the PIT method may be more suitable for forming solid lipid nanoparticles, since the lipid phase can be melted at high temperatures ($>$ PIT), but crystallized at lower temperatures after the nanoemulsion has formed.

Similarities can also be observed between the two main isothermal low energy methods, spontaneous emulsification (SE) and emulsion phase inversion (EPI). When a system of 8% VE + 2% MCT as the oil phase was used to produce nanoemulsions using SE and EPI similar results were observed: droplet diameter decreased with an increase in

surfactant concentration and Tween 80 was the most suitable surfactant. Based on this, it is likely that there is a common underlying mechanism that dictates the two methods and perhaps knowledge gained from one method could also apply to the other. Additionally, because EPI goes through a spontaneous emulsification step in its proposed mechanism, it is likely that small droplets can only be formed by EPI if the same system is successful using SE. Because smaller droplets could be formed by spontaneous emulsification in this optimized system ($d \approx 55$ nm for SE and $d \approx 88$ nm for EPI), SE may be better suited for nanoemulsion production [16]. This is advantageous for large scale manufacturing as the organic phase is a smaller volume in oil-in-water emulsions. As spontaneous emulsification involves the addition of the organic phase into the aqueous phase it is likely to be easier to implement than emulsion phase inversion which involves the addition of the aqueous phase into the organic phase.

2.7.2. Comparison of low versus high energy methods

Some researchers have found that in certain surfactant-oil-water systems high energy methods produce smaller particle sizes than low energy methods. For example, when using grape seed oil and orange oil, emulsions made with a microfluidizer were smaller than those made in the same conditions using spontaneous emulsification [47]. Additionally, a much higher concentration of surfactant is required to produce comparable particle size. In a study of 20 wt% MCT oil-in-water emulsions, it was found that a small mean droplet radius ($r < 100$ nm) could be achieved with a surfactant-to-oil ratio (SOR) < 0.1 when using microfluidization but an SOR > 1 was required with the same system utilizing spontaneous emulsification [35]. When comparing to emulsion phase inversion method, an SOR > 0.7 was required to achieved particle points similar to microfluidization at an SOR = 0.1 [7]. This high amount of synthetic surfactant is

undesirable from a cost, taste and toxicity standpoint. High energy methods require much less surfactant to achieve small droplet size when compared with EPI [7, 16]. If low-levels of surfactants are not a necessity then EPI may be a viable option. Additionally, low energy methods like EPI have the added advantage of being inexpensive, energy efficient, and easy to implement [7, 16].

In some cases, such as in the production of Solid Lipid Nanoparticles (SLNs), low energy methods may actually be better at producing small particle sizes than high energy methods. For example, when comparing high-pressure homogenization (HPH) to the low energy phase inversion temperature (PIT) method, smaller particle size was achieved using the PIT method. Additionally, since heating is already required for the melting of the solid lipid phase, the PIT method is likely more cost effective than HPH [65]. Each system may need to be investigated to determine if a high or low energy method would be more appropriate.

2.7.3. Advantages of low energy methods

There are many situations in which low-energy methods may be preferred over high-energy methods. For instance, if the initial capital cost of high-energy equipment may be too large to overcome, low-energy methods may be the solution. Additionally, in certain situations the major drawback of the low-energy methods (high use of synthetic surfactants) may be overcome by significant dilution of the initial emulsion, *e.g.*, in beverages where the final oil and surfactant concentrations are very low (< 0.1%). Furthermore, some bioactive compounds cannot be encapsulated using high-energy methods due to the rise in temperature caused by the high amount of energy. There are certain remedies to this, such as the use of ice to surround a homogenizer, but these

cooling methods will ultimately contribute more to the cost of using high-energy methods. In these cases, isothermal methods may be useful to encapsulate compounds that are heat sensitive since no high temperatures are required. This means that SE or EPI methods may be useful for these types of encapsulates.

2.7.4. Disadvantages of low energy methods

While low-energy methods have some advantages over high-energy methods, the types of oils and emulsifiers that can be used often limit them. Previous studies suggest that best type of oils to use to form nanoemulsions using low energy methods are medium chain triglycerides (MCT). It is often difficult to produce very small droplets using long chain triglycerides (LCT) using low energy methods, which limits this method for many applications, e.g., fish or algal oils. It is sometimes possible to overcome this problem by mixing LCT oils with other oils (such as flavor oils) that facilitate nanoemulsion formation. The LCT oils have the additional advantages of inhibiting Ostwald ripening in nanoemulsions formed from fairly polar oils, such as flavor or essential oils [88]. Oil solubility increases with decreasing droplet size so that large droplets grow at the expense of smaller ones. There is believed to be a linear relationship between the cube of the radius and time, according to LSW (Lifshitz-Slezov-Wagner) theory [59]. Because LCT are not suitable for use with low-energy methods [7, 15], alternative strategies to combat Ostwald ripening will have to be considered.

Currently, low-energy methods have only been shown to work with synthetic surfactants, such as Tweens and Spans. . Additionally, relatively high concentrations of synthetic surfactants are required to form nanoemulsions (often around $SOR = 1$) which could be limiting for many applications due taste, safety and economic reasons [7, 33,

89]. There is hope that future research could successfully use natural emulsifiers, such as lecithin, when utilizing low energy methods. The use of natural emulsifiers with low energy methods could potentially make the processes much more appealing.

2.7.5. Water-in-oil emulsion formation

Water-in-oil emulsions have been made by low energy methods with non-food grade components by adding oil into a stirring water and surfactant mixtures [90, 91] or by the phase inversion temperature method [67, 92]. While oil-in-water emulsions are most common in food systems, it may be of interest to investigate if water-in-oil emulsions can be made by low energy methods with food grade components.

2.8. Applications of low energy methods

Low-energy methods are unsuitable for the formation of food products that contain relatively high levels of fat, such as salad dressings or mayonnaise, since there would be high levels of surfactant present in the final product. Conversely, they are suitable for applications that only require a low amount of oil in the final product, such as fortified waters and soft drinks, since the total amount of surfactant in the final product is relatively low, even though the surfactant-to-oil ratio is high.

2.8.1. Bioactive delivery systems

Numerous studies have shown that hydrophobic bioactives, such as vitamins, nutrients, and nutraceuticals, can be incorporated into nanoemulsions produced by low-energy methods. For example, studies have shown that vitamin D [33], vitamin E [39] and carotenoids [65] can be encapsulated in oil-in-water nanoemulsions. Studies have also shown that the nanoemulsions are rapidly digested under simulated gastrointestinal

conditions, and form mixed micelles that can solubilize the hydrophobic bioactives [93]. Recent studies have shown that nanoemulsions formed by the spontaneous emulsification may be a viable means of fortifying food gels with low levels of [46]. Small lipid droplets could be incorporated into gelatin gels without appreciably affecting their rheology or appearance, which may be useful for incorporating lipophilic bioactive agents into transparent hydrophilic products.

2.8.2. Antimicrobial delivery systems

One area that holds a lot of promise for low-energy methods is in the production of antimicrobial delivery systems [36, 94]. For example, nanoemulsions produced using the SE method with an oil phase of carvacrol and MCT were proven to be effective at controlling growth of *Salmonella enterica* and *E. coli* on mung beans and alfalfa seeds [95]. Similarly, antimicrobial nanoemulsions formed by the SE method have shown to be effective against acid-resistant spoilage yeasts [96]. Production of antimicrobial nanoemulsions can be made by low energy methods in a simple cost effective manner, which may facilitate their application.

2.9. Conclusion

Nanoemulsions are of interest because their small size leads to high optical clarity and good stability, two important qualities for incorporation into foods. However, their production often requires high amounts of energy which may make them cost ineffective. Therefore, there is interest in investigating low energy methods to produce nanoemulsions either with or without the help of elevated temperatures. All the low energy methods may share a common mechanism where the surfactant moves from the

oil phase into the aqueous phase in order to form fine emulsion droplets at the oil-water interface. The major disadvantage for the low energy methods for nanoemulsion production is the requirement for high amounts of surfactant. However, for certain applications the cost of the extra surfactant may be less than the initial capital cost for the high-energy methods (**Table 9**). Between 10,000 and 100,000 kilograms of 10% oil-in-water emulsion can be produced before it approaches the cost of high energy equipment (\approx \$10,000-200,000). Therefore, if taste and toxicity can be controlled by dilution, the low-energy methods hold a lot of promise for certain applications within the food industry. Future research should focus on investigating the use of natural emulsifiers as well as different food systems and encapsulates. In addition, methods of reducing the surfactant-to-oil ratio and the range of different oils that could be homogenized would also be advantageous.

Table 9. Estimated surfactant cost (Tween 80) for 10% oil-in-water emulsions made by low and high energy methods.

			Surfactant Cost for varied weights of 10% oil Emulsion (\$)					
Method	Typical SOR	Surfactant amount (kg) in 1 kg of 10% o/w emulsion	1 kg	10 kg	100 kg	1,000 kg	10,000 kg	100,000 kg
Low Energy	1	0.1	1.49	14.88	148.80	1,488.00	14,880.00	148,800.00
High Energy	0.1	0.01	0.15	1.49	14.88	148.80	1,488.00	14,880.00
Difference			1.34	13.39	133.92	1,339.20	13,392.00	133,920.00

CHAPTER 3

OPTIMIZATION OF ISOTHERMAL LOW-ENERGY NANOEMULSION FORMATION: HYDROCARBON OIL, NON-IONIC SURFACTANT, AND WATER SYSTEMS

3.1. Abstract

Nanoemulsions can be fabricated using either high-energy or low-energy methods, with the latter being advantageous because of ease of implementation, lower equipment and operation costs, and higher energy efficiency. In this study, isothermal low-energy methods were used to spontaneously produce nanoemulsions using a model system consisting of oil (hexadecane), non-ionic surfactant (Brij 30) and water. Rate and order of addition of surfactant, oil and water into the final mixture were investigated to identify optimal conditions for producing small droplets. The emulsion phase inversion (EPI) and spontaneous emulsion (SE) methods were found to be the most successful, which both require the surfactant to be mixed with the oil phase prior to production. Order of addition and surfactant-to-oil ratio (SOR) influenced the particle size distribution, while addition rate and stirring speed had a minimal effect. Emulsion stability was strongly influenced by storage temperature, with droplet size increasing rapidly at higher temperatures, which was attributed to coalescence near the phase inversion temperature. Nanoemulsions with a mean particle diameter of approximately 60 nm could be produced using both EPI and SE methods at a final composition of 5% hexadecane and 1.9% Brij 30, and were relatively stable to droplet growth at temperatures $< 25^{\circ}\text{C}$.

3.2. Introduction

Oil-in-water (O/W) emulsions are utilized in a wide range of industries to encapsulate, protect, and/or deliver lipophilic components, *e.g.*, pharmaceuticals, cosmetics, foods, agrochemicals, and petrochemicals. Emulsions are formed when one of two immiscible liquids is dispersed in the other liquid as small spherical droplets [4, 58]. The resulting systems are thermodynamically unstable and may breakdown through a variety of instability mechanisms, including gravitational separation, coalescence, flocculation, and Ostwald ripening. Nanoemulsions are emulsions whose droplet diameter typically falls in the range of 20-200 nm [1]. Unlike microemulsions, which may have similar particle sizes, nanoemulsions are also thermodynamically unstable systems that have a tendency to breakdown over time. There has been growing interest in the formation, stabilization and utilization of nanoemulsions due to their novel physicochemical properties, high optical clarity, good stability to gravitational separation and aggregation, and ability to increase the bioavailability of encapsulated active ingredients [6, 10, 97].

Nanoemulsions can be fabricated using both high energy and low energy approaches. High energy approaches utilize specialized equipment (“homogenizers”) capable of generating intense mechanical forces that disrupt and intermingle the oil and water phases. The main variables that impact nanoemulsion characteristics using high energy methods are the energy intensity and duration, the surfactant type and concentration, and the physicochemical properties of the oil and water phases [98]. In contrast, low energy approaches rely on the spontaneous formation of emulsions based on the phase behavior of certain surfactant, oil, and water systems [3]. There is interest in

using lower energy techniques in the emulsion formation process due to economic benefits [58] and increasing amounts of research have been conducted to investigate the utility of different low-energy approaches [3, 39, 99, 100]. However, the goal of using low energy in a high product throughput industry setting has yet to be fully realized [80] with few studies investigating the effect of scaling-up from a laboratory setting [101].

Low energy approaches can be broadly categorized as either thermal or isothermal methods. Thermal methods rely on emulsion formation due to changes in surfactant properties with temperature, whereas isothermal methods rely on emulsion formation due to changes in local system composition at a fixed temperature. The spontaneous emulsification (SE) and emulsion phase inversion (EPI) methods fall into the category of isothermal methods [39, 99], while the phase inversion temperature (PIT) method is an example of a thermal method [58]. In the SE method, an emulsion is formed when an oil-surfactant mixture is added to water, whereas in the EPI method, an emulsion is formed when water is added to an oil-surfactant mixture [101]. In the PIT method, an emulsion is formed when a surfactant-oil-water mixture is rapidly cooled below the phase inversion temperature (PIT) with continuous mixing [102].

One of the main objectives of the current study was to investigate the formation of nanoemulsions by low energy isothermal methods using a well-defined model system: hydrocarbon oil, non-ionic surfactant, and water. A substantial amount of research has already been carried out on optimizing emulsion formation by emulsion phase inversion [7] and spontaneous emulsification [39, 103] methods, but few studies have directly compared these two approaches with each other and with other possible isothermal methods [1, 3, 101, 104]. In principle, there are six different methods of forming

nanoemulsions from surfactant (S), oil (O) and water (W) by injecting one liquid into another liquid at fixed temperature: (SO)→W; (W)→SO; (SW)→O; (O)→SW; (OW)→S; and, (S)→OW. Here, the material in parentheses is initially in an injector, while the other material is initially in a reaction vessel. In this study, we investigated all six possible methods of forming nanoemulsions using this approach. Previous studies have compared two or three of these methods. Forgiarini reported that nanoemulsions could be formed using the (W)→SO method but not with the (O)→SW method [105]. Studies using similar surfactants, oils, and water phases have reported differences in the size of the particles produced by the (W)→SO method (EPI) and the (SO)→W method (SE) [39, 99]. We looked to further investigate if differences could be seen between the two methods. In addition to examining order of addition effects, we also investigated the influence of surfactant-to-oil ratio, addition rate, stirring speed, and storage temperature on the formation and stability of emulsions formed by isothermal low energy methods.

3.3. Materials and methods

3.3.1. Materials

Hexadecane (Sigma–Aldrich, St. Louis, MO) was used as the hydrocarbon oil phase. Polyoxyethylene (4) lauryl ether (Brij 30) (Acros, Thermo Fisher Scientific, NJ) was used as the non-ionic surfactant. Distilled and deionized water was used as the aqueous phase to prepare all solutions and emulsions (Milli-Q®). For convenience, we use the symbols S to refer to surfactant, O to refer to oil, and W to refer to water in the remainder of the chapter.

3.3.2. Emulsion preparation

3.3.2.1. Influence of order of addition

Emulsions were prepared by simple addition of 1 or 2 components (S, O and/or W) from an automated electronic pipette (Rainin SE4, Mettler Toledo, Oakland, CA) into a beaker containing 1 or 2 components (S, O, and/or W) and stirring at 700 rotations per minute (RPM) using a magnetic stir bar at room temperature ($\sim 20^{\circ}\text{C}$). All combinations of water, oil and surfactant were tested for a total of 6 methods: (SO) \rightarrow W; (W) \rightarrow SO; (SW) \rightarrow O; (O) \rightarrow SW; (OW) \rightarrow S; and, (S) \rightarrow OW (**Figure 5**). The material in parentheses was initially in the pipette (injector), while the other material was initially in the beaker (reaction vessel). The titration was done over 20 minutes and the sample was allowed to stir for an additional 5 minutes for a total mixing time of 25 minutes. Method (SO) \rightarrow W is also known as Spontaneous Emulsification (SE), while Method (W) \rightarrow SO is also known as Emulsion Phase Inversion (EPI). Prior to emulsion production, initial phases containing two components were mixed for a minimum of 30 minutes at 500 RPM. These experiments were carried out at a fixed surfactant-to-oil ratio (SOR) of 0.375. These preliminary experiments indicated that only the SE and EPI methods were able to produce very fine droplets, and so only these two methods were used in later studies.

The aliquot volume, interval time, and dispense speed of the electronic pipette used to titrate the systems were controlled. All pipetted aliquots were divided into 100 increments and the interval time was varied to attain the desired addition time. To obtain the same final SOR, the aliquot and total volumes had to be adjusted for each system.

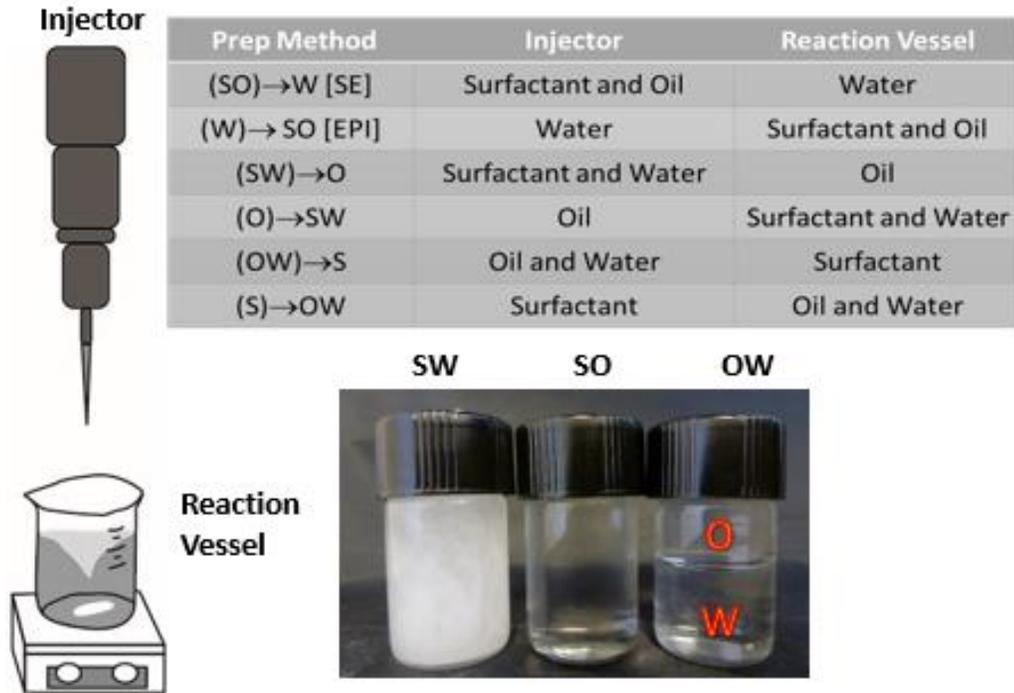


Figure 5. Visual representation of the order of addition screening study. Table shows the six different preparation methods tested. The photographs show vials containing various two component mixtures: surfactant and water formed a gel; surfactant and oil formed a homogenous solution; and, oil and water were immiscible (oil phase on top of water phase).

3.3.2.2. Influence of surfactant-to-oil ratio

The influence of surfactant concentration was investigated by varying the surfactant-to-oil ratio (SOR). The total oil content in the final systems was held constant at 5%, while the SOR was varied by altering the amounts of surfactant and water content in the final system:

$$\text{SOR} = m_s/m_o \quad (3.1)$$

$$m_w = 100 - m_o - m_s \quad (3.2)$$

Here, m_s , m_o and m_w are the masses of surfactant, oil and water, respectively. SORs tested included 0.1, 0.25, 0.375, 0.5, 0.625, 0.75, 1, 1.25 and 1.5. Total mixing time was

held constant at 25 minutes (20 minutes for titration and 5 additional minutes), stir speed was held constant at 700 RPM, and the experiments were conducted at room temperature (~20°C).

3.3.2.3. Influence of addition rate

The addition rate was tested by varying the interval time of the electronic pipette. All experiments were carried out at a fixed SOR of 0.375 because the smallest particle size was achieved here. Addition times tested were 0.25, 0.75, 1.5, 5 and 20 minutes. After all the components were mixed together the samples were allowed to stir for an additional 5 minutes before being removed. All samples were stirred at 700 RPM and experiments were conducted at room temperature (~20°C).

3.3.2.4. Influence of stirring speed

The effect of stirring speed was tested by changing the rotational speed of the stirrer throughout production of the emulsions. Pre-established optimized conditions (SOR = 0.375; addition time = 5 minutes) were used for these tests. The stirring speeds tested include 0, 60, 150, 300 and 700 rotations per minute (RPM). All experiments were conducted at room temperature (~20°C).

3.3.3. Emulsion stability tests

After optimizing the production of the nanoemulsions, their storage stability at three different temperatures (25, 30, and 35°C) was tested. These experiments were carried out using pre-established optimized parameters: SOR = 0.375; addition time = 5 minutes; stirring speed = 700 RPM. Emulsions were tested for both particle size and turbidity throughout 8 hours storage. Temperature scanning of selected nanoemulsions was also carried out. The turbidity of the nanoemulsions was measured over the temperature range 15 to 50 °C, at a scan rate of 0.5°C/minute.

3.3.4. Emulsion characterization

3.3.4.1. Particle size analysis

Dynamic light scattering: Systems containing relatively small droplets ($d < 3000$ nm) were analyzed by dynamic light scattering (DLS). The particle size distribution, mean particle diameter, and polydispersity index (PDI) were measured using a commercial DLS instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Samples were diluted prior to measurement by adding 10 μ L of emulsion into 5 mL of water. Initial experiments showed that dilution did not influence the measured particle size (Table 10).

Table 10. Effect of dilution on mean particle diameter and attenuator value for dynamic light scattering methods. Samples were analyzed as prepared (undiluted) or after dilution with water.

Name	Mean Diameter (nm)	Attenuation
Undiluted	55.0 \pm 0.6	3.0 \pm 0.0
Diluted	58.1 \pm 0.7	7.8 \pm 0.4

Static light scattering: Systems containing relatively large droplets ($d > 1000$ nm) were analyzed by static light scattering (SLS). The particle size distribution and mean particle diameter (D[3,2]) were measured using a commercial SLS instrument (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). A refractive index of 1.33 was used for the water phase and 1.43 for the oil phase. Samples were diluted in water prior to analysis to avoid multiple scattering effects.

3.3.4.2. Turbidity measurements

The turbidity of selected emulsions was measured at 600 nm using a UV–visible spectrophotometer with temperature scanning capabilities (Evolution Array, Thermo Scientific, Waltham, MA).

3.3.4.3. Optical microscopy/microstructure analysis

A Nikon optical microscope (C1 80i Digital Eclipse, Tokyo, Japan) with a 60x objective lens and 10x eyepiece was used to capture the images of emulsions produced immediately after production and after a thirty minute delay. Approximately 5 μ L of emulsion was placed between slide and coverslip and observed by optical microscopy equipped with a cross-polarized lens. The cross-polarized lens allowed the presence of any non-isotropic structures to be determined, such as crystals or liquid crystals. The images were analyzed using image analysis software (Nikon, Melville, NY, U.S.).

3.3.5. Experimental design

All measurements were performed on two freshly prepared samples in triplicate. The mean and standard deviations were calculated from this data. Statistical analysis was performed through subsection of the data to analysis of variance (ANOVA) using commercial statistics software (Minitab 16.2.4, Minitab Inc., State College, PA). Means were subject to Tukey's test and a *P*-value of <0.05 was considered statistically significant.

3.4. Results and discussion

3.4.1. Influence of order of addition

Initially, the effect of order of addition on the nature of the emulsion formed was investigated. Very different particle sizes and emulsion stabilities were obtained by

changing the preparation method used, *i.e.*, which components were initially in the injector and which were initially in the reaction vessel. (SO)→W and (W)→SO, or SE and EPI respectively, produced nanoemulsions with a surface-weighted mean diameter ($D_{[3,2]}$) of approximately 0.1 μm . All other methods produced mean particle diameters about 2 orders of magnitude higher, *i.e.*, around 20 to 30 μm (**Figure 6**). The difference in particle size between SE and EPI methods compared to all other preparation methods was statistically significant, while the difference between the SE and EPI methods was non-significant ($p < 0.05$). All further experiments were therefore carried out using only the SE and EPI methods since these were the only ones capable of producing very fine droplets. Indeed, the nanoemulsions formed using these methods were transparent with a slight blue appearance, similar to results reported in other studies [105].

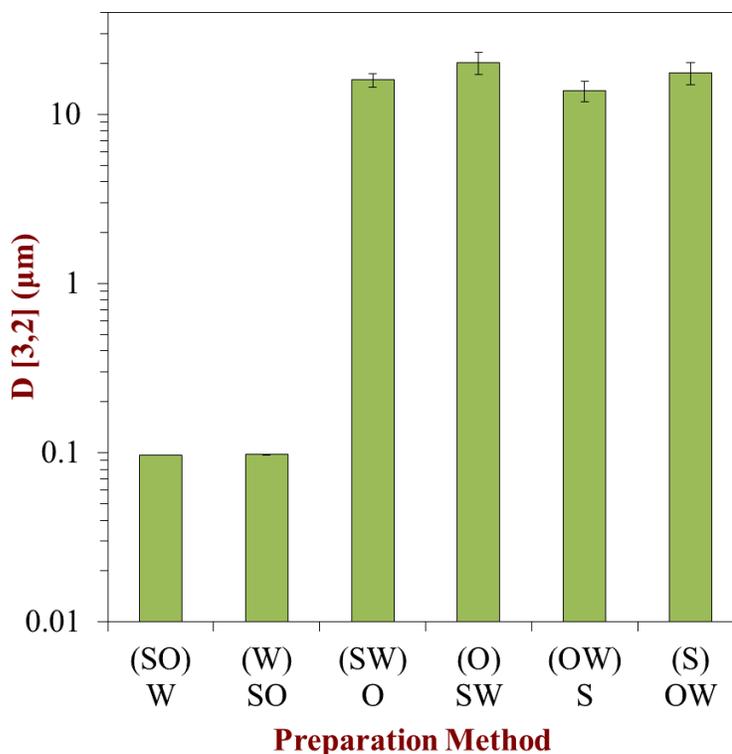


Figure 6. Effect of preparation method on initial mean droplet diameter for six different low energy isothermal preparation methods.

The main factor distinguishing the SE and EPI methods from all the other preparation methods was that the oil and surfactant phases were intimately mixed together prior to combining them with water. There are 12 carbon atoms in the hydrocarbon chain of Brij 30 and 16 carbon atoms in hexadecane, which enabled them to form a molecular dispersion by simply mixing [103]. In contrast, water and hexadecane were completely immiscible due to the hydrophobic effect, and so when WO was in either the pipette or the beaker it did not form a homogenous molecular dispersion. Additionally, when water and Brij 30 were mixed together they formed a gel, indicating that these two components did not form a homogeneous molecular dispersion either. These results suggest that in order for a successful nanoemulsion to be formed using isothermal low-energy methods there must be miscibility among the two mixed components prior to production.

The surfactant used to form the nanoemulsions is also important because it must be capable of moving from the oil phase into the aqueous phase when they come into contact. As already mentioned, we were able to form nanoemulsions with Brij 30 using the (W)→SO or (SO)→W methods, however we were not able to form them with Tween 80 under similar conditions. This phenomenon may be related to the fact that Tween 80 was more hydrophilic than Brij 30, and therefore could not be successfully dispersed into the oil phase. Indeed, a clear solution was formed when Brij 30 was mixed with hexadecane, but a solution with two distinct phases was formed when Tween 80 was mixed with hexadecane. Similar to our work, other researchers have also reported that nanoemulsions could be formed using the (W)→SO method but not with the (O)→SW method, again highlighting the importance of having the surfactant and oil phases

intimately mixed prior to introduction of the water phase [105]. Some studies have reported differences in the size of the particles produced by the (W)→SO method (EPI) and the (SO)→W method (SE) [39, 99], which suggests that the pathway taken is important for certain systems even if the oil and surfactant are intimately mixed prior to their interaction with water.

A number of previous studies indicate that the pathway taken through the SOW composition phase diagram plays an important role in determining the final size of the droplets formed [1, 3, 101, 104]. These studies suggest that nanoemulsions containing ultrafine droplets can be formed by spontaneous emulsification when the pathway rapidly crosses through a SOW composition consisting of an oil-in-water microemulsion, but not when it crosses a SOW composition that exists as a lamellar liquid crystalline phase [104]. This effect was attributed to the extremely high viscosity of lamellar liquid crystalline phases, which may retard molecular motion and inhibit the spontaneous formation of ultrafine droplets. On the other hand, nanoemulsions can be formed by emulsion phase inversion if water is added very slowly to a lamellar liquid crystalline phase containing surfactant and oil, but not when it is added rapidly [104]. These studies highlight the importance of both the pathway for nanoemulsion formation, as well as the preparation conditions.

3.4.2. Influence of SOR on particle size

Surfactant-to-oil ratio (SOR) had a significant effect on particle size with the smallest droplets being formed at an SOR of 0.375 for both preparation methods (**Figure 7**). At a lower SOR than 0.375, the particle size was appreciably higher. Other researchers have reported similar results using other surfactants with lower surfactant

concentrations leading to larger particle sizes using isothermal low energy methods [106]. At SORs higher than 0.375, the final emulsion became highly viscous and gel-like, which suggests that a liquid crystalline phase may have been formed at these surfactant, oil, and water compositions. Additionally at the higher SORs a bimodal particle size distribution was observed (**Figure 8**). Both spontaneous emulsification (SE) and emulsion phase inversion (EPI) methods exhibited similar dependencies of particle size on SOR. Indeed, there was a non-significant difference in particle diameter between SE and EPI methods at all SORs. All further experiments were therefore carried out at an SOR of 0.375 since this gave the smallest droplet size.

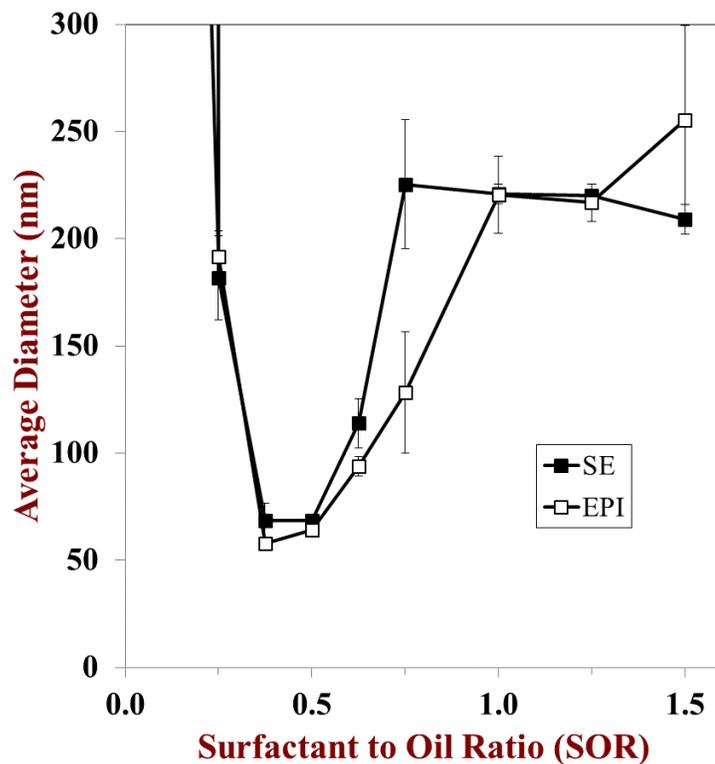
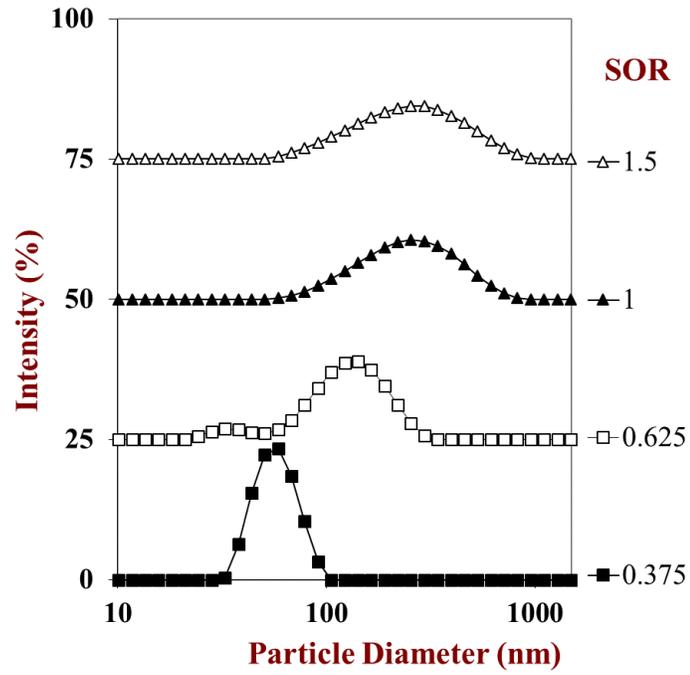


Figure 7. Effect of surfactant-to-oil ratio (SOR) on the initial mean droplet diameter formed by spontaneous emulsification (SE) and emulsion phase inversion (EPI) methods.

a)



b)

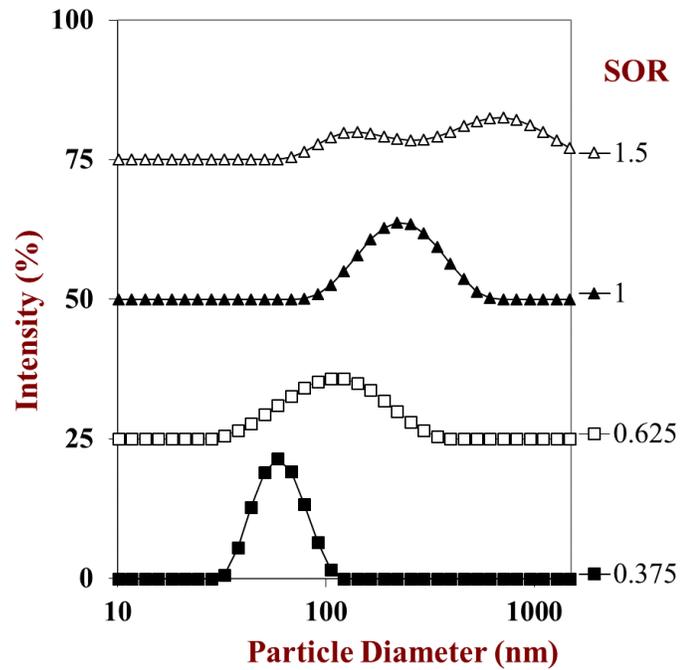


Figure 8. Effect of surfactant-to-oil ratio (SOR) on the particle size distributions of emulsions formed by a) spontaneous emulsification and b) emulsion phase inversion methods.

A possible reason for the close similarity in the droplet size versus SOR dependence for the spontaneous emulsification and emulsion phase inversion methods may be the nature of the physicochemical processes involved. In the SE method, an oil-in-water (O/W) nanoemulsion is directly formed when the SO mixture is titrated into the water phase. On the other hand, in the EPI method a water-in-oil (W/O) emulsion is initially formed when water is titrated into an SO mixture. However, at higher water contents the system converts to an oil-in-water-in-oil ($O_1/W/O_2$) emulsion that then breaks down to an oil-in-water system upon further water addition. It has been proposed that the internal oil phase (O_1) forms the droplets in the final O/W nanoemulsion created at the end of the titration process [56, 107]. These internal oil droplets (O_1) are likely to be formed by the spontaneous emulsification process at the boundary between the SO and water phases in the initial W/O emulsion, and therefore depends on similar factors as the SE method.

3.4.3. Influence of addition rate on particle size

The addition rate used to titrate one phase (SO or W) into the other phase (W or SO) appeared to have a minimum effect on particle size (**Figure 9**). For the EPI method, no significant difference was found between all addition times. For the SE method, a significant difference could only be seen at 0.25 minutes; all other preparation times were not significantly different. Comparing the SE and EPI methods, significant differences were observed between the two preparation methods at addition times of 0.25, 0.75 and 1.5 minutes. However, at longer addition times (5 and 20 minutes) no significant difference was observed between the two methods. All further experiments were

therefore carried out using an addition time of 5 minutes since this time produced small droplets using both methods.

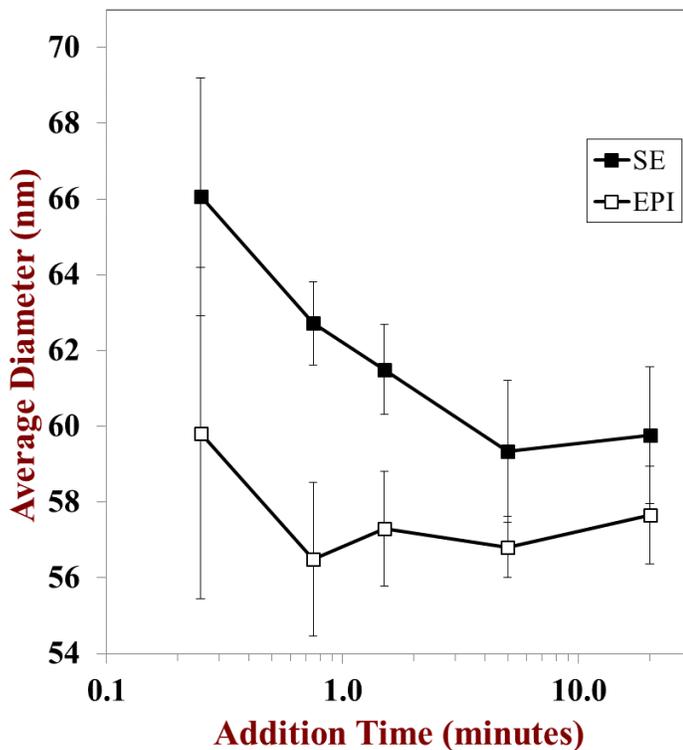


Figure 9. Effect of addition rate on initial mean particle diameter of emulsions formed by spontaneous emulsification (SE) and emulsion phase inversion (EPI).

These results are important because they imply it is possible to form emulsions with small particle sizes (≈ 60 nm) in a short time. Additionally, it shows that there are no added benefits to producing emulsions over long periods of time which has positive repercussions for industrial manufacturing of emulsion products. In general, one might expect that each surfactant, oil, water combination would have a different optimum rate of addition depending on factors such as the SOW phase diagram and phase properties (such as rheology and microstructure) [68]. In this case, it may be necessary to optimize the preparation conditions for each surfactant-oil-water combination used.

3.4.4. Influence of stir speed on particle size

Stirring speed had an appreciable effect on the mean particle diameter produced depending on the preparation method used (**Figure 10**). For the EPI method there was no significant difference in mean particle diameter until the stirring speed was reduced to 0 RPM. The differences between 60, 150, 300 and 700 RPM were all non-significant ($p < 0.05$). In contrast, the particle size produced by the SE method was much more dependent on stirring speed. Production at 700 RPM using the SE method produced nanoemulsions with the smallest droplet diameters ($d \approx 58$ nm). Production at 60, 150 and 300 RPM produced nanoemulsions with mean particle diameters around 80 nm, with no significant difference between these three speeds. At 0 RPM no emulsion could be produced using the EPI technique, instead the oil-surfactant phase simply sat on top of the water phase (**Figure 11**).

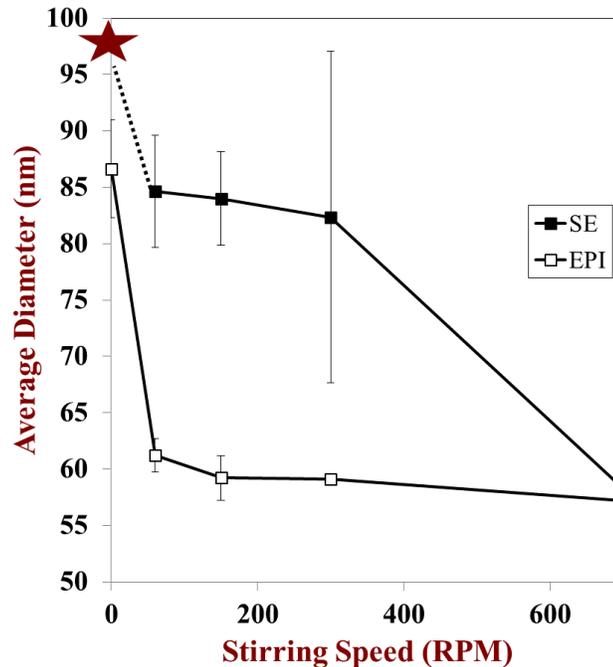


Figure 10. Effect of stirring speed on initial mean particle diameter of emulsions formed by spontaneous emulsification and emulsion phase inversion methods. The star indicates that this emulsion was highly unstable and its size could not be reliably measured using DLS.

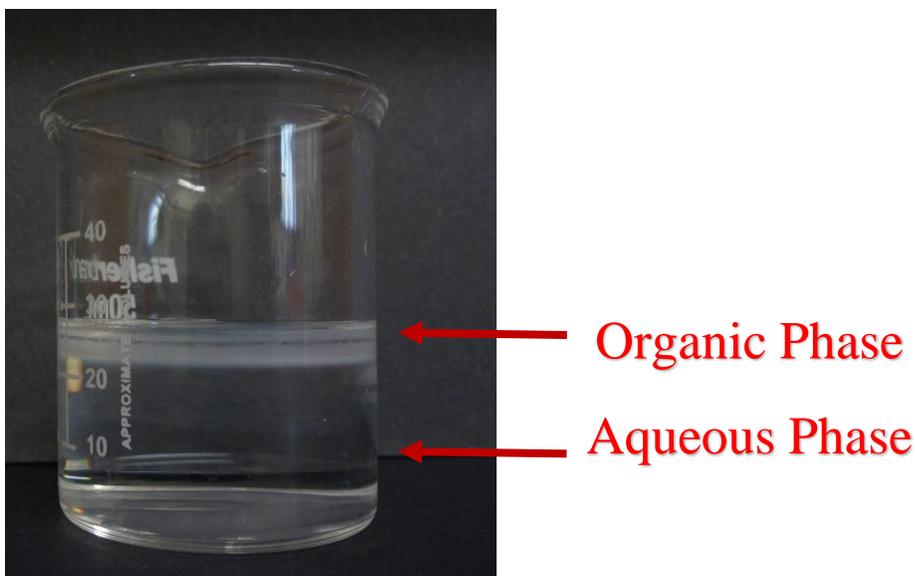


Figure 11. Photograph of surfactant-oil-water system when preparing emulsion by emulsion phase inversion method at 0 rpm. No emulsion could be formed with these conditions; the organic phase (surfactant and oil) simply sat atop the aqueous phase.

There are practical benefits from being able to produce nanoemulsions at lower stirring speeds due to cost savings associated with energy input, which is an important factor for the scale-up of this process to an industrial setting [80]. These experiments suggest that the EPI method produces smaller droplets than the SE method at lower stirring speeds, and may therefore be more suitable for industrial applications.

A possible explanation for the observed differences between the two methods is associated with differences in the amount of titrant that needed to be added to the reaction vessel. The final composition of the system used in these experiments was 5% hexadecane, 1.9% Brij 30, and 93.1% water. For the EPI method, a large volume of water is titrated into a small volume of surfactant-oil. However, for the SE method, a small volume of surfactant-oil is titrated into a large volume of water. At lower mixing speeds, the force from the addition of the titrant into the reaction vessel may provide enough mixing for an emulsion to be formed. This was seen clearly at 0 RPM where an emulsion

could still be formed using the EPI technique but no emulsion was formed using the SE technique. There may also be differences due to the droplet formation mechanism. In the SE method, small oil droplets should be formed as soon as the SO mixture is added to the water. On the other hand, in the EPI method, a W/O emulsion, then O₁/W/O₂ emulsion, and lastly O/W nanoemulsion is formed. The spontaneous formation of fine oil droplets within the W/O emulsion (EPI method) may be less sensitive to addition rate and stirring speed than their formation when S/O is directly titrated into water (SE method).

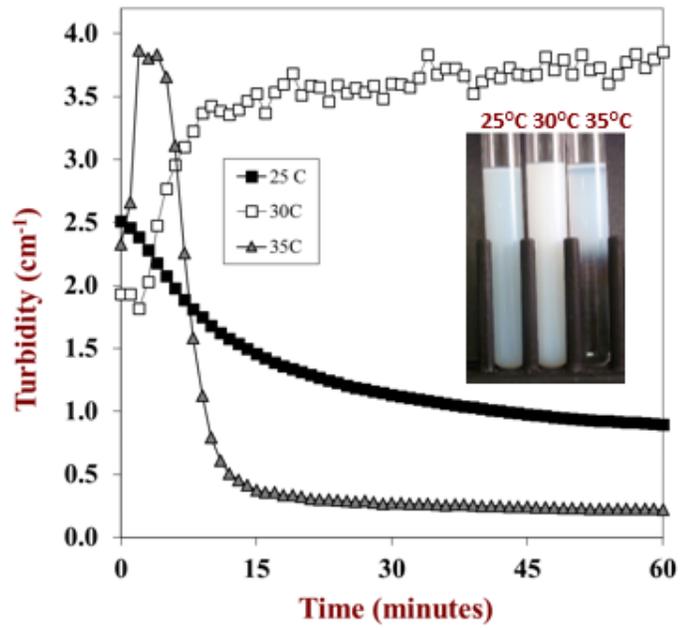
Some researchers have reported that mixing rate has no effect on emulsion properties when using low-energy processes due the driving force being self-assembly of surfactant molecules [68]. This is in direct contrast to the results we observed with our system, where stirring speed did have a major influence on droplet size. Some researchers have reported that too high mixing rates can lead to the promotion of droplet coalescence [101], a result that was not observed with our system. These results suggest that every system should be investigated individually since factors such as the phase behavior of the surfactant-oil-water system and the physicochemical properties of the components greatly impact the effect of variables like stirring or mixing speed.

3.4.5. Effect of isothermal storage

In practical applications it is important that nanoemulsions have a sufficiently long shelf life after they have been formed, and so we examined their storage stability. The storage temperature (25, 30 or 35°C) had a significant effect on the turbidity of nanoemulsions prepared using both the SE and EPI methods (**Figure 12**). The figures only show the first 60 minutes of storage because after this point the turbidity remained fairly constant.

The change in turbidity with time was strongly dependent on isothermal storage temperature for emulsions prepared using the SE method. At 25°C, the nanoemulsions remained homogeneous during storage but there was a decrease in turbidity during the first 30 minutes after preparation, and then the appearance remained fairly stable. This effect was attributed to the initial formation of surfactant-oil-water structures (liquid crystals) in the samples that broke down over time, as observed by cross-polarized optical microscopy (**Figure 13**). At 30 °C, the turbidity of the nanoemulsion increased steeply with time during the first 15 minutes, and then remained optically opaque. At 35°C the turbidity of the nanoemulsion rapidly increased during the first 2 minutes of storage, and then decreased rapidly. The decrease observed at later times was attributed to phase separation, *i.e.*, upward movement of the droplets leading to clearing at the bottom (**Figure 12.a**). We hypothesize that the droplets were highly unstable to coalescence at elevated temperatures, which led to an increase in mean particle diameter, rapid creaming, and phase separation. Indeed, optical microscopy measurements indicated the presence of relatively large droplets when samples were stored at elevated temperatures.

a)



b)

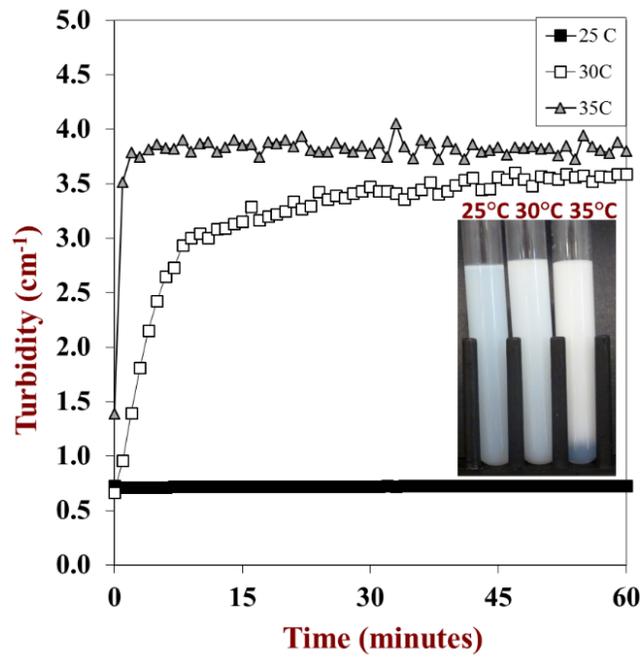
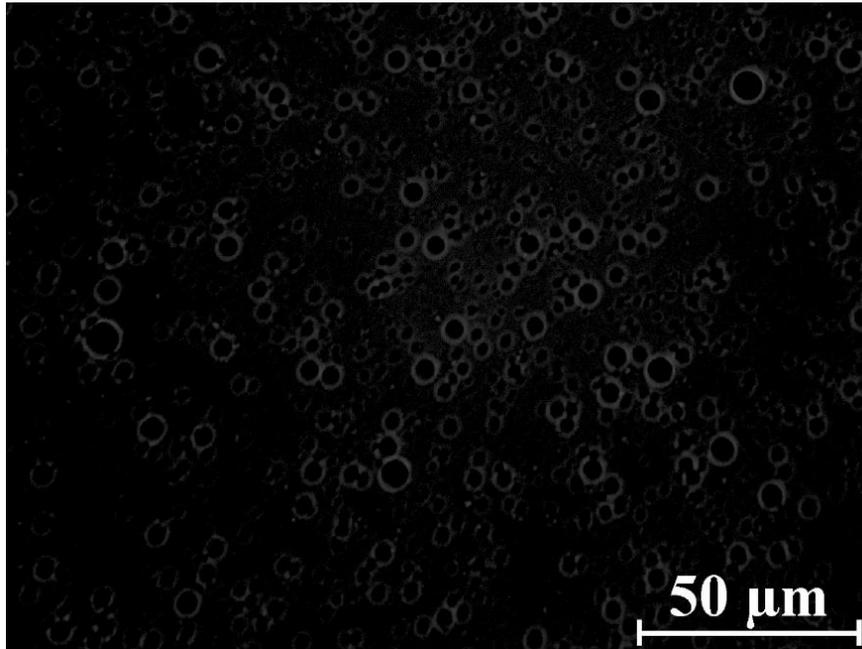


Figure 12. Effect of isothermal storage temperature on turbidity of nanoemulsions formed by a) spontaneous emulsification and b) emulsion phase inversion

a)



b)

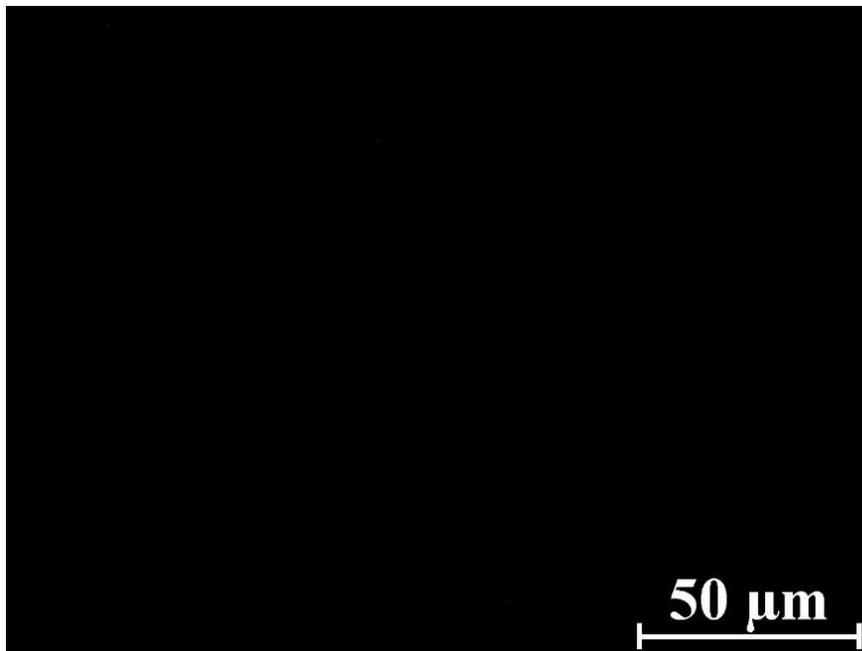
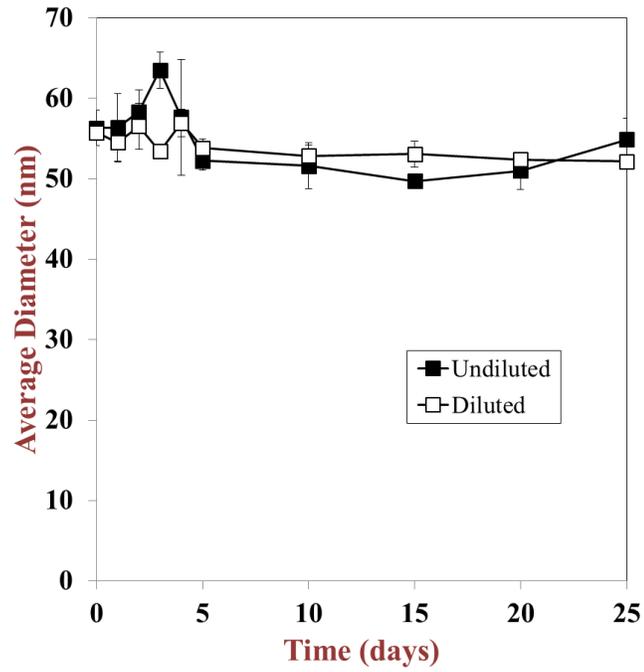


Figure 13. Optical microscopy images using a polarized lens of emulsions prepared by the spontaneous emulsion technique a) immediately after production and b) thirty minutes after production. The presence of large structures can be seen initially but disappear with time.

The nanoemulsions prepared using the EPI method behaved somewhat similarly to those prepared using the SE method (**Figure 12.b**): at 25°C the turbidity remained relatively low; at 30°C the turbidity increased steadily with time; at 35°C the turbidity increased very rapidly with time. Some phase separation was observed in the nanoemulsions prepared using the EPI method after storage at 35°C, but it was not as extreme as that observed for those prepared using the SE method. In this case, the height of the clear serum phase at the bottom of the test tubes did not reach the height of the light beam, and so there was no reduction in turbidity. The origin of this difference in the behavior of the nanoemulsions produced by SE and EPI methods is currently unknown, but it suggests that they initially had different structures.

In order to understand the influence of storage temperature more in depth, we stored emulsions made by EPI at temperatures corresponding to refrigeration (5°C) and room temperature (20 °C) for one month; no significant change in particle size (**Figure 14**) or turbidity (**Figure 15**) was observed. In fact, turbidity actually decreased with time at both storage temperatures which may correspond to a dissolution of liquid crystals with time. Additionally, dilution had minimal effect on storage stability. Other researchers have found emulsions to be formed by EPI to be sensitive to droplet instability at higher storage temperatures as well [55]. These results suggest that the emulsions made by isothermal low energy methods are highly unstable to coalescence when stored at elevated temperatures but may be appropriately stored in refrigeration or room temperature storage conditions either diluted or undiluted.

a)



b)

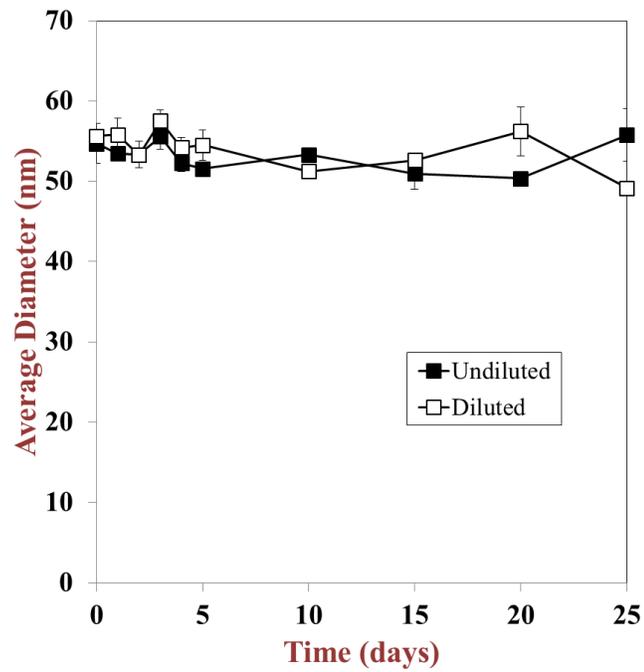
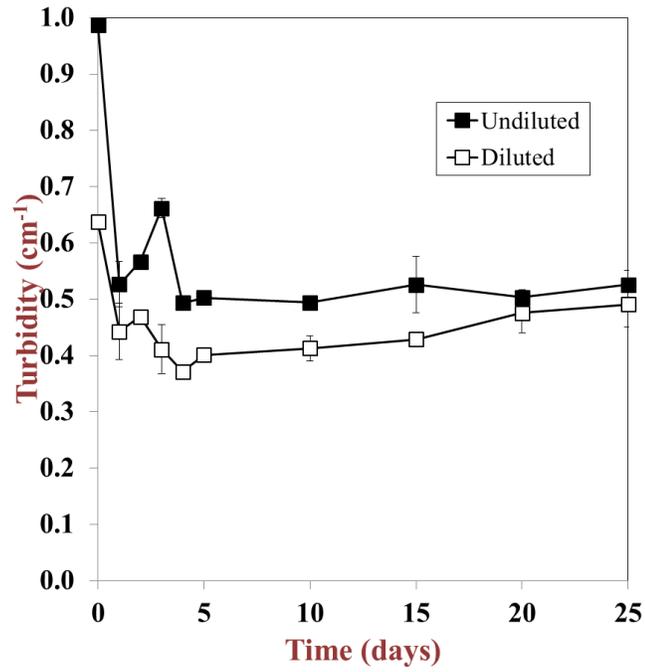


Figure 14. Effect of isothermal storage temperature and dilution on particle diameter of emulsions made by the emulsion phase inversion technique when stored at a) 5°C or b) 20°C.

a)



b)

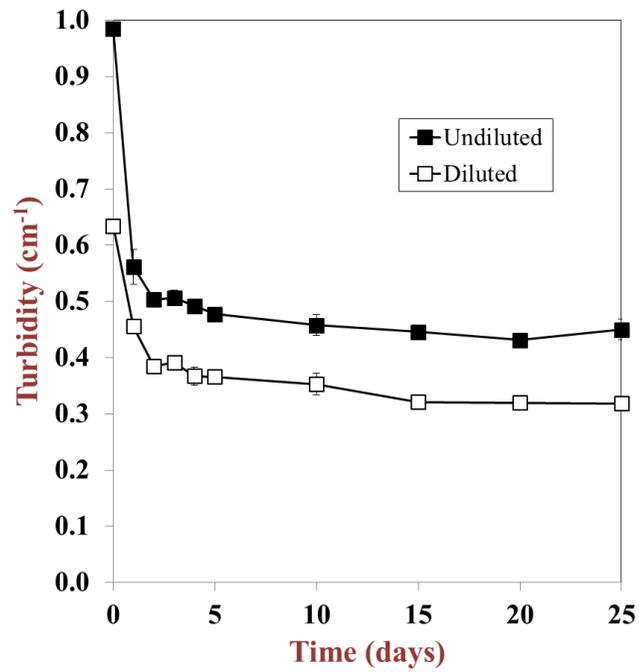


Figure 15. Effect of isothermal storage temperature and dilution on turbidity of emulsions made by the emulsion phase inversion technique when stored at a) 5°C or b) 20°C.

3.4.6. Effect of temperature scanning

In commercial applications it is often important to establish the influence of thermal treatments on the stability of nanoemulsions and so we used temperature-scanning turbidity measurements to obtain further information about their thermal stability (**Figure 16**). Three nanoemulsions samples were compared: (i) those prepared using the EPI method (EPI); (ii) those prepared using the SE method directly after preparation (initial SE); (iii) those prepared using the SE method 30 minutes after preparation (delayed SE). Two different nanoemulsions were prepared using the SE method because of the appreciable decrease in turbidity that occurred during the first 30 minutes of storage (**Figure 12** and **Figure 13**). The EPI nanoemulsions were relatively stable to droplet growth (no appreciable increase in turbidity) from 15 to 25 °C, but then became highly unstable at higher temperatures as indicated by a large increase in turbidity. The subsequent decrease in turbidity observed above 32 °C was due to phase separation. The delayed SE nanoemulsions behaved similarly to the EPI nanoemulsions, except that the initial turbidity was slightly higher at lower temperatures, which can be attributed to larger droplet sizes. The initial SE nanoemulsions behaved somewhat differently: the turbidity was relatively high from 15 to 25 °C, fell steeply from 25 to 28 °C, and then increased in a similar manner to the EPI and initial SE nanoemulsions at higher temperatures. The initial decrease in turbidity is similar to that observed in the isothermal experiments and suggests that there was some breakdown of structures formed during nanoemulsion preparation. We hypothesize that during preparation of the nanoemulsions using the SE method a SOW composition was passed in which large structures were formed (such as liquid crystals) that slowly broke down over time or upon

heating. Presumably, this SOW composition was not passed when using the EPI method to prepare the nanoemulsions thereby leading to lower droplet sizes for this latter method.

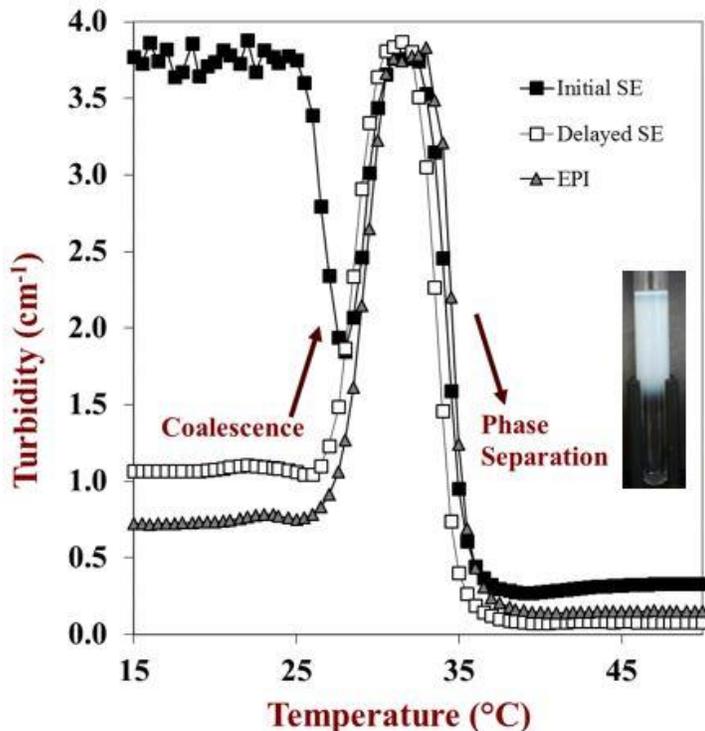


Figure 16. Effect of Temperature on Turbidity of nanoemulsions produced by spontaneous emulsification (SE) and emulsion phase inversion (EPI) methods. SE produced nanoemulsions were measured immediately after production and 30 minutes after production due to the difference in turbidity. All samples saw an increase in turbidity around 25-30°C likely due to coalescence and a decrease in turbidity above 30°C attributed to phase separation.

The origin of nanoemulsion stability at elevated temperatures can be attributed to progressive dehydration of the non-ionic surfactant head group at elevated temperatures. Head group dehydration alters the optimum curvature of the surfactant monolayer and changes the solubility of the surfactant in the oil and water phases [68]. Non-ionic surfactants, especially those based on polyoxyethylene like Brij 30, are very susceptible to the effects of temperature [108]. Hence, nanoemulsions produced using low-energy methods and non-ionic surfactants are likely to be thermally sensitive, which could be a

major drawback to their production. In these cases, effective strategies may need to be developed to stabilize the nanoemulsions after they have been formed, such as adding co-surfactants that alter the effective HLB number, optimum curvature, or colloidal interactions in the system [66].

3.5. Conclusions

There is increasing interest in the fabrication of nanoemulsions by isothermal low-energy methods, but there is still a lack of knowledge about the major factors influencing their formation and stability [3, 58, 68, 85]. In particular, there is a lack of understanding about how the formation pathway influences the final droplet size. In this study, we therefore systematically examined some of the major factors influencing the formation and stability of nanoemulsions produced by isothermal low-energy methods. The order of surfactant (S), oil (O), and water (W) addition was found to be critical in successfully producing nanoemulsions, which is in agreement with previous studies on selected formation pathways [105]. However, in this study we examined all possible combinations of combining the different components (S, O, W) together by titrating one liquid into another. Nanoemulsions with ultrafine droplets could only be produced from systems where the surfactant and oil phase were mixed together prior to interaction with the aqueous phase, and in which the surfactant and oil were miscible. These methods included the spontaneous emulsification (SE) method where a surfactant-oil mixture is titrated into water [(SO)→W], and the emulsion phase inversion (EPI) method where water is titrated into a surfactant-oil mixture [(W)→SO].

An innovative aspect of this work is that we compare SE and EPI methods together to establish the factors that impacted droplet formation and stability, and to highlight similarities and differences in these two approaches. The optimum surfactant-to-oil ratio (SOR) was found to be one where the surfactant concentration was high enough to spontaneously form nanoemulsions but not too high that it led to a large increase in viscosity (presumably due to formation of liquid crystals that were difficult to disperse). An optimum SOR of 0.375 was observed for both SE and EPI methods, which is in agreement with previous studies that have found similar SOR values for SE and EPI methods using similar surfactant, oil, and water components [39, 99]. Addition rate was not particularly important for both SE and EPI methods, as long as the total addition time was above a critical limit. Stirring speed was much more important for the SE method than for the EPI method, which may have been due to differences in the pathway of droplet formation, e.g., the need to disrupt the liquid crystalline structures formed in the SE method.

Finally, we found that temperature greatly affected the stability of the nanoemulsions after formation, independent of the preparation method. At temperatures less than 25 °C the emulsions had good long term stability. However, at higher temperatures the emulsion became more turbid due to droplet growth and even exhibited phase separation at higher temperatures (35 °C). These effects were attributed to the fact that the system approached the phase inversion temperature at these elevated temperatures, which promoted rapid droplet coalescence, as was established by Shinoda and co-workers many years ago for emulsions stabilized by non-ionic surfactants [109]. Overall these results are useful for the rational design of nanoemulsions-based delivery

systems using isothermal low-energy preparation methods. In particular, it highlights the importance of producing small initial droplet sizes, and then stabilizing these droplets against growth during storage.

In principle it should be relatively simple to scale-up this method for commercial applications since it simply requires metering one liquid into another at a controlled rate with stirring. This type of process is already commonly used in the food and other industries and therefore should be relatively easy to implement. On the other hand, the major disadvantage of this method is that it requires relatively high amounts of synthetic surfactant, which may be unsuitable for some applications.

3.6. Acknowledgements

This material is based upon work supported by a United States Department of Agriculture, NRI and AFRI Grants.

CHAPTER 4

LOW-ENERGY FORMATION OF EDIBLE NANOEMULSIONS BY SPONTANEOUS EMULSIFICATION: FACTORS INFLUENCING PARTICLE SIZE

4.1. Abstract

Nanoemulsions are used as delivery systems in food, pharmaceutical, and personal care applications for a variety of lipophilic active components, e.g., antimicrobials, flavors, colors, preservatives, vitamins, nutraceuticals, and drugs. In this study, we examined the effect of system composition and preparation conditions on the production of edible nanoemulsions using spontaneous emulsification (SE). SE is a low-energy method that simply involves addition of an organic phase (oil + surfactant) into an aqueous phase. The influence of surfactant-to-oil ratio (SOR), surfactant type, surfactant location, and oil type were tested. The droplet size produced decreased with increasing SOR, and was smallest when the non-ionic surfactant Tween 80 was used. Smaller droplets were formed when the surfactant was initially dispersed in the oil phase rather than the aqueous phase. Ten food-grade oils were tested and we found that droplet size followed the order: medium chain triglycerides < flavor oils < long chain triglycerides. No correlation was found between droplet size and the physicochemical characteristics of the oil phase (refractive index, density, interfacial tension, and viscosity). Results obtained by spontaneous emulsification were correlated to those obtained by emulsion phase inversion on similar systems suggesting a common underlying physicochemical mechanism.

4.2. Introduction

Nanoemulsions have been defined as emulsions with mean droplet diameters < 200 nanometers, *i.e.*, radius < 100 nm [1]. They are of particular interest in the food, supplement, and pharmaceutical industries because their small particle size can lead to delivery systems with high optical clarity, good kinetic stability, and high oral bioavailability [6, 30, 110]. Nanoemulsions can be fabricated using either high-energy or low-energy approaches, which can be distinguished based on the physicochemical mechanisms involved. High-energy approaches rely on specialized equipment (“homogenizers”) to disrupt and intermingle the oil and water phases, thus forming small droplets [98]. In contrast, low-energy approaches require no special equipment and utilize the properties of the surfactant, oil, and water system to spontaneously form nanoemulsions based on simply mixing procedures or by changing system conditions such as temperature [3, 111].

Low energy methods are of interest due to their low cost and ease of implementation [51], which has led to an increasing amount of research into the development and application of various low energy approaches [7, 12, 16, 39, 51]. Low energy approaches can be broadly divided into isothermal and thermal methods. Isothermal methods, such as spontaneous emulsification (SE) and emulsion phase inversion (EPI), do not require any temperature changes to form nanoemulsions [51]. Instead, they are based on the spontaneous formation of ultrafine droplets at the boundary between an organic and aqueous phase of specific composition when they are brought into contact. The spontaneous formation of nanoemulsions by isothermal methods can be achieved using various methods: (1) simply mixing oil, water, and water-miscible solvent

together [31]; (2) contact of an oil, hydrophobic surfactant, and water-miscible solvent mixture with an aqueous phase [30]; and (3) addition of an oil and hydrophilic surfactant mixture into an aqueous phase [28]. In food grade systems, process (3) is of particular interest due to the fact that a solvent is not necessary. The proposed mechanism for spontaneous emulsification by this method is the rapid diffusion of hydrophilic surfactant from the organic phase to the aqueous phase when they come into contact [28] (**Figure 17**).

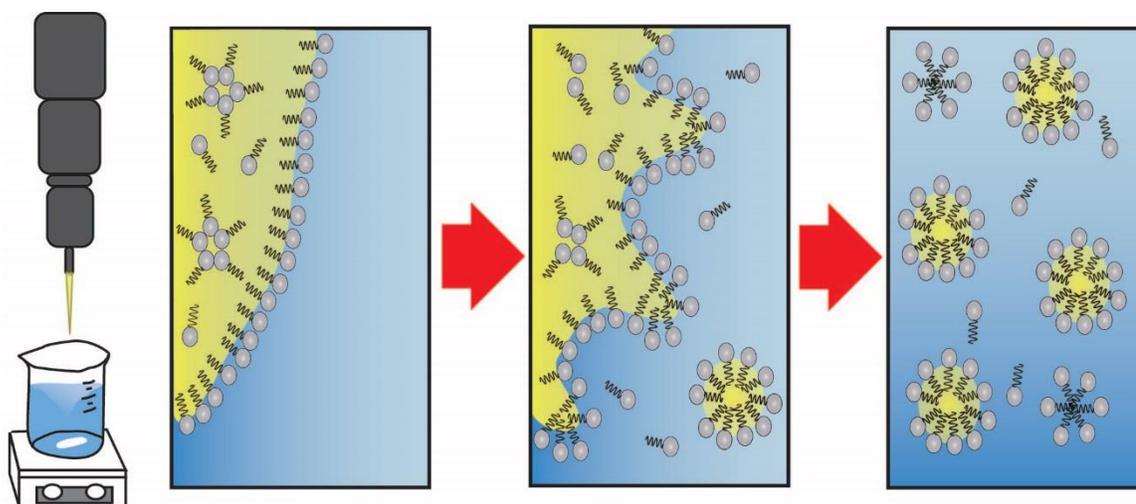


Figure 17. Schematic representation of the spontaneous emulsification method. An organic phase (oil + surfactant) was added in intervals from a pipette into a constantly stirring aqueous phase (buffer solution) in a glass beaker using a magnetic stirrer. A proposed molecular view is included.

Other researchers have examined the factors affecting the spontaneous emulsification process using non-food grade components and solvents [30]. Our laboratory has previously examined the factors affecting the size of oil droplets produced using the EPI method with food grade components [7]. The EPI method involves titrating an aqueous phase into an organic phase containing oil and hydrophilic surfactant. This

process initially leads to the formation of a water-in-oil emulsion (W/O), then an oil-in-water-in-oil emulsion (O/W/O) and then an oil-in-water emulsion (O/W). The goal of the current research was to determine the factors affecting the size of the droplets produced using the SE method with food grade components. The SE method is also an isothermal low-energy method, but it involves titrating an organic phase containing oil and hydrophilic surfactant into an aqueous phase. Previous research has suggested that there are some similarities and differences between these two different isothermal low-energy methods [7, 39, 99]. One of the aims of this study was to compare the size of the droplets produced using the SE and EPI methods on similar surfactant-oil-water systems. The experiments were therefore intentionally designed so that a direct comparison could be drawn between the two methods.

4.3. Materials and methods

4.3.1. Materials

Ten different oil phases were used to prepare the emulsions (**Table 11**). Medium chain triglycerides (MCT, Miglyol 812N, Warner Graham Company, Cockeysville, MD), orange oil (10×, Item No. 49024, The Chemistry Store, Cayce, SC), and Mineral Oil (Sigma-Aldrich, St Louis, MO) were bought from chemical suppliers. Lemon oil (3×, Citrus & Allied Essences, Lake Success, NY) and fish oil (Ropufa 30 *n*-3 food oil, DSM Nutritional Products Ltd, Basel, Switzerland) were kindly donated. Grapeseed oil, toasted sesame oil, canola oil, peanut oil, and extra virgin olive oil were bought from a local grocery store. A variety of non-ionic surfactants were used (**Table 12**) including Span® 20, Tween® 20, 40, 60, 80 and 85 (Sigma-Aldrich, St. Louis, MO). The aqueous phase

for all emulsions was a sodium phosphate buffer solution (5 mM; pH 7.0). Distilled and deionized water (Milli-Q®) was used to prepare all solutions and emulsions.

Table 11. Physical properties of oils used to prepare emulsions by the SE method, and mean particle diameters (d_{32}) produced using Tween 80 (SOR=2.0). The physiochemical properties were measured at ambient temperature ($\approx 20^\circ\text{C}$). The correlation coefficients (R^2) were calculated from linear plots of the mean particle diameter versus the physiochemical property of interest.

Oil Type	Refractive Index	Density (kg m⁻³)	Interfacial tension (mN m⁻¹)	Viscosity (mPa s)	d_{32} (μm)
Canola Oil	1.473	912 \pm 2	21.1 \pm 0.8	74.1 \pm 0.3	8.5 \pm 0.4
Fish Oil	1.481	905 \pm 2	24.4 \pm 0.3	49.2 \pm 0.4	5.6 \pm 0.3
Grapeseed Oil	1.4755	912 \pm 1	25.2 \pm 0.2	68.0 \pm 0.0	6.7 \pm 0.8
Lemon Oil	1.476	868 \pm 4	9.2 \pm 0.8	4.1 \pm 0.0	0.9 \pm 0.3
MCT	1.445	937 \pm 1	28.2 \pm 0.1	31.9 \pm 0.1	0.10 \pm 0.1
Mineral Oil	1.467	844 \pm 0	61.4 \pm 1.2	219.2 \pm 0.2	7.5 \pm 0.7
Olive Oil	1.469	904 \pm 1	20.3 \pm 0.9	83.7 \pm 0.3	10.5 \pm 1.0
Orange Oil	1.4715	847 \pm 1	18.1 \pm 0.2	4.2 \pm 0.5	1.3 \pm 0.1
Peanut Oil	1.470	889 \pm 3	28.2 \pm 0.6	81.7 \pm 2.6	9.0 \pm 1.4
Sesame Oil	1.473	901 \pm 0	9.0 \pm 0.7	75.8 \pm 2.9	7.3 \pm 0.6
Correlation (R²)	0.012	0.012	0.047	0.362	

Table 12. Properties of the surfactants used to prepare emulsions by the SE method, and mean particle diameters (d_{32}) produced using MCT (SOR=2.0). The values with asterisk were calculated as a weighted average. The correlation coefficients (R^2) were calculated from linear plots of the mean particle diameter versus the physiochemical property of interest.

Non-Ionic Surfactant	Chemical Structure	Molecular Weight (g/mol)	HLB Number	Critical Micelle Conc (mM)	d_{32} (μm)
Span 20	Sorbitan-monolaurate	346	8.6	-	59.1±4.4
Tween® 20	Polyoxyethylen-20-sorbitan-monolaurate	1228	16.7	0.050 ^a	1.46±0.09
Tween® 40	Polyoxyethylene-20-sorbitan-monopalmitate	1277	15.6	0.023 ^a	0.117±0.001
Tween® 60	Polyoxyethylene-20-sorbitan-monostearate	1312	14.9	0.021 ^a	0.23±0.04
Tween® 80	Polyoxyethylen-20-sorbitan-monooleate	1310	15.0	0.010 ^a	0.101±0.004
Tween® 85	Polyoxyethylene-20-sorbitan-trioleate	1836	11.0	0.00029 ^a	2.65±0.13
Tween®20,80,85 mixture	1:1:1 T20:T80:T85	1458*	14.2*	-	0.110±0.003
Correlation (R^2)			0.644		

^aValues from [112]. Measured at 298 K.

4.3.2. Methods

4.3.2.1. Emulsion preparation

Emulsions were prepared by spontaneous emulsification which involves titrating an organic phase into an aqueous phase. In most experiments, the organic phase consisted of oil and surfactant. The experiments were performed in a 50 ml beaker at ambient

temperature ($\approx 20\text{ }^{\circ}\text{C}$). The experiments were designed so that the final emulsion always had a total mass of 25 g including 2.5 g of oil (*i.e.*, 10 wt% oil). Initially, an organic phase was prepared by adding the surfactant and oil to the beaker and then mixing using a magnetic stirrer (500 rpm) for a minimum of 30 minutes. The thoroughly mixed organic phase was then added to a stirring aqueous phase (750 rpm) over 5 minutes using a programmable automated pipette (Rainin SE4, Mettler Toledo, Oakland, CA). An additional 5 minutes was allowed for mixing to bring the total preparation time to 10 minutes. Previous studies with a model system showed that there was no added benefit to increasing the processing time further [51].

4.3.2.2. Variables tested

Four main variables were tested: surfactant-to-oil ratio, type of surfactant, surfactant location, and oil type.

4.3.2.2.1. Influence of surfactant-to-oil ratio

The influence of surfactant concentration was investigated by varying the surfactant-to-oil ratio (SOR). The total oil content in the final systems was held constant at 10%, while the SOR was varied by altering the amounts of surfactant and water in the final system:

$$\text{SOR} = m_s/m_o \quad (4.1)$$

$$m_w = 100 - m_o - m_s \quad (4.2)$$

Here, m_s , m_o and m_w are the mass percentages of surfactant, oil and water, respectively. SORs tested included 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2. All these tests were carried out using medium chain triglycerides (MCT) as the oil and Tween 80 as the surfactant.

4.3.2.2.2. Influence of surfactant type

A number of nonionic surfactants were used to stabilize the emulsions (**Table 12**) including Tween[®] 20, 40, 60, 80 and 85 as well as Span[®] 20. SOR was held constant at 2 and MCT was used as the oil phase for all experiments. Tween surfactants consist of a polyoxyethylene head and a fatty acid tail of various lengths with the two moieties being linked together via a sorbitol. Tween 20 has a monolaurate tail (C_{12:0}), Tween 40 has a monopalmitate tail (C_{16:0}), Tween 60 has a monostearate tail (C_{18:0}), Tween 80 has a monooleate tail (C_{18:1}), and Tween 85 has a trioleate tail (3 x C_{18:1}). Span surfactants have a fatty acid tail of various lengths connected to a sorbitol. Span 20 has a monolaurate tail (C_{12:0}). A mixed surfactant system ($\frac{1}{3}$ Tween 20, $\frac{1}{3}$ Tween 80 and $\frac{1}{3}$ Tween 85) was also tested.

4.3.2.2.3. Influence of surfactant location

The effect of surfactant location was tested by varying the relative amounts of surfactant in the aqueous and organic phases. A fixed composition was used for these experiments based on the optimized conditions established in earlier experiments: oil = MCT; surfactant = Tween[®] 80; SOR = 2.0. The amount of the surfactant initially in the organic phase was varied from 0 to 100% in 25% intervals, with the remainder of the surfactant initially being incorporated into the aqueous phase.

4.3.2.2.4. Influence of oil type

The effect of oil type was tested by varying the nature of the oil incorporated into the organic phase. A constant surfactant-to-oil ratio (SOR = 2.0) and surfactant type (Tween[®] 80) were used. The surfactant and oil were thoroughly mixed for a minimum of 30 minutes when preparing the organic phase.

4.3.2.3. Oil characterization

The physiochemical properties of the oils used in this study were measured to determine if a correlation could be made between them and the size of the droplets formed by spontaneous emulsification.

4.3.2.3.1. Refractive index

Refractive index was measured using a refractometer (Abbe 3L, Bausch & Lomb, Rochester, NY) at ambient temperature (~20°C). The results found for refractive index can be found in **Table 11**, and are in agreement with literature values [113]. These refractive index values were also used to determine the particle size distribution using the light scattering methods [114].

4.3.2.3.2. Density

The density was measured by weighing samples in a controlled temperature environment (20 °C). 10 mL samples were injected into a container using a calibrated pipette (Rainin Classic, PR-10, Mettler Toledo, Oakland, CA) and then accurately weighed (SI-234, Denver Instrument, Bohemia, NY). The measured densities found are reported in **Table 11**, and are in good agreement with reported values [30, 113]. These measurements were also used in the calculation of the interfacial tension [115].

4.3.2.3.3. Interfacial tension

The interfacial tension at the oil-water interface was measured using a droplet shape analysis device (DSA 100, Krüss GmbH, Hamburg, Germany). For oil droplet formation a hook-needle with a diameter of 1.463 mm was used to create a pendant drop. The pendant drop was extruded into a quartz cell containing buffer solution (sodium phosphate, 5 mM, pH 7.0). Each sample was a composite of measurements made every 0.1 seconds for 5 minutes. Digital images were also captured using the device's camera

function. Interfacial tension values were calculated based on the Young-Laplace equation by the drop shape analysis program supplied by the instrument manufacturer.

4.3.2.3.4. Viscosity

The viscosity was measured using a cup and bob configuration on a rotational rheometer (Kinexus pro+, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Approximately 17.61 mL of sample was loaded into the cup. Low viscosity samples (lemon oil and orange oil) were tested through a range of shear rates (100-500 s⁻¹, with 20 samples per decade). All other samples were tested at shear rates of 25-100 s⁻¹, with 20 samples per decade. Measurements were carried out at a temperature of 20 °C. Viscosity was calculated by finding the slope of a linear best fit line of shear stress (mPa) versus shear rate (s⁻¹) using Microsoft Excel (Microsoft Office Professional Plus 2013, Microsoft Corporation, Redmond, WA). The values obtained were in good agreement with those reported in the literature [7, 30].

4.3.2.4. Emulsion characterization

4.3.2.4.1. Particle size analysis

The particle size distribution and mean particle diameter (d_{32}) were measured using a commercial static light scattering (SLS) instrument (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in buffer solution prior to analysis to avoid multiple scattering effects.

4.3.2.5. Experimental design

All measurements were performed on two freshly prepared samples in triplicate. The mean and standard deviations were calculated from this data. Statistical analysis was performed through subsection of the data to analysis of variance (ANOVA) using commercial statistics software (Minitab 16.2.4, Minitab Inc., State College, PA). Means

were subject to Tukey's test and a P-value of <0.05 was considered statistically significant.

4.4. Results and discussion

4.4.1. Influence of surfactant to oil ratio

Initially, the effect of surfactant-to-oil ratio (SOR) on the size of the droplets produced by the SE method was investigated. The influence of varying SOR on both the particle size distribution (**Figure 18**) and mean particle diameters (**Figure 19**) of emulsions produced using Tween 80 as the surfactant and MCT as the oil phase was examined. Particle size was highly dependent on SOR, with larger droplets being formed at lower SORs (0.05-0.25) and smaller droplets being formed at higher SORs (0.5-2). While the particle diameter continued to decrease with increasing SOR, the difference was not appreciable at $\text{SOR} \geq 0.5$. The light scattering results indicated that the nanoemulsions formed at high SOR were monomodal with narrow particle size distributions, which may be advantageous for certain commercial applications. All further experiments were carried out using an SOR of 2, where the smallest particle diameter was achieved ($d_{32} \approx 0.1 \mu\text{m}$), so as to compare the results obtained using the SE method with previous results obtained using the EPI method [7]. Decreasing particle size with increasing SOR has also been reported by other researchers using spontaneous emulsification with Tween 85 and MCT [116], and with the EPI technique using MCT and Tween 80 [7].

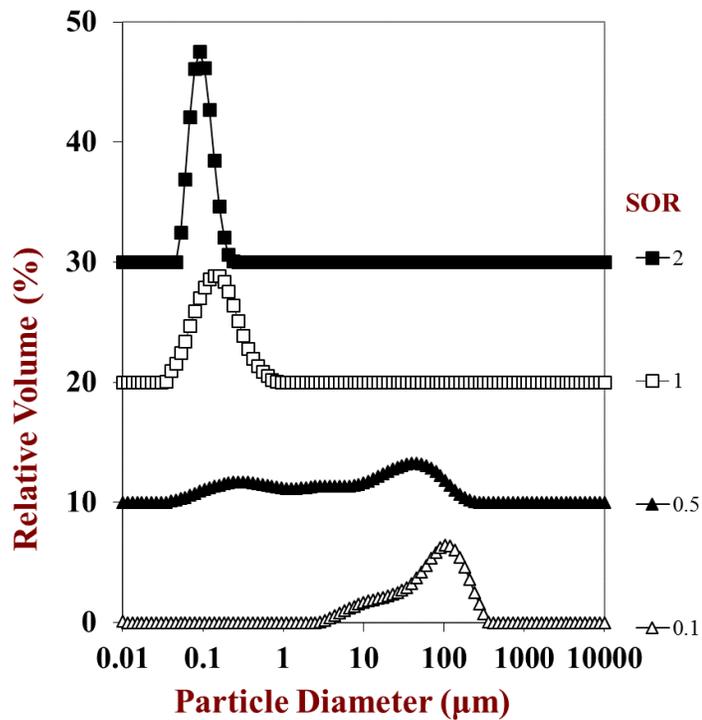


Figure 18. Particle size distributions of 10 wt% oil-in-water emulsions with different surfactant-to-oil ratios (SOR) produced by the SE method. The surfactant used was Tween 80.

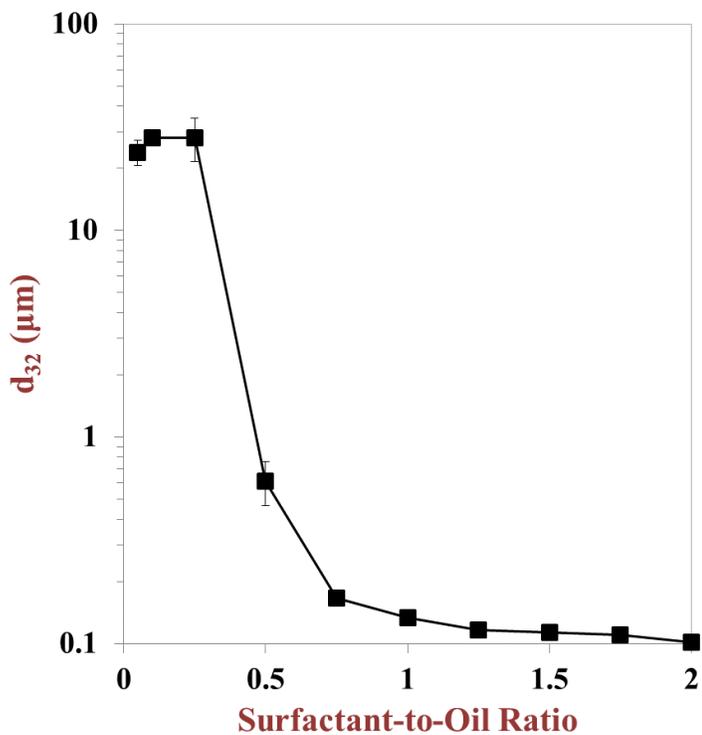


Figure 19. Mean particle diameters (d_{32}) of 10 wt% oil-in-water emulsions with different surfactant-to-oil ratios (SOR) produced by the SE method.

The emulsions appeared visibly less turbid with increasing SOR, which can be attributed to the reduction of light scattering by smaller droplets [2]. This property would be beneficial in commercial applications where delivery systems that are optically transparent are required, such as waters or soft drinks fortified with oil-soluble nutrients (*e.g.*, vitamins or nutraceuticals).

A number of physicochemical phenomena may account for the observed reduction in particle size with increasing surfactant concentration. Surfactants adsorb to the surfaces of oil droplets forming a protective coating that inhibits droplet aggregation [6]. The specific surface area of an emulsion increases with decreasing droplet size, thus requiring a larger surfactant concentration to stabilize the droplets formed. If there is insufficient surfactant present to cover all of the droplet surfaces formed, then the droplets will tend to coalesce after colliding with each other [28]. In addition, the phase behavior of a surfactant-oil-water (SOW) system is likely to influence the spontaneous formation of oil droplets at the boundary between organic and aqueous phases. Only certain SOW compositions may lead to the spontaneous formation of ultrafine droplets. If the surfactant level is too high, then a further increase in surfactant concentration may actually increase the particle size by inhibiting the self-emulsification process, which has been attributed to the formation of liquid crystals that are difficult to disrupt [51, 116, 117]. This concentration was not reached in the present study which is why a continual decrease in particle size was observed with the increasing addition of surfactant rather than a “U” shaped curve reported in other studies [51, 116, 117].

To provide further insights into the role of SOR on droplet formation we investigated its influence on the interaction of organic and aqueous phases using droplet

shape analysis (**Figure 20**). In these experiments an organic phase containing surfactant and oil was injected into an aqueous buffer solution, and video images were captured. When no surfactant was present in the organic phase ($SOR = 0$), a stable oil drop was formed at the end of the tip. In the presence of small amounts of surfactant ($SOR=0.05$), the oil drops formed were much smaller and they would not stay attached to the hook. Instead they quickly detached and moved to the surface of the water phase due to gravity. At an intermediate surfactant concentration ($SOR=0.5$), the oil phase formed a continuous stream of oil droplets that moved upwards with no distinct droplet shape. At higher surfactant concentrations ($SOR = 1$ to 2), a 3-dimensional gel-like structure was formed that had some rigidity. As the organic phase (containing oil and surfactant) was injected into the aqueous phase a fairly rigid structure was formed that began to fold into itself when the injection pressure was released. This can be seen in the image for $SOR = 2$ where the structure formed appears wrinkled in the middle. The droplet shape analysis images show that the SOR composition had a direct effect on the nature of the structures formed when the organic and aqueous phases came into contact, which is likely to influence the spontaneous emulsification process. However, it should be noted that there was no stirring in the drop shape analysis device, which is different from the spontaneous emulsification method used to produce the nanoemulsions.

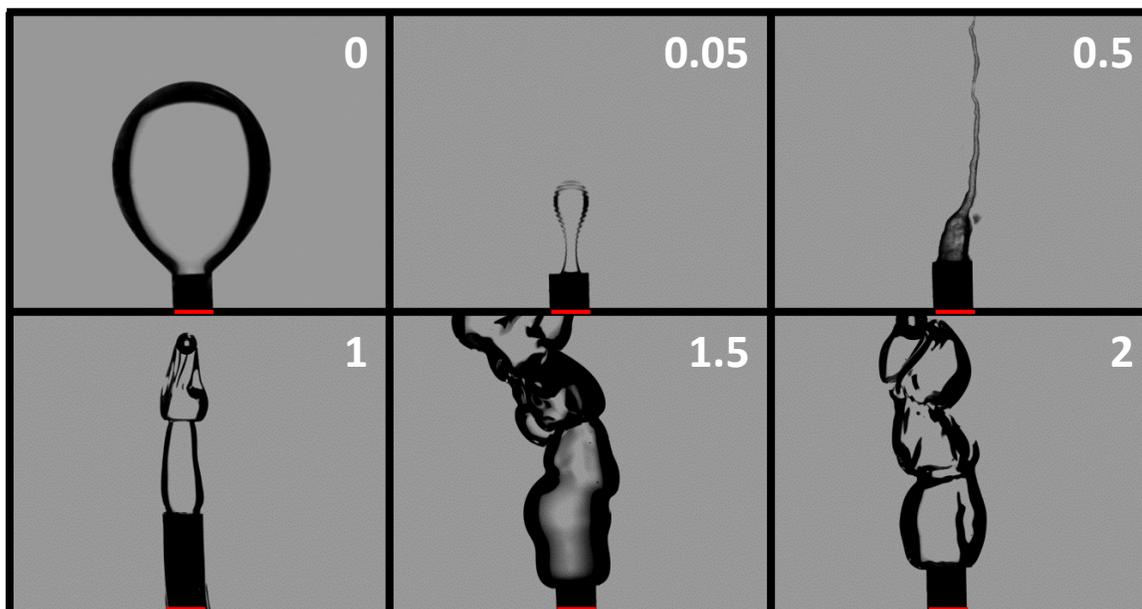


Figure 20. Droplet shape analysis images of varying surfactant-to-oil ratios (SOR). The organic phase (oil + surfactant) was slowly added into the aqueous phase (buffer solution). Images were captured to show the qualitative difference observed with varying amounts of surfactant. Red line shows the width of the hook (1.463 mm) and number indicates SOR. Droplet started off rounded with zero surfactant. As surfactant increased to an SOR of .05 and 0.5 the droplet was unable to be formed. At higher surfactant amounts a 3-dimensional gel-like structure was formed.

4.4.2. Influence of surfactant type

The size of the droplets formed by spontaneous emulsification was greatly influenced by surfactant type (**Figure 21**). The smallest droplets were formed when Tween 80 was used ($d_{32} \approx 0.10 \mu\text{m}$) but fine droplets were also formed when using Tween 40 ($d_{32} \approx 0.12 \mu\text{m}$), Tween 60 ($d_{32} \approx 0.23 \mu\text{m}$) and the mixed surfactant system ($d_{32} \approx 0.11 \mu\text{m}$). Much larger droplets were formed using Span 20 ($d_{32} \approx 59 \mu\text{m}$), Tween 20 ($d_{32} \approx 1.4 \mu\text{m}$), and Tween 85 ($d_{32} \approx 2.7 \mu\text{m}$).

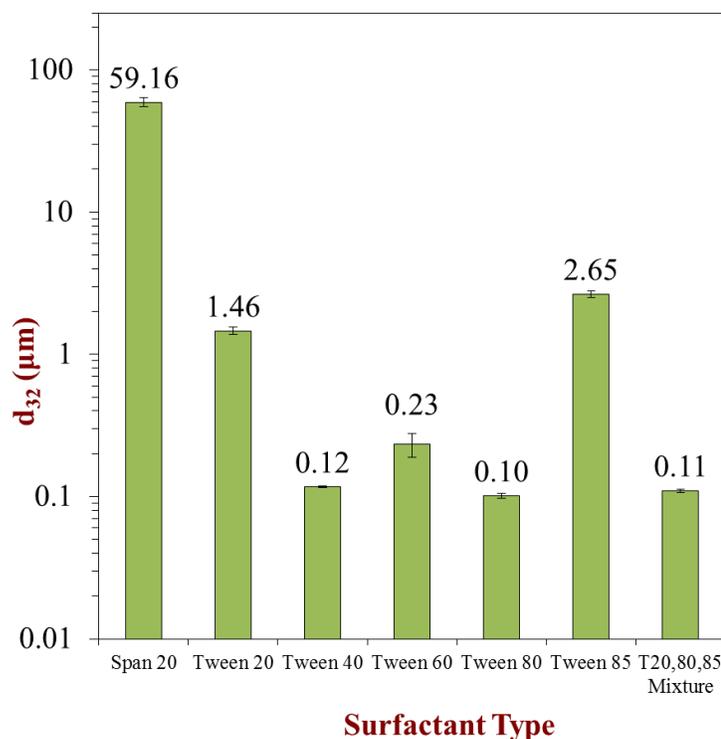


Figure 21. Mean particle diameters (d_{32}) of 10 wt% oil-in-water emulsions with constant surfactant-to-oil ratio (SOR = 2.0) produced by the SE method using different types of surfactant. The oil used was MCT.

Anton and co-workers reported that the affinity of a surfactant for the hydrophobic phase plays an important role in nanoemulsion formation [28]. Our results support this proposal to a certain degree as the HLB number is related to the hydrophobic affinity [118]. The smallest particle size was achieved when the surfactants had intermediate HLB values around 15. This is in agreement with what was found for nanoemulsions produced with similar system compositions using the emulsion phase inversion method [7] and spontaneous emulsification [23], but varied from other studies that found that the most efficient surfactants for nanoemulsion formation had HLB values around 11 [116]. In addition, Bouchemal and co-workers reported that the mean particle size decreased with increasing HLB [30], a trend that we also observed. We propose that

the surfactant should be predominantly hydrophilic (and should therefore have a high HLB number), but it should not be too hydrophilic (otherwise it will not be soluble in the oil phase). In addition, the molecular geometry of the surfactant is also important since this will affect interfacial curvature and flexibility, which would be expected to impact spontaneous oil droplet formation.

4.4.3. Influence of initial surfactant location

The movement of the surfactant from the organic phase into the aqueous phase is the proposed mechanism for the formation of fine droplets in the spontaneous emulsification process [28]. Because of this, we investigated the influence of the initial surfactant location on the size of the droplets produced. Our results showed that the initial location significantly impacted the mean particle diameter (**Figure 22**). When surfactant was originally located in the aqueous phase, the droplets were significantly larger than when it was initially in the organic phase. We had previously seen a similar result in a model system consisting of hexadecane and Brij 30 [51]. These results support the notion that it is the movement of the surfactant from the organic phase to the aqueous phase that drives the spontaneous production of ultrafine droplets, rather than the final composition of the system. When there was no surfactant initially in the organic phase the production of fine droplets was not possible (**Figure 22**). However, with even a quarter of the total surfactant present in the oil phase, droplets less than 1 μm could be achieved.

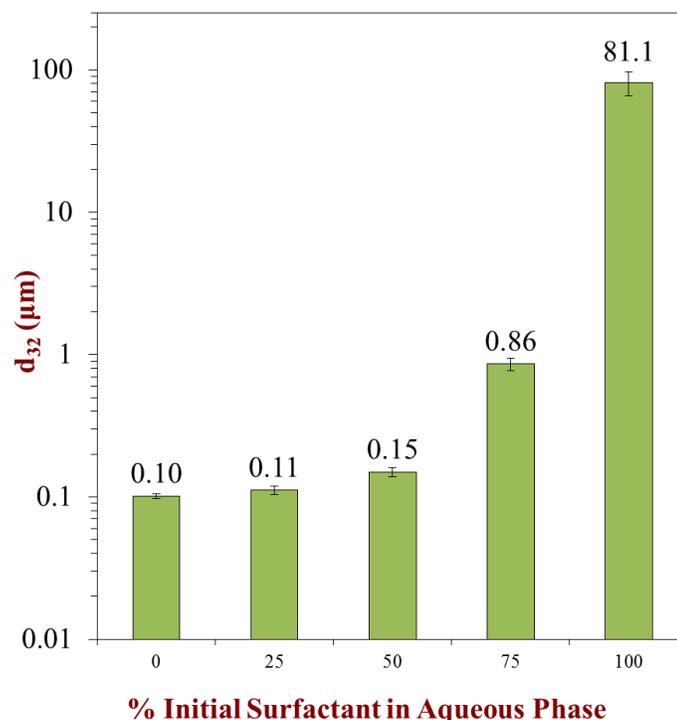


Figure 22. Mean particle diameters (d_{32}) of 10 wt% oil-in-water emulsions with constant surfactant-to-oil ratios (SOR = 2.0) produced by the SE method using MCT as the oil and Tween 80 as the surfactant location was varied. The percentage of the Tween 80 initially in the aqueous phase was varied from 0% to 100%.

4.4.4. Influence of oil type

From a practical point of view it is important to establish which oils are suitable for forming nanoemulsions using the spontaneous emulsification method. The mean particle diameter produced varied greatly depending on the type of oil in the organic phase (**Figure 23**). The smallest particles were produced using medium chain triglycerides (MCT), and then flavor oils (lemon and orange), and then long chain triglycerides (LCT). However, only large droplets ($d_{32} > 5 \mu\text{m}$) could be formed with mineral oils and LCT oils. This is in good agreement with what was found using the emulsion phase inversion technique to produce nanoemulsions using similar components (**Figure 24**) [7].

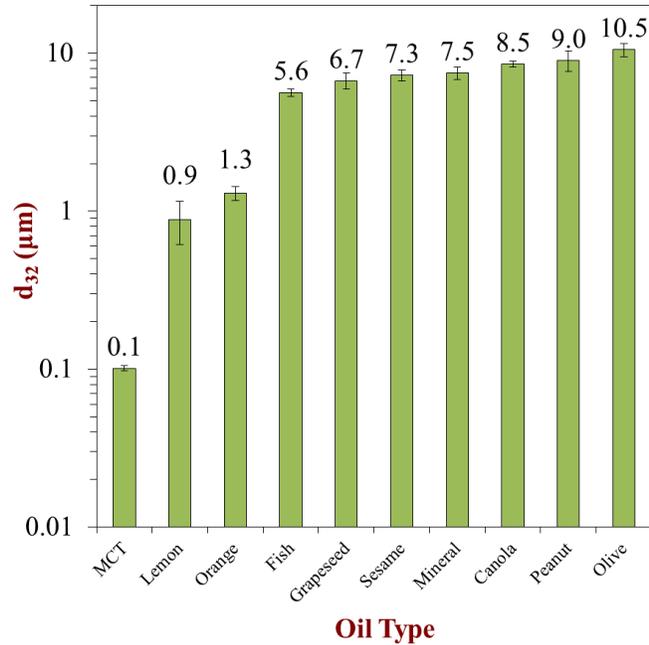


Figure 23. Mean particle diameters (d_{32}) of 10 wt% oil-in-water emulsions with constant surfactant-to-oil ratios (SOR = 2.0) produced by the SE method using different types of oil. The surfactant used was Tween 80.

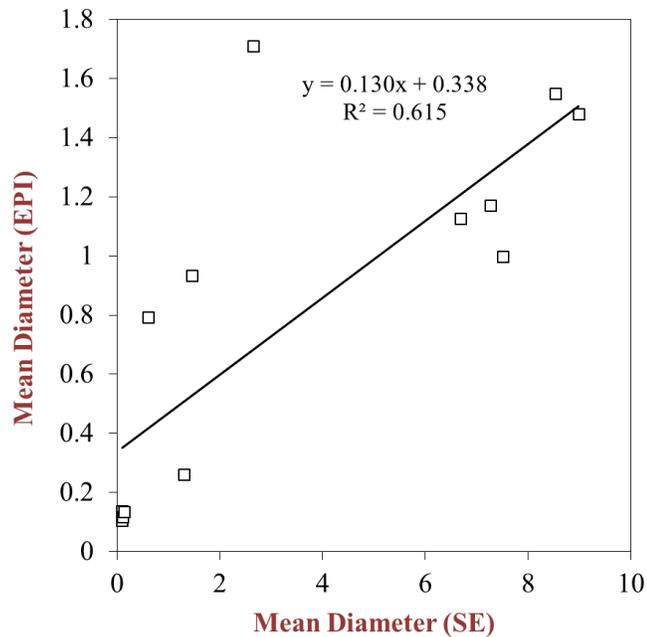


Figure 24. Comparison of the mean particle diameter (d_{32}) of emulsions produced using spontaneous emulsification (SE) and emulsion phase inversion (EPI) low-energy methods. The particle sizes were compared on similar systems with varying oil type, surfactant type, and surfactant to oil ratio. Data were taken from this study and that by Ostertag et al., 2012 [7].

We originally hypothesized that there would be a correlation between the bulk physicochemical properties of the oil used and the final particle size produced, based on the fact that one might expect oil viscosity, density or interfacial tension to impact the spontaneous formation of oil droplets at the organic phase/aqueous phase boundary [119]. Indeed, Bouchemal and co-workers reported that smaller droplets were produced by spontaneous emulsification as the oil viscosity increased, however they also stated that this was not a sufficient condition since some low viscosity oils also produced fine droplets [30]. In our study, we correlated the mean droplet diameter with a number of physicochemical properties of the oils used, *i.e.*, refractive index, density, interfacial tension, and viscosity. We found that there was not a good correlation ($r^2 < 0.4$) between any of these parameters and the mean droplet diameter (**Table 11**). These results suggest that knowledge of the bulk physicochemical properties of food-grade oils does not provide a good prediction of their ability to form small droplets using spontaneous emulsification. Instead, the phase behavior of the surfactant-oil-water system is likely to be more important.

4.4.5. Comparison of SE and EPI methods

Finally, we compared the size of the droplets produced using the spontaneous emulsification method in this study with those produced using the emulsion phase inversion method in a previous study [7]. As mentioned earlier, the main difference between these two methods is that SE involves titration of an organic phase into an aqueous phase, whereas EPI involves titration of an aqueous phase into an organic phase. In the SE method, oil droplets are believed to form spontaneously at the boundary created when an organic phase comes into contact with an aqueous phase. In the EPI method, a W/O, then O/W/O, and then O/W emulsion is formed as increasing amounts of aqueous

phase are titrated into the organic phase. The internal oil droplets in the O/W/O emulsion are believed to form the oil droplets in the final O/W emulsions. These internal oil droplets may form spontaneously at the boundary between the aqueous and organic phases within the W/O emulsion. We therefore hypothesized that there may be some correlation between the size of the droplets produced using these two methods because of the potential similarities in the physicochemical mechanisms of droplet formation. The droplet size produced using the SE method is plotted against the droplet size produced using the EPI method on similar SOW systems (*i.e.*, same oil type, surfactant type, and SOR) (**Figure 24**). Only data for emulsions containing droplets with diameters $< 10 \mu\text{m}$ were compared, since systems with higher droplet sizes were highly unstable to creaming within the particle size analyzer. In general, there was some correlation ($r^2 > 0.61$, $n = 14$) between the size of the droplets produced using the two different low-energy methods, which suggests that there was some common underlying mechanism. In addition, the general trends in the data were similar for both methods when examining a particular attribute, such as oil type, surfactant type, or SOR. Nevertheless, the EPI method appeared to consistently give smaller droplets than the SE method on similar SOW systems, which suggests that it may be more efficient at producing emulsions or nanoemulsions. The physicochemical origin of this difference is currently unknown and will require further studies.

4.5. Conclusions

Previous work on spontaneous emulsification has examined specific systems [12, 39] or used materials not suitable for food-grade applications [30, 31, 51]. In this study

we investigated the influence of food-grade surfactants and oils on the formation of nanoemulsions using the spontaneous emulsification approach. We have shown that nanoemulsions ($d_{32} < 200$ nm) can be produced by simple addition of an organic phase (oil and surfactant) to a stirring aqueous phase. The size of the droplets depended on surfactant-to-oil ratio, surfactant type, initial surfactant location, and oil type. We found no simple correlation between bulk physicochemical properties (refractive index, density, interfacial tension and viscosity) of the oil and droplet diameter. These results suggest that the spontaneous emulsification method may be useful for producing food-grade nanoemulsions for only a limited number of oils and surfactants. However, the molecular or physicochemical parameters that determine which oils and surfactants are most suitable still needs to be established. The main disadvantage of the spontaneous emulsification method is that one requires high levels of synthetic surfactants, which is undesirable for many food applications due to cost, flavor, and regulatory concerns. Nevertheless, this technology is still useful for applications where small amounts of lipophilic components need to be incorporated into clear aqueous-based products, such as flavors, nutraceuticals, vitamins, or antimicrobials. In future studies, it would be advantageous to establish whether the same procedure could be used to form nanoemulsions using low levels of natural surfactants.

4.6. Acknowledgements

This material is based upon work supported by United States Department of Agriculture, NRI Grants (2011-03539 and 2013-03795).

CHAPTER 5

FOOD-GRADE NANOEMULSION FILLED HYDROGELS FORMED BY SPONTANEOUS EMULSIFICATION: OPTICAL PROPERTIES, RHEOLOGY, AND STABILITY

5.1. Abstract

Nanoemulsions may be used as delivery systems for lipophilic bioactive components in foods and beverages, such as oil-soluble vitamins, nutraceuticals, flavors, and antimicrobials. In this study, we examined the possibility of incorporating nanoemulsions into clear hydrogels to form optically translucent hydrogels. The effect of preparation and storage conditions on the formation and stability of nanoemulsion-filled gelatin hydrogels was studied. Nanoemulsions were produced using the spontaneous emulsification (SE) method, which simply involves addition of an organic phase (oil + surfactant) to an aqueous phase. Droplet size decreased and optical clarity increased when the SE method was performed at an elevated temperature (60 °C) rather than at ambient temperature. Translucent filled hydrogels could be formed by incorporating the nanoemulsions into the gelatin gels. The optical and rheological properties of a model gelatin gel and a commercial gelatin dessert did not change appreciably after the nanoemulsion droplets (1%) were incorporated. This approach may therefore be useful for the incorporation of various types of lipophilic bioactive agents into functional food gels.

5.2. Introduction

Hydrogels are soft-materials consisting of polymeric networks with pores on the nanoscale that trap substantial quantities of water inside [120]. Hydrogels are important constituents of many foods, including yogurts, desserts, spreads, and some meat products, where they provide desirable appearance, texture, flavor, and stability characteristics. They may also be utilized in the development of delivery systems to encapsulate, protect and release bioactive molecules [121]. In the food industry, the most commonly used polymers to form hydrogels are proteins and polysaccharides. The nature of the polymer used determines the physicochemical and functional properties of the hydrogel formed, such as its optical, rheological, stability and release properties [120]. Gelatin is one of the most commonly used proteins for hydrogel formation currently used in the food industry [121], although other proteins are also available, including those from eggs, milk, and plants.

Gelatin is typically obtained by acid or alkaline hydrolysis of pig skin, bovine hide, or pork and cattle bones [122]. Gelatin from pork is currently the most widely used in the food industry because of its characteristic “melt-in-the mouth” property, which is especially important in popular gelatin desserts [123]. For this reason, gelatin derived from pork skin was used in our studies. Gelatin desserts typically have between 1-3% gelatin with a lower percentage leading to a more tender product [124]. Additional ingredients in gelatin desserts include sweeteners, water, flavors, colors and pH balancing ingredients [123]. Gelatin desserts are widely consumed by both children and adults, and therefore they may be a suitable candidate for fortification with health-promoting bioactive agents, such as nutraceuticals or vitamins. However, many of these bioactive

agents are highly lipophilic substances that cannot easily be dispersed within aqueous-based food products [125, 126].

There has been considerable interest in the utilization of nanoemulsions as delivery systems for lipophilic bioactive agents in foods because of their high optical clarity, good physical stability, and ability to increase bioavailability [76, 127]. The transparency of nanoemulsions can be attributed to the relatively small size of the droplets they contain compared to the wavelength of light [2]. Optical clarity is important in food applications where the final product should appear clear, such as gelatin desserts. In general, nanoemulsions can be produced using either high or low energy approaches, with high energy approaches requiring specialized equipment and low energy approaches relying on the physiochemical properties of the system [51]. Low-energy methods are easy to incorporate into a manufacturing setting because they are very cheap and simple to implement. The low energy approach used in the current study was spontaneous emulsification (SE), which involves the addition of an organic phase (surfactant and oil) into an aqueous phase (water) with continuous stirring. The surfactant should be slightly hydrophilic so that it diffuses from the organic phase into the aqueous phase, thus causing a budding action at the oil-water interface that leads to the spontaneous generation of very fine oil droplets [28].

The aim of this study was to look at the possibility of incorporating nanoemulsions into a gelatin dessert by use of the spontaneous emulsification process. The resulting system falls under the general category of emulsion-filled gels, which has been reviewed recently [128]. This paper serves as a proof of concept for the formation of translucent nanoemulsion filled hydrogels by spontaneous emulsification. These

nanoemulsion-filled hydrogels may be useful for incorporating a range of different lipophilic bioactive agents into gelled food products.

5.3. Materials and methods

5.3.1. Materials

Medium chain triglycerides (MCT, Miglyol 812N, Warner Graham Company, Cockeysville, MD) were used as a model oil and Tween® 80 (Sigma-Aldrich, St. Louis, MO) was used as a model surfactant for emulsion preparation. The aqueous phase for all systems was distilled and deionized water (Milli-Q®). Gelatin (200 Bloom from Porksin, 8 Mesh, Gelita, Sergeant Bluff, IA) was kindly donated. For the model gelatin dessert system, sugar-free Jell-O® ready-to-eat snacks were bought from a local grocery store in strawberry and lemon-lime flavors (Kraft Foods, Chicago, IL).

5.3.2. Methods

5.3.2.1. Model emulsion system

Emulsions were prepared by the spontaneous emulsification process which consists of the addition of oil and surfactant into stirring water [28]. Two temperatures were tested, ambient temperature (≈ 20 °C) and gelation temperature (60 °C). The elevated temperature was used to ensure that the gelatin was in the sol state prior to nanoemulsion addition. The organic phase (containing oil and surfactant) was added over 5 minutes with an additional 5 minutes of mixing for a total of 10 minutes [46]. The organic phase was added to the aqueous phase using a programmable automated pipette (Rainin SE4, Mettler Toledo, Oakland, CA). The aqueous phase was stirred at 700 rotations per minute (RPM) using a magnetic stir bar. All emulsions had a surfactant-to-oil ratio of 2 and consisted of 1% MCT, 2% Tween 80 and 97% water by weight. In this

study we used the designation “E20” to refer to the emulsion prepared at 20 °C and “E60” to refer to the emulsion prepared at 60 °C.

5.3.2.2. Model gelatin gel system

All solutions were initially heated and stirred at 60 °C for 10 minutes prior to dissolution of the gelatin. The preparation conditions used in this study were based on those reported previously: powdered gelatin was gradually added to a stirring beaker placed on a hot plate at 60°C [129]. Samples were left to stir for an additional 10 minutes before spontaneous emulsification was carried out. Control samples without emulsion were also kept stirring for 10 minutes so that all samples had a total of 30 minutes on the hot plate at 60 °C (**Figure 25**). Samples were left to equilibrate at room temperature and gel for at least one hour prior to measurements. All gelatin systems contained 2% gelatin by volume. Hydrogel formation relied on a simple, easy to implement thermal transition [121]. “GE60/filled hydrogel” refers to the gelatin containing emulsion prepared at 60 °C.

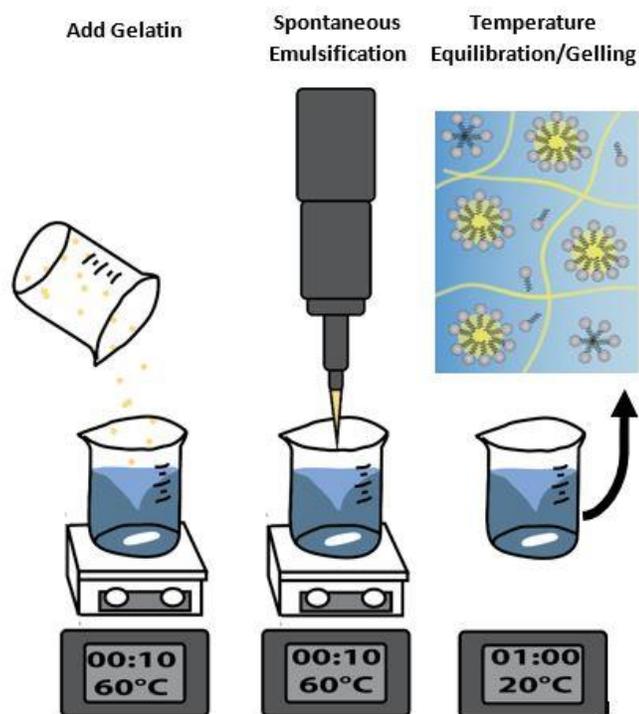


Figure 25. Schematic of model gelatin experimental methods. Temperature was first set and allowed to equilibrate for ten minutes. If gelatin was to be incorporated, it was added and allowed to stir for ten minutes. Next, the spontaneous emulsification process took place over ten minutes by adding oil and surfactant into the stirring water (and gelatin if incorporated). Lastly, the sample was allowed to equilibrate at room temperature ($\approx 20^{\circ}\text{C}$) for one hour prior to any measurements. The final product was a hydrogel matrix filled with emulsion droplets.

5.3.2.3. Model gelatin dessert system

To test a more realistic system, a commercial gelatin dessert was also tested. Jell-O[®] snack cups (Kraft Foods, Chicago, IL) were weighed and heated to 60 °C for ten minutes to melt the gel. Organic phase (surfactant and oil) was then titrated into the hot gelatin solution over a 10 minute period to perform the spontaneous emulsification process or a comparable amount of water was added as a control for dilution. Rheology and colorimetry tests were then conducted. Filled Jello hydrogels and Jello hydrogel were used to refer to the samples with and without the emulsion.

5.3.2.4. Sample characterization

5.3.2.4.1. Particle size analysis

The particle size distribution and mean particle diameter were measured using a commercial dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Samples were measured undiluted at 25 °C. Refractive indices of 1.333 and 1.445 were used for the continuous and dispersed phases respectively.

5.3.2.4.2. Temperature scanning analysis

The effect of temperature cycling was tested by use of a temperature scanning spectrophotometer (Cary 100 UV-Vis Biomelt, Agilent Technologies, United States). Emulsions prepared at room temperature, gelation temperature, and those containing gelatin were heated from 20 °C to either 60 or 90 °C, then cooled back to 20 °C at a rate of 1 °C/min. Turbidity was measured as the absorbance at 600 nm.

5.3.2.4.3. Temperature stability analysis

Samples were kept in storage for 1 week at 5, 20, and 55°C prior to size and turbidity analysis. Turbidity was measured using a UV/visible spectrophotometer (Ultraspec 3000 pro, Biochrom Ltd. Cambridge, England) at 600 nm.

5.3.2.4.4. Rheology

The influence of the nanoemulsion incorporation on the rheology of the samples was tested using a dynamic shear rheometer. Gelatin solutions and gelatin desserts were both tested using small deformation rheological measurements. Dynamic oscillatory measurements were performed on a rotational rheometer (Kinexus pro+, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Approximately 17.6 mL of sample was loaded into a cup at 60 °C. A strain sweep test revealed that 1% strain at 1 Hz frequency

was within the linear viscoelastic region (LVR) for the simple gelatin gels while the gelatin dessert required 0.1% strain and 1 Hz frequency to be in the LVR.

The measurements were carried out in three stages [130, 131]:

- a) Cooling from 60-5°C at a rate of 1°C/min
- b) Gelling/Annealing at 5°C for 55 minutes
- c) Heating from 5-60°C at a rate of 1°C/min

Data was analyzed as complex shear modulus (G^*) versus temperature and phase angle (δ) versus temperature. The complex shear modulus is defined as $\sqrt{G''^2 + G' ^2}$ and provides information about the overall stiffness of the sample. The phase angle ranges from 0 to 90°, with 0° being for a purely elastic material and 90° for a purely viscous fluid [132].

5.3.2.4.5. Color analysis

The tristimulus color coordinates ($L^*a^*b^*$) of the systems were measured using a colorimeter (ColorFlez EZ, HunterLab, Reston, Virginia, U.S.). L^* represented the lightness of the samples while a^* and b^* provide color coordinates. For the a^* scale, $+a^*$ is the red direction while $-a^*$ is the green direction. For the b^* scale, $+b^*$ is the yellow direction while $-b^*$ is the blue direction [133]. All samples were measured in the sol state. The samples were placed in an optical measurement cell and then a white or black plate was placed behind the samples. The lightness was calculated from the light reflected from the surface of the samples.

5.3.2.5. Experimental Design

All measurements were performed on two freshly prepared samples in at least duplicate. The mean and standard deviations were calculated from this data. Statistical

analysis was performed through subsection of the data to analysis of variance (ANOVA) using commercial statistics software (Minitab 16.2.4, Minitab Inc., State College, PA). Means were subject to Tukey's test and a P-value of <0.05 was considered statistically significant.

5.4. Results and Discussion

5.4.1. Preparation and characterization of nanoemulsions

Initially, the influence of preparation temperature on the properties of the nanoemulsions formed by spontaneous emulsification was measured. Nanoemulsions were either prepared at ambient temperature ($\approx 20^{\circ}\text{C}$) or at an elevated temperature (60°C) corresponding to the conditions used during gelatin incorporation. The mean particle diameter was approximately 83 nm for nanoemulsions prepared at ambient temperature, but only 42 nm for those prepared at 60°C , with both systems having monomodal particle distributions (**Figure 26**). In addition, the nanoemulsions prepared at 60°C had a higher optical clarity than those prepared at ambient temperature, which can be attributed to a reduction in light scattering with decreasing droplet size [2]. These results suggest that it is advantageous to produce nanoemulsions at an elevated temperature if one wants to produce optically transparent systems. However, the preparation temperature should not be too high otherwise droplet coalescence will occur. The mean particle diameter for a 1 wt% solution of Tween 80 micelles measured by dynamic light scattering was around 9 nm, which highlights the fact that the particles observed in the nanoemulsions were oil droplets rather than swollen micelles.

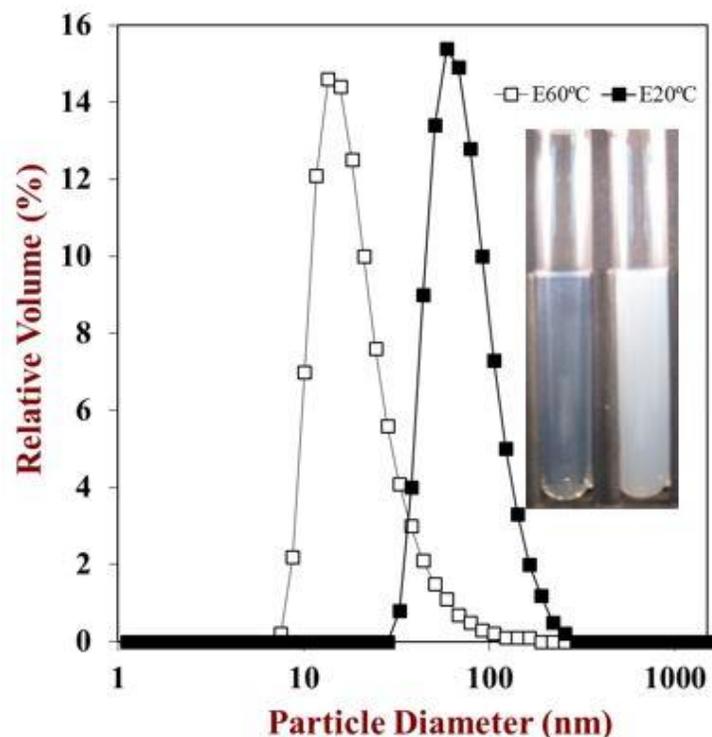


Figure 26. Particle size distribution for emulsions made at room temperature ($\approx 20^{\circ}\text{C}$) and gelation temperature ($\approx 60^{\circ}\text{C}$). Emulsions consisted of 1% medium chain triglycerides (MCT), 2% Tween 80, and 97% water. Inset picture shows the appearance of the emulsions prepared at room temperature and gelation temperature.

Previous studies have also reported that the size of the droplets in nanoemulsions produced using low energy methods depends on the preparation temperature. For example, the droplet size of nanoemulsions produced by spontaneous emulsification has been shown to decrease when the temperature was increased [28, 39]. There are a number of possible physiochemical mechanisms that may account for the decrease in droplet size with increasing preparation temperature. The underlying principle governing spontaneous emulsification is the movement of surfactant molecules from the organic to the aqueous phase [28]. The viscosity of the oil phase decreases appreciably with increasing temperature [134], which may facilitate the diffusion of surfactant molecules through the

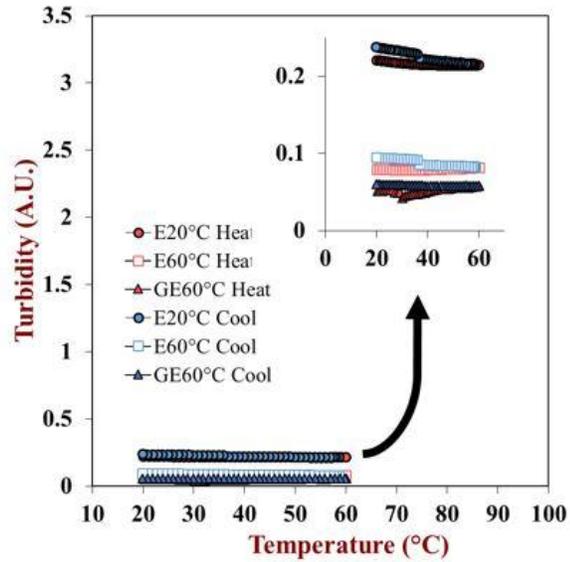
oil phase and therefore the spontaneous formation of small droplets. In addition, surfactant head groups become progressively dehydrated as the temperature is increased, which alters the optimum curvature of the surfactant monolayer [118]. As the phase inversion temperature (PIT) is approached there is a reduction in interfacial tension. A lower interfacial tension may also favor the spontaneous formation of ultrafine droplets at the boundary between the organic and aqueous phases. However, if the preparation temperature is too close to the PIT, then extensive droplet coalescence may occur (see below), and the droplet size may increase again [135].

Commercially, it is important to establish the range of temperatures over which a product will remain stable. We therefore measured the change in turbidity with temperature for the two nanoemulsions (**Figure 27**). Samples were either heated from 20 to 60 °C and then cooled back to 20 °C (**Figure 27a**), or they were heated from 20 to 90 °C and then cooled back to 20 °C (**Figure 27b**). Prior to heating, the nanoemulsions had relatively low turbidities due to their small particle sizes: $\tau = 0.08$ and 0.22 cm^{-1} for the nanoemulsions prepared at 60 °C and ambient temperature, respectively. The turbidities of these samples remained relatively low when they were heated to 60 °C and then cooled down (**Figure 27a**), and there was little change in particle size after heating (**Figure 28**). These results suggested that the nanoemulsions were relatively stable to droplet growth over this temperature range (20 to 60 °C). On the other hand, there was a large irreversible increase in turbidity of both samples after they were heated to 90 °C (**Figure 27b**), which can be attributed to an increased in droplet diameter (**Figure 28**). During the heating stage, the turbidity started to increase appreciably around 74 °C, which can be credited to droplet coalescence as the surfactant-oil-water systems approached the PIT

[135]. The PIT of the SOW system used in this study could not be accurately established as it was above the highest temperature used (i.e., 90 °C). As mentioned above, the head groups of the surfactant molecules become progressively dehydrated as the temperature is raised, which leads to an ultra-low interfacial tension, and therefore high susceptibility to coalescence [118, 135, 136]. Instability at elevated temperatures is often a concern for nanoemulsions formed using low energy methods, and must be taken into account when developing food-grade delivery systems based on this approach [51].

Commercially, samples may be stored at different temperatures for extended periods. We therefore examined the stability of the nanoemulsions after 1 week storage at three different holding temperatures: 5, 20 and 55 °C (**Figure 29**). These results showed that there was an appreciable increase in sample turbidity and mean particle size for the nanoemulsions stored at the highest holding temperature (55 °C), but only a slight or negligible increase at lower temperatures. These results suggest that the samples should remain relatively stable when stored under refrigerator or ambient temperatures.

a)



b)

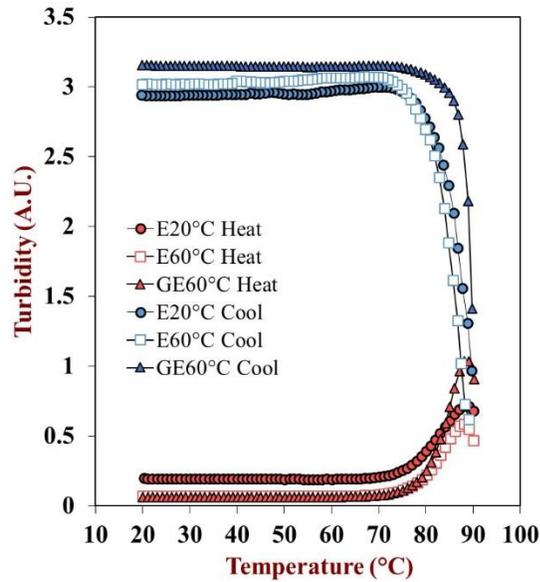


Figure 27. Temperature scans of samples prepared at room temperature (E20°C), gelation temperature (E60°C), and with gelatin (GE60°C). Emulsion (E) samples consist of 1% medium chain triglycerides (MCT), 2% Tween 80, and 97% water, while GE also contains 2% w/v gelatin. The temperature scans consisted of a) 20 to 60 to 20°C and b) 20 to 90 to 20°C, with the heating cycle occurring first followed by the cooling cycle. In figure a) the inset picture shows a zoomed in image to help better see what is occurring between the individual samples.

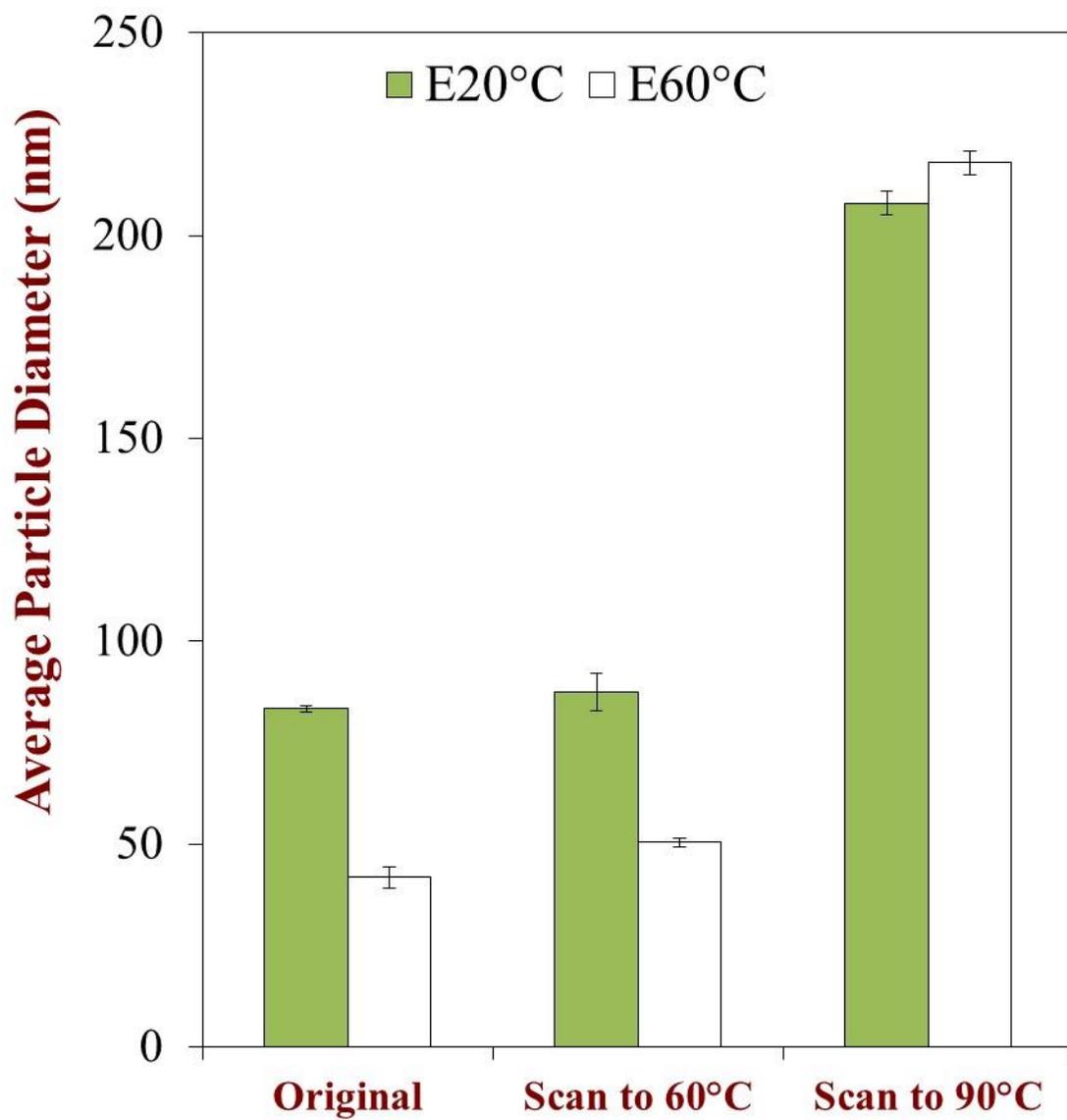
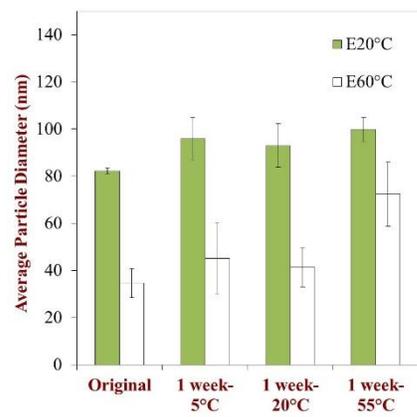
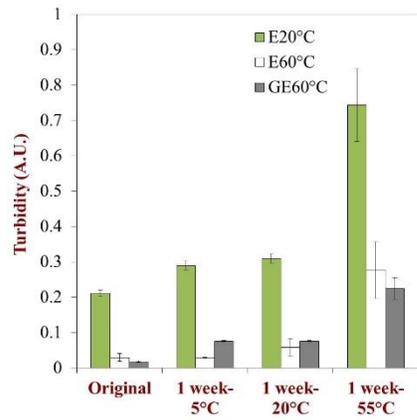


Figure 28. Particle size as a function of maximum temperature scanned to. Samples were measured 1 hour after preparation (either at 20 or 60°C) and 1 hour after temperature cycling (either to 60 or 90°C and back to 20°C) was complete.

a)



b)



c) From left to right, E20°C, E60°C, Pure gelatin, GE60°C

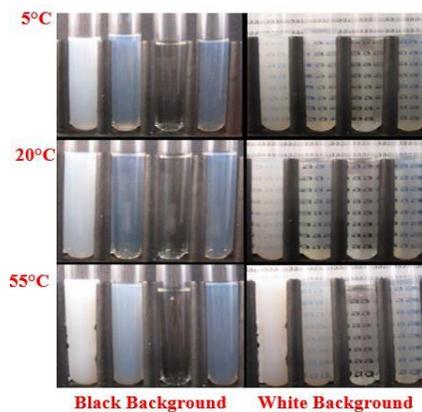


Figure 29. Particle size (a), turbidity (b), and physical appearance (c) of nanoemulsions and gels as a function of storage temperature. Samples were measured 1 hour after preparation (either at 20 or 60°C) and after 1 week of isothermal storage (either 5, 20 or 55°C) was complete. The samples containing gelatin remained in the test tubes after they were turned upside down.

5.4.2. Preparation and characterization of model gelatin systems

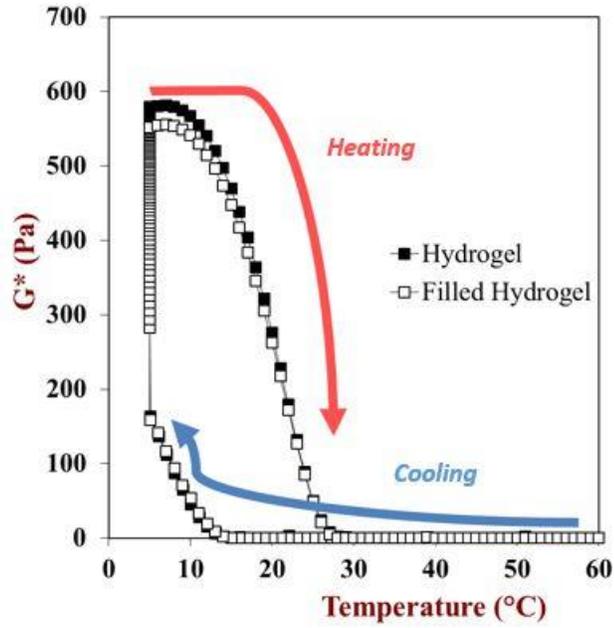
The nanoemulsions were then incorporated into a simple model hydrogel system consisting of 2% gelatin dissolved in water. Measurements of the temperature-dependence of their optical properties indicated that the nanoemulsions dispersed in gelatin had a significantly lower turbidity across all temperatures than the equivalent nanoemulsions alone (**Figure 27**). The average turbidity of nanoemulsions with gelatin was 0.055 cm^{-1} compared to 0.084 cm^{-1} for those without gelatin. The lower turbidity likely corresponds to a decrease in particle size, although this could not be directly measured using traditional light scattering methods (because the aqueous phase was gelled) or seen under a light microscope (because the droplets were too small). A possible mechanism for this effect is that the presence of the gelatin increases the aqueous phase viscosity, which reduces droplet coalescence during the SE process. The presence of the gelatin in the aqueous phase might also be expected to inhibit droplet coalescence and creaming during storage [6]. However, we did not find any major differences between the turbidities or appearances of the nanoemulsions in the absence or presence of gelatin after storage (**Figure 29**). These results again suggest that it would be beneficial to store samples at refrigerated or ambient temperatures, which are temperatures commonly used for the storage of commercial gelatin desserts.

The effect of nanoemulsion incorporation on the rheological properties of the gelatin gel was also measured (**Figure 30**). These results clearly show that introduction of the lipid nanoparticles into the hydrogels had little effect on their rheological characteristics. As expected, the gelatin formed gels upon cooling, which melted upon heating. The gelation and melting temperatures were determined from the phase angle data as $14 \text{ }^{\circ}\text{C}$ and $27 \text{ }^{\circ}\text{C}$ respectively for both the hydrogel and filled hydrogel systems.

It was assumed that a sol-to-gel transition occurred when the phase angle decreased below 45° , and vice versa. The fact that the lipid nanoparticles had little effect on the rheology of the gels can be attributed to the relatively low droplet concentration (1%) and the fact that the small particles easily fit into the pores in the gel.

Colorimetry was used to investigate the effect of the nanoemulsion on the optical properties of the gelatin gels (**Figure 31**). The tristimulus (L^*, a^*, b^*) values of the samples was measured against both a white background and a black background. There was a significant difference in all the color coordinates when a black background was used (**Figure 31a**), but only for the b^* values when a white background was used (**Figure 31b**). This phenomenon can also be seen visually in the photographs of the samples, where the samples looked appreciably different when observed against a black background but less so when observed against a white background. When the samples were observed in front of a white background, they had a slight blue tint, which is supported by the negative b^* value. This effect can be attributed to preferential scattering of light of different wavelengths by the nanoemulsion droplets, i.e., blue light (shorter wavelength) is scattered more than red light (longer wavelength) [137]. The fact that the letters written on the white background could still be observed highlights the fact that the samples were still translucent. Overall, these results show that the optical properties of the gelatin hydrogels are altered somewhat by the presence of the nanoemulsions, which can be attributed to light scattering effects, but that they are still translucent. In practice, much lower amounts of lipophilic agents (such as vitamins or nutraceuticals) may need to be incorporated into hydrogels than used in this study (1%), which would increase the optical clarity further.

a)



b)

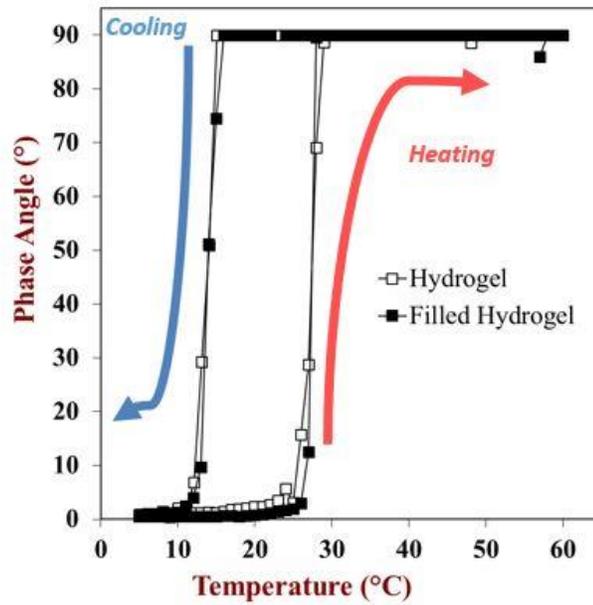
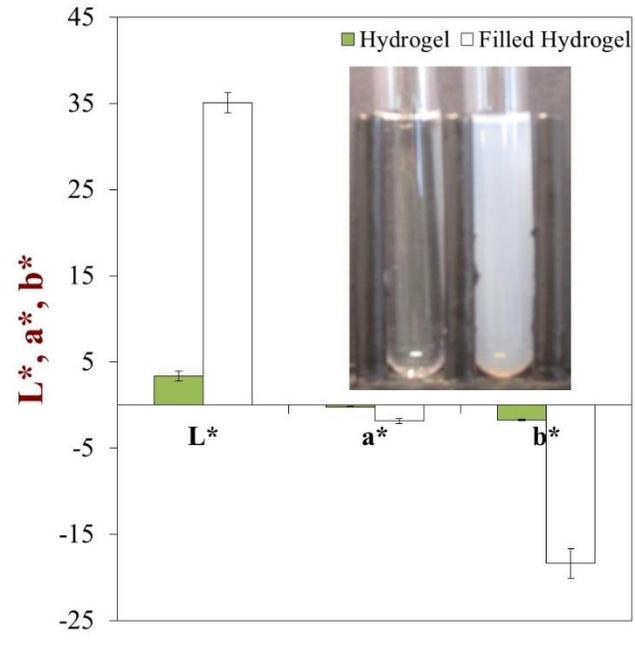


Figure 30. The effect of nanoemulsion addition and temperature on the rheology of model gelatin systems. The samples were cooled from 60-5°C, held at 5°C then heated from 5-60°C. Data was plotted as a) complex shear modulus (G^*) versus temperature and b) phase angle versus temperature.

a) Black background



b) White background

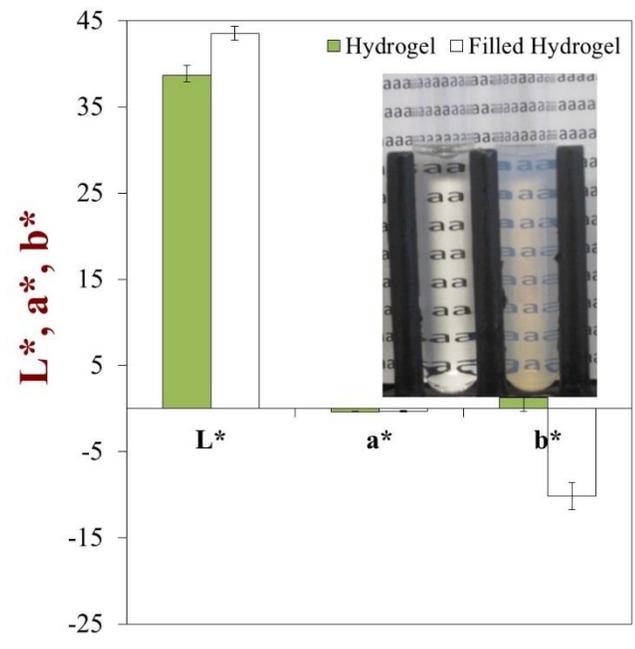


Figure 31. Influence of nanoemulsion addition and background color on the appearance and color coordinates of model gelatin hydrogels. Inset shows sample with same background color.

5.4.3. Preparation and characterization of gelatin dessert system

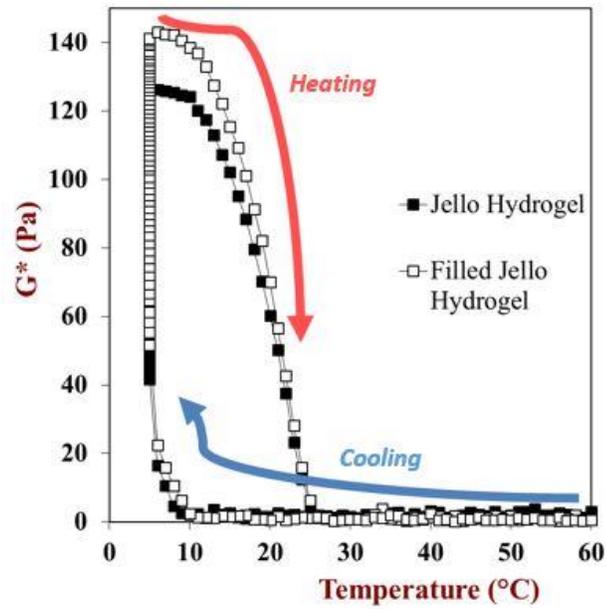
The results found in this simple hydrogel system encouraged us to test a more complex commercial gelatin system. In addition to gelatin this system also contained ingredients commonly found in gelatin desserts including acids, sweeteners, salt, colors and flavors. The rheology results for the model gelatin dessert (**Figure 32**) were fairly similar to those observed in the model gelatin systems, *i.e.*, addition of nanoemulsion did not appreciably change gelling or melting behavior. We did observe a slightly higher complex shear modulus for the gelatin sample containing nanoemulsion after the annealing stage but the gelling (10 °C) and melting (27 °C) temperatures were similar in the presence and absence of lipid nanoparticles. The phase angle data for the commercial gelatin gels (**Figure 32b**) showed more variation (“noise”) than for the model gelatin systems (**Figure 30**), which is likely because the commercial sample had a lower gelatin concentration and therefore aqueous phase viscosity and elastic modulus.

Colorimetry was used to characterize the optical properties of the gelatin desserts. Two flavors (strawberry and lemon-lime), and thus two corresponding colors (red and green), were tested. Nanoemulsions contain relatively small particles that preferentially scatter certain wavelengths on light. Indeed, in the absence of added dyes, they often have a bluish color. It was therefore interesting to determine the influence of nanoemulsion addition on the optical properties of commercial hydrogels of different colors. Similar results were observed for both red and green colored Jello (**Figure 33**). In both cases, more appreciable color changes (L^* , a^* , and b^* values) were observed with a black background than with a white background. These results are consistent with those obtained for the model gelatin system. It may be possible to reduce the influence of the nanoemulsions on the appearances of the commercial hydrogels by reducing the particle

size or reducing the oil content. Our systems were all tested with 1% oil in the system, which is on the high end of what would need to be incorporated to get a daily value of most lipophilic bioactive compounds. For example, the recommended dietary allowance (RDA) for Vitamin D in young people is 600 IU, or 15 μg [138]. An average serving size of gelatin dessert is approximately 100 grams. On a pure weight basis, that means only $1.5 \times 10^{-6}\%$ of the gelatin dessert has to be Vitamin D to reach the daily required value. Typically Vitamin D is dissolved in a carrier oil (such as MCT) and therefore total level of oil droplets present may be somewhat higher than this value. Even considering the use of a carrier oil, it is easy to see that the final oil content, and subsequent surfactant concentration, will likely be lower than the maximum we tested. It should be noted that if a nanoemulsion is diluted too much so that the surfactant concentration falls below the critical micelle concentration (CMC) it may become unstable, which should be taken into account when formulating appropriate delivery systems using this method.

Lastly, the development of new colors using filled hydrogels may actually be advantageous in the food industry. Consumers are likely not able to differentiate the difference of the filled and unfilled Jello hydrogels when the products are not directly next to one another. Additionally, there is currently interest in moving towards natural colors for health and safety reasons [139]. As there is a limited number of approved colors, the use of developing new colors by incorporation of colloidal particles, such as nanoemulsions, is a real possibility [140].

a)



b)

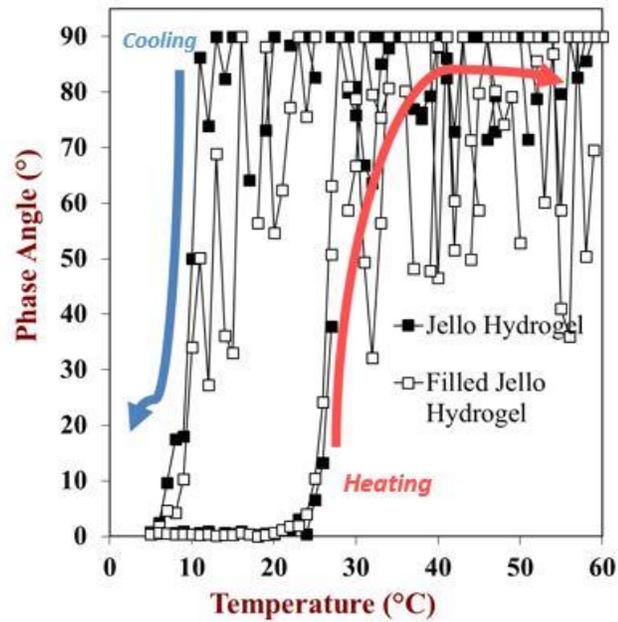
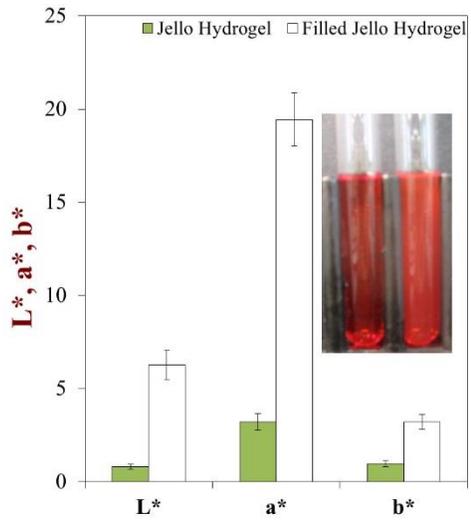
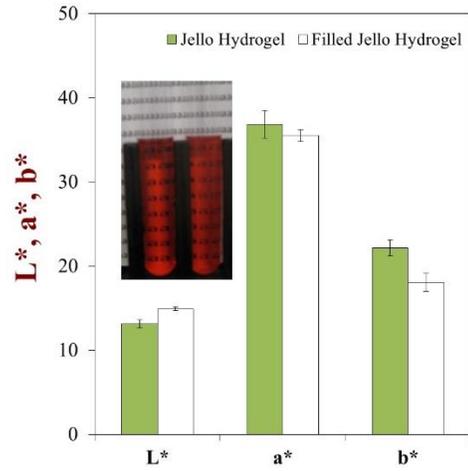


Figure 32. Influence of nanoemulsion addition on the rheology of commercial gelatin hydrogels. The system was cooled from 60-5°C, held at 5°C then heated from 5-60°C. Data was plotted as a) complex shear modulus (G^*) versus temperature and b) phase angle versus temperature.

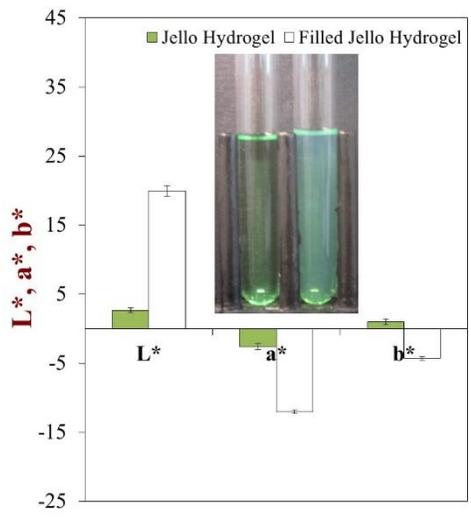
a) Red Jello, Black Background



b) Red Jello, White Background



c) Green Jello, Black Background



d) Green Jello, White Background

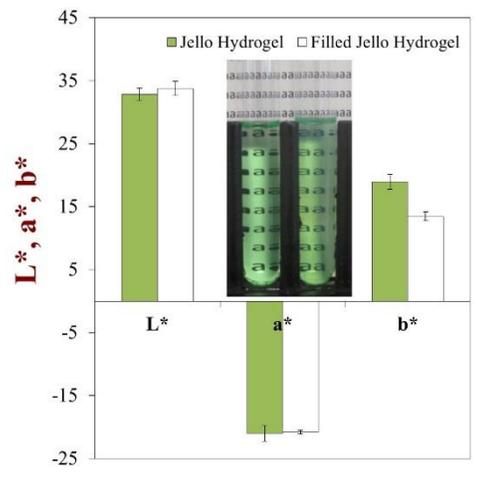


Figure 33. Influence of nanoemulsion addition and background color on the appearance and color coordinates of two commercial gelatin hydrogels ("Jello"). Inset shows sample with same background color. Jello hydrogel is on left and filled jello hydrogel is on right for all inset pictures.

5.5. Conclusions

Previous work on spontaneous emulsification has focused on beverage applications [12, 39] while research related to emulsion gels has mostly focused on protein stabilized systems, specifically milk proteins [128, 141]. In this study we investigated the incorporation of nanoemulsions into a gelatin dessert using spontaneous emulsification. We have shown that nanoemulsions ($d < 100$ nm) can be produced by simple addition of an organic phase (oil and surfactant) to a stirring aqueous phase, with an elevated temperature leading to a smaller particle size. Additionally we showed that nanoemulsion incorporation into a model gelatin system and a gelatin dessert had little effect on their rheological characteristics, and only caused slight changes in their optical properties (but still gave translucent hydrogels). These results suggest that the spontaneous emulsification method may be useful for incorporating fat-soluble compounds, such as vitamins or nutraceuticals, into a gelatin matrix. The main disadvantage of the spontaneous emulsification method is that it requires high levels of synthetic surfactants. However, with such small amounts of oil being required for a product such as gelatin desserts this may be overcome. In future studies, it would be advantageous to establish whether the same procedure could be used with incorporation of specific bioactive ingredients.

It should be noted that lipophilic bioactive ingredients may also be solubilized into micelle or microemulsion systems, which could then be incorporated into hydrogels. These systems have the advantage that they are optically clear and thermodynamically stable, but they typically require higher surfactant concentrations than nanoemulsions.

5.6. Acknowledgements

This material is based upon work supported by a United States Department of Agriculture, NRI and AFRI Grants (2011-67021, 2014-67021, 2011-03539, 2013-03795).

CHAPTER 6

FORMATION OF OIL-IN-WATER EMULSIONS FROM NATURAL EMULSIFIERS USING SPONTANEOUS EMULSIFICATION: SUNFLOWER PHOSPHOLIPIDS

6.1. Abstract

This study examined the possibility of producing oil-in-water emulsions using a natural surfactant (sunflower phospholipids) and a low-energy method (spontaneous emulsification). Spontaneous emulsification was carried out by titrating an organic phase (oil and phospholipid) into an aqueous phase with continuous stirring. The influence of phospholipid composition, surfactant-to-oil ratio (SOR), initial phospholipids location, storage time, phospholipid type, and preparation method were tested. The initial droplet size depended on the nature of the phospholipid used, which was attributed to differences in phospholipid composition. Droplet size decreased with increasing SOR and was smallest when the phospholipid was fully dissolved in the organic phase rather than the aqueous phase. The droplets formed using spontaneous emulsification were relatively large ($d > 10 \mu\text{m}$), and so the emulsions were unstable to gravitational separation. At low SORs (0.1 and 0.5), emulsions produced with phospholipids had a smaller particle diameter than those produced with a synthetic surfactant (Tween 80); but at a higher SOR (1.0), this trend was reversed. High-energy methods (microfluidization and sonication) formed significantly smaller droplets ($d < 10 \mu\text{m}$) than spontaneous emulsification. The results from this study show that low-energy methods could be utilized with natural surfactants for applications where fine droplets are not essential.

6.2. Introduction

Oil-in-water emulsions can be found in a variety of food and beverage products, including creams, desserts, dressings, dips, milks, sauces and soft drinks [142]. These emulsions can be formed using either high-energy or low-energy methods. High-energy approaches, such as colloid mills, high pressure homogenizers, sonicators, and microfluidizers, rely on specialized equipment to disrupt and intermingle the oil and water phases thereby forming small droplets [98]. In contrast, low-energy approaches require no specialized equipment and utilize the physicochemical properties of the surfactant, oil, and water system to spontaneously generate emulsion droplets based on simple mixing procedures or by simply changing environmental conditions such as temperature [3, 111]. High-energy methods are currently the most commonly used in the food industry because they are already well-established, capable of large-scale production, and can produce emulsions and nanoemulsions from a range of components [76]. Low-energy methods, however, are of growing interest due to their low cost and ease of implementation [51].

A major drawback of high-energy methods is the requirement for relatively expensive specialized equipment, such as colloid mills, sonicators, high pressure homogenizers, or microfluidizers [143]. Sonication has been used to form emulsions from a variety of different oils and surfactants [144-147]. . It has advantages such as requiring low surfactant concentrations, being fairly energy-efficient, having low production costs, and being easy to operate, clean, and control [98]. However, scaling-up from the laboratory to an industrial-scale food processing operation has been a major challenge [85]. High pressure homogenization can be achieved using specialized

equipment such as high pressure valve homogenizers (HPVHs) and microfluidizers. HPVHs are currently the most common method of producing fine emulsions in the food industry and involve forcing a coarse emulsion through a narrow gap at high pressure. Microfluidizers have been shown to be one of the most efficient systems for producing fine emulsions [148], and are therefore gaining increasing application within the food industry. Inside a microfluidizer, an emulsion is split into two channels and then the two channels are directed towards each other in an interaction chamber. As a result, intense disruptive forces are generated within the interaction chamber that lead to highly efficient droplet fragmentation [76].

While high-energy approaches are based on the utilization of specialized mechanical homogenizers, low-energy approaches only require a simple low-intensity mixer. Numerous low-energy methods are available that can be broadly categorized into two classes: *thermal methods*, which rely on a change in temperature; and, *isothermal methods*, which rely on a change in system composition [51]. On an industrial scale, the isothermal methods are likely to be easy to implement since rapidly changing the temperature of large volumes of fluids, which is required for the thermal methods, may be difficult and expensive. Of the isothermal methods, *spontaneous emulsification* has the most potential for commercial applications. When making an oil-in-water emulsion, the volume of the organic phase is usually less than that of the aqueous phase. In spontaneous emulsification, where the organic phase is added into the aqueous phase, this makes the technique easier to implement than a method like *emulsion phase inversion* where the aqueous phase is added to the organic phase.

However, a major limitation to the widespread use of the spontaneous emulsification method is the requirement for high concentrations of synthetic surfactants [76]. High amounts of surfactant can lead to cost, taste, and toxicity concerns [15]. In addition, as consumers become more label conscious, there is a movement away from synthetic and towards natural ingredients, such as natural emulsifiers [149, 150]. Depending on which technique is used for emulsion formation, emulsifiers can serve different purposes with the common trend being that they concentrate at the oil-water interface and thus lower the interfacial tension [151]. For high-energy methods, emulsifiers facilitate droplet fragmentation within the homogenizer, as well as providing stability to the droplets after they have been formed [152]. For low-energy methods, the emulsifier assists in the spontaneous formation of the emulsion droplets, as well as providing long-term stability to droplets after formation [76]. Therefore it is critical that an emulsifier be present but it is preferred that it comes from a natural source and is used at a low level.

There are many different natural emulsifiers available for utilization within foods, including proteins, polysaccharides, and phospholipids [153]. Phospholipids are of particular interest because they contain both hydrophilic and hydrophobic groups making them easily able to orient at the oil-water interface [154]. There are a variety of sources that phospholipids can come from including soybeans, rapeseeds, or sunflowers [151]. In the food industry, the term “lecithin” refers to a mixture of different types of phospholipids, whereas in some other industries it is mainly used to refer to phosphatidylcholine [153]. While phospholipids from soybean may be relatively cheap [155], they are difficult to find from a non-genetically modified source [151] and need to

be declared as an allergen on food labels [156]. Therefore, interest in phospholipids from sunflowers is growing because they are not genetically modified [151, 152, 157, 158] and contain no declared allergens [155, 156]. The major disadvantage of sunflower phospholipids is they are more expensive and difficult to extract [155]. However, with public awareness of food allergens and genetically modified organisms growing [156], the use of sunflower phospholipids may become more prevalent for certain applications.

The main objective of the current study was to investigate the possibility of forming food-grade oil-in-water emulsions using a natural surfactant (sunflower phospholipids) and a low-energy method (spontaneous emulsification). A substantial amount of research has been carried out on optimizing this process with synthetic surfactants [15, 30, 46, 51] and using the resulting emulsions as delivery systems [12, 33, 40, 47, 48]. Recently researchers in the pharmaceutical industry have reported that emulsions can be formed by spontaneous emulsification using phospholipids as emulsifiers, but cosolvents were also required [49]. In the current study, we examined the effect of different types of sunflower phospholipids, surfactant-to-oil ratio (SOR), initial surfactant location, and storage stability. Additionally, we prepared emulsions with a synthetic surfactant and with high-energy methods so direct comparisons could be drawn.

6.3. Materials and methods

6.3.1. Materials

Four different natural phospholipid surfactants derived from sunflower oil were kindly donated by a commercial supplier (Perimondo, New York, USA) (**Table 13**). Tween[®] 80 (Sigma-Aldrich, St. Louis, USA) and medium chain triglycerides (MCT,

Miglyol 812N, Warner Graham Company, Cockeysville, USA) were bought from chemical suppliers. The aqueous phase for all emulsions was a sodium phosphate buffer solution (5 mM; pH 7.0). Distilled and deionized water obtained from a water purification system (Milli-Q[®], Millipore, Darmstadt, Germany) was used to prepare all solutions and emulsions.

Table 13. Properties of the sunflower phospholipids used in this study (as provided by manufacturer).

a) Phospholipid Information

Phospholipid	Weight %			
	Sunlipon TM 50	Sunlipon TM 65	Sunlipon TM 75	Sunlipon TM 90
Phosphatidylcholine	58	65	74	90
1-Lysophosphatidylcholine	1	1	1	0.5
2-Lysophosphatidylcholine	3	5	4	2
Phosphatidylinositol	1	<1	-	-
Lysophosphatidylinositol	-	-	-	-
Phosphatidylserine-Na	-	-	-	-
Lysophosphatidylserine	-	-	-	-
Sphingomyelin	-	-	-	-
Phosphatidylethanolamine	5	4	0.5	-
Lysophosphatidylethanolamine	1	<1	-	-
acyl- Phosphatidylethanolamine	3	2	2	-
Phosphatidylglycerol	1	1	-	-
Phosphatidic acid	-	<1	-	-
Lysophosphatidic acid	0.2	-	-	-
Other	2	1	1	0.1

-: Not observed

b) Fatty Acid Information (not provided for Sunlipon™ 75)

Fatty Acids	Weight %		
	Sunlipon™ 50	Sunlipon™ 65	Sunlipon™ 90
Total Fatty Acids	59.3	62.8	62.6
14:0 Myristic	0.1	0.1	0.1
15:0 Pentadecanoic	0.02	0.03	0.02
16:0 Palmitic	5.8	6.5	6.1
16:1 Palmitoleic	0.04	0.1	0.1
17:0 Heptadecanoic	0.1	0.1	0.04
18:0 stearic	1.6	1.5	1.5
18:1 Oleic	9.8	10.6	11.1
18:2 Linoleic	40.8	43.3	43.3
18:3 α -Linolenic	0.1	0.2	0.1
20:0 Arachidic	0.1	0.1	0.1
20:1 Eicosenoic	0.1	0.1	0.1
22:0 Behenic	0.2	0.2	0.1
24:0 Lignoceric	0.1	0.1	0.03

6.3.2. Methods

6.3.2.1. Emulsion preparation

Emulsions were prepared by spontaneous emulsification by titrating an organic phase into an aqueous phase (**Figure 34a**). In most experiments the organic phase consisted of oil and surfactant. The oil used in all experiments was medium chain triglycerides (MCT) and the surfactant used was Sunlipon™ 90 (unless specified otherwise). The titrations were performed in a 100 mL beaker at a temperature of 60 °C. The experiments were designed so that the final emulsion always had a total mass of 50 g including 2.5 g of oil (*i.e.*, 5 wt% oil). Initially, an organic phase was prepared by adding the surfactant and oil to the beaker and then mixing using a magnetic stirrer (500 rpm) for a minimum of 30 minutes. A temperature of 60 °C was utilized to facilitate the dispersion of the surfactant in the oil phase [154]. The thoroughly mixed organic phase was then added to a stirring aqueous phase (750 rpm) slowly over 5 minutes. An additional 5 minutes was allowed for mixing to bring the total preparation time to 10 minutes. Emulsions were allowed to equilibrate for one hour prior to initial analysis.

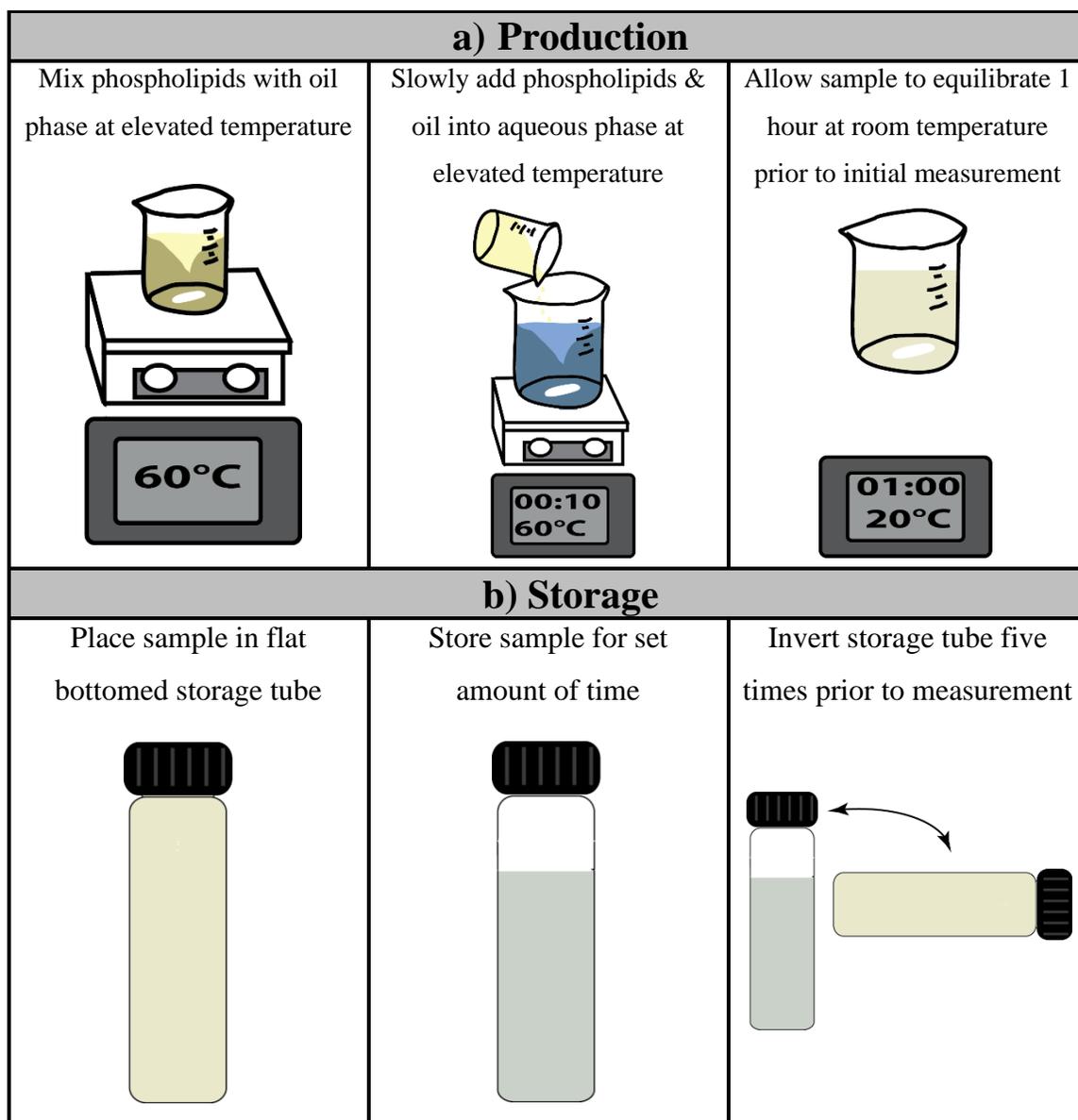


Figure 34. a) Schematic of spontaneous emulsification process using phospholipids as the surfactant phase and b) the storage protocol. Heat is required to disperse the phospholipids in the oil phase prior to conducting the spontaneous emulsification. Emulsions were transferred to flat bottomed storage tubes so that creaming could be measured. Prior to measurement, samples were inverted to re-disperse the oil droplets.

6.3.2.2. Variables tested

Six main variables were tested: phospholipid composition, surfactant-to-oil ratio, surfactant location, storage time, surfactant type, and preparation method.

6.3.2.2.1. Phospholipid composition

As stated in the materials, four different phospholipids were tested (Sunlipon™ 50, 65, 75, and 90), which mainly differ in their ratio of phosphatidylcholine (Table 1). All further experiments were conducted with Sunlipon™ 90.

6.3.2.2.2. Surfactant-to-oil ratio

The influence of surfactant concentration was investigated by varying the surfactant-to-oil ratio (SOR). The total oil content in the final systems was held constant at 5%, while the SOR was varied by altering the amounts of surfactant and buffer in the final system:

$$\text{SOR} = \frac{m_s}{m_o} \quad (6.1)$$

$$m_b = 100 - m_o - m_s \quad (6.2)$$

Here, m_s , m_o and m_b are the mass percentages of surfactant, oil and buffer in the overall system, respectively. SORs tested for all variables included 0.1, 0.5, and 1.

6.3.2.2.3. Surfactant location

The effect of surfactant location was tested by varying the relative amounts of surfactant in the aqueous and organic phases. The amount of the surfactant initially in the aqueous phase was varied from 100 to 0% in 25% intervals, with the remainder of the surfactant being incorporated into the organic phase. Thus, all the systems had the same final composition.

6.3.2.2.4. Storage time

To observe how the emulsions behaved over the span of a week, a shelf-life study was conducted (**Figure 34b**). After production and initial measurement, samples were placed in flat bottomed storage tubes. Each tube held 15 mL of emulsion. At set intervals of time (1-7 days), samples were analyzed for particle size, visual appearance, and creaming index. Prior to particle size analysis measurement, storage tubes were inverted five times to ensure the sample was homogenous. Separate storage tubes were used for particle size analysis and visual appearance/creaming index samples. On day 0 and day 7 microscopy analysis was also conducted.

The increase in droplet diameter over time was represented by *droplet growth* (%), which was calculated according to the following equation:

$$G = \frac{d_f - d_i}{d_i} \times 100 \quad (6.3)$$

where G is droplet growth (%), d_f is the final emulsion diameter, and d_i is the initial emulsion diameter.

6.3.2.2.5. Surfactant type

The effect of surfactant type was investigated by preparing emulsions using either a synthetic surfactant (Tween® 80) or a natural surfactant (Sunlipon™ 90). Tween® 80 was chosen as the synthetic surfactant based on previous research that showed it formed the smallest particle size using the spontaneous emulsification method out of a range of synthetic surfactants tested [15].

6.3.2.2.6. Preparation method

The influence of preparation method was tested by preparing emulsions using either a low-energy technique (spontaneous emulsification) or high-energy technique

(microfluidization or sonication). With both high-energy methods, Sunlipon™ 90 was dissolved in the aqueous phase for 30 minutes at room temperature rather than being mixed in with the organic phase.

Spontaneous emulsification: Described in 6.3.2.1.

Microfluidization: Coarse emulsions were prepared by blending both organic (MCT) and aqueous phases (5 mM sodium phosphate, pH 7.0 and Sunlipon™ 90) together using a high-speed blender (Bamix, Switzerland) for 2 minutes at room temperature. The coarse emulsions were then passed through a high pressure homogenizer (Microfluidics M110L, Newton, MA, USA) for 3 cycles at 12,000 psi.

Sonication: The organic (MCT) and aqueous (5 mM sodium phosphate, pH 7.0 and Sunlipon™ 90) phases were combined prior to being placed in the sonicator. Samples were sonicated for 2 minutes at an amplitude of 70% and pulse of 5 seconds on followed by 1 second off (Model 505 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA).

6.3.2.3. Emulsion characterization

Emulsions and components were analyzed using a variety of techniques including particle size analysis, droplet shape analysis, zeta potential analysis, visual observation, creaming index measurement, and optical microscopy.

6.3.2.3.1. Particle size analysis

The particle size distribution and mean particle diameter ($D[4,3]$) were measured using a commercial static light scattering (SLS) instrument (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in double distilled water prior to analysis to avoid multiple scattering effects. The diameter is presented as

the volume-weighted mean diameter ($D[4,3] = \sum n_i d_i^4 / \sum n_i d_i^3$) rather than the surface-weighted mean diameter ($D[3,2] = \sum n_i d_i^3 / \sum n_i d_i^2$) because the mean-volume weighted diameter is more sensitive to fat droplet growth through coalescence and/or flocculation [158, 159]. Here, n_i is the number of droplets with diameter d_i in the i^{th} size category.

6.3.2.3.2. Droplet shape analysis

The interfacial tension at the organic-aqueous interface was measured using a droplet shape analysis device (DSA 100, Krüss GmbH, Hamburg, Germany). For oil droplet formation a hook-needle with a diameter of 1.463 mm was used to create a pendant drop. The pendant drop was extruded into a quartz cell containing buffer solution (sodium phosphate, 5 mM, pH 7.0). Each sample was a composite of measurements made every 0.1 seconds for 15 seconds. Interfacial tension values were calculated based on the Young-Laplace equation by the drop shape analysis program supplied by the instrument manufacturer. Digital images were also captured using the device's camera function.

6.3.2.3.3. ζ -potential analysis

The electrical charge (ζ -potential) on the droplets was measuring using particle electrophoresis (Zetasizer Nano ZS-90, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in buffer solutions prior to measurement to avoid multiple scattering effects.

6.3.2.3.4. Visual observation

Samples were observed visually and recorded using a digital camera (Lumix ZS8, Panasonic, Osaka, Japan). All pictures were taken against a black background in a photo box using ambient light.

6.3.2.3.5. Creaming index

Creaming of stored samples (15 mL) was monitored at room temperature. At set time intervals (0-7 days) the height of the serum layer (H_s) and cream layer (H_c) were measured with a ruler. From this, the total height of emulsion (H_t) and creaming index (CI) could be calculated:

$$H_t = H_s + H_c \quad (6.4)$$

$$CI = \frac{H_s}{H_t} \times 100 \quad (6.5)$$

6.3.2.3.6. Microscopy

An optical microscope (C1 80i Digital Eclipse, Nikon, Tokyo, Japan/Melville, NY, U.S.) with a 20× objective lens and 10× eyepiece was used to capture the images of emulsions produced initially after production and after seven days of storage. The cream layer of stored samples was also observed in addition to the homogenous emulsion, created by inverting the storage tube five times prior to placing on the slide. Pictures were analyzed using image analysis software provided by the manufacturer.

6.3.2.4. Experimental design

All measurements were performed on two freshly prepared samples in triplicate. The mean and standard deviations were calculated from this data. Statistical analysis was performed through subsection of the data to analysis of variance (ANOVA) using commercial statistics software (Minitab 16.2.4, Minitab Inc., State College, PA). Means were subject to Tukey's test and a P -value of <0.05 was considered statistically significant.

6.4. Results and discussion

6.4.1. Influence of phospholipid composition

The influence of four phospholipids at three surfactant-to-oil ratios on the properties of oil-in-water emulsions formed using the spontaneous emulsification method was tested (**Figure 35**). For each phospholipid type, a similar general trend of decreasing particle size with increasing surfactant concentration was observed, although at the higher SOR levels (0.5 and 1.0) these differences were not significant for most systems. Nevertheless, phospholipid type also had an appreciable influence on particle size. For example, SunliponTM 75 produced significantly larger droplets than the other phospholipids at all SOR values studied. Other researchers have also observed a decrease in particle diameter with an increase in lecithin concentration using various homogenization methods [152, 154, 158, 160]. For example, the smallest droplet diameter ($D[4,3]$) produced using a homogenizer was reported to be about 40 μm for oil-in-water emulsions containing lecithin at a SOR of 0.11 [160]. In comparison, emulsions formed using the spontaneous emulsification method at an SOR of 0.1 using SunliponTM 50, 65, 75, and 90 had mean droplet diameters ($D[4,3]$) of 26, 23, 68, and 40 μm . These results suggest that the low-energy method (spontaneous emulsification) may be a viable alternative to high-energy methods (homogenizers) when using sunflower phospholipids.

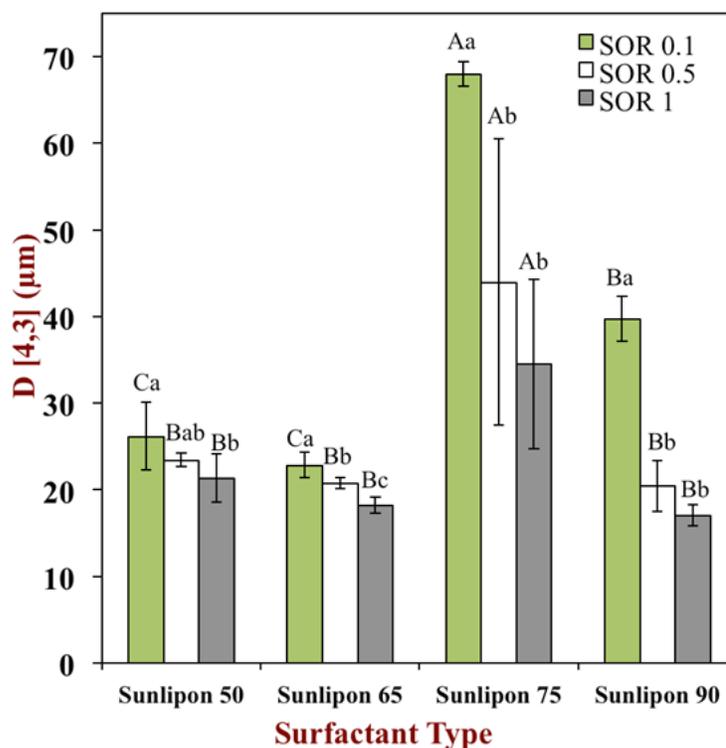


Figure 35. Effect of SOR and surfactant type on particle size using sunflower phospholipids. Different capital letters (A, B, C) mean statistical differences in the particle diameter of a given surfactant-to-oil ratio (i.e. the effect of phospholipid type). Different lower case letters (a, b, c) mean statistical differences in the particle diameter of a given phospholipid type (i.e., the effect of SOR).

Generally, the major phospholipids in sunflower oil include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA) [154]. The phospholipids tested in this study were mainly composed of PC, with the ratio increasing from SunliponTM 50 to 90 (**Table 13**). The phospholipid composition of the lecithin ingredients would be expected to influence their functional performance as emulsifiers. Previous studies have reported that PC can form well-ordered lamellar monolayers or bilayers around lipid droplets that can facilitate emulsion formation and increase emulsion stability. On the other hand, PE tends to assemble into reversed hexagonal structures, which are more difficult to form around lipid droplets and which

are more sensitive to pH because of their zwitterionic nature [153]. When used in combination, studies have shown that the highest emulsion stability occurs when there is a high ratio of PC to PE [158]. Lecithin ingredients enriched with PC have previously been shown to have the best emulsifying properties and to be most effective at stabilizing lipid droplets [152]. Our results are in agreement with these previous studies, since the smallest initial mean droplet size was observed for emulsions prepared with Sunlipon™ 90, which had the highest PC content. Having said this, the emulsions prepared with Sunlipon™ 75 had the largest droplet size, even though they had the second highest PC content. This result suggests that the PC content of the lecithin ingredients was not the only important factor influencing their ability to form emulsions. In general, the formation of small droplets using the spontaneous emulsification method depends on the ability of the surfactant-oil-water system to form a bicontinuous microemulsion at the oil-water interface, which can breakdown and form small droplets. In addition, the subsequent stability of the droplets to aggregation depends on the ability of the emulsifiers to generate strong repulsive interactions between the droplets, such as steric or electrostatic repulsion. It is likely that phospholipid type may influence both emulsion formation and stability in a complex manner.

Phospholipid composition also affected the electrical characteristics of the oil droplets produced by spontaneous emulsification, with the ζ -potential becoming less negative as the amount of PC in the phospholipid ingredients increased, *i.e.*, moving from Sunlipon™ 50 to 90 (**Table 14**). The electrical charge of the droplets will influence their aggregation stability by altering the strength of the electrostatic repulsion between them.

In addition, the electrical charge will influence the interaction of the droplets with other ionized species, such as mineral ions, proteins, or polysaccharides.

Table 14. Zeta Potential of emulsions made with Sunlipon™ 50, 65, 75, and 90 at a surfactant-to-oil ratio of 1. Different lower case letters (a, b, c, d) mean statistical differences in the zeta potential of different phospholipid compositions.

Sunlipon™ 50	Sunlipon™ 65	Sunlipon™ 75	Sunlipon™ 90
-45.3±4.04 ^a	-30.45±1.88 ^b	-26.18±1.56 ^c	-0.10±0.66 ^d

6.4.2. Influence of surfactant-to-oil ratio

In the remainder of the studies we only used Sunlipon™ 90 as an emulsifier since it produced the smallest droplets using the low-energy method. Initially, we examined the influence of SOR on emulsion formation and stability in more detail. Droplet shape analysis was used to provide some insight into the influence of surfactant concentration on droplet formation (**Figure 36**). When no surfactant was present a well-defined oil droplet formed when the organic phase was injected into the aqueous phase. When a relatively low level of surfactant was present (SOR = 0.1 and 0.5), the oil droplet formed became much smaller and gel-like. As expected, the presence of the surfactant greatly decreased the interfacial tension: from 29.7 mN m⁻¹ with no surfactant to 0.6 and 0.5 mN m⁻¹ for SOR of 0.1 and 0.5, respectively. At a higher surfactant concentration (SOR = 1), the oil droplet formed was highly irregular in shape and so the interfacial tension could not be measured. These results are in good agreement with those obtained when a synthetic small molecule surfactant (Tween® 80) was studied using the same methods [15].

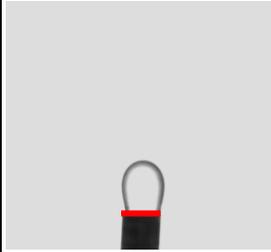
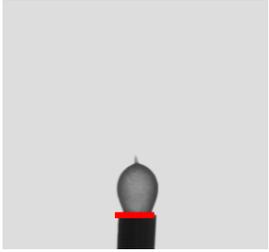
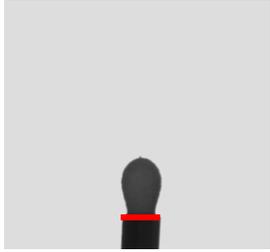
No Surfactant	SOR 0.1	
		
29.7 ± 0.4^a	0.60 ± 0.07^b	
SOR 0.5		SOR 1
		
0.49 ± 0.04^b		No interfacial tension measured

Figure 36. Droplet shape analysis of samples containing varying surfactant-to-oil ratios (SOR). The organic phase (MCT + Sunlipon™ 90) was slowly added into the aqueous phase (buffer solution) using an inverted hook (diameter 1.463 mm). The measured interfacial tension (mN m^{-1}) is displayed under the droplet shape picture (in mN m^{-1}). No interfacial tension could be measured for SOR 1 because of the irregular droplet shape formed. Two images are shown for the SOR 0.1 and 0.5 samples to highlight that the drops changed from light to dark over time, possibly due to liquid crystalline formation by the surfactant and oil phase. Different lower case letters (a, b) mean statistical differences in the interfacial tension.

The surfactant-oil mixtures injected into the aqueous solution initially appeared transparent (light) but became opaque (darker) with time (**Figure 36**). Interestingly, this change did not cause an appreciable alteration in the measured interfacial tension. The origin of this effect may be due to the formation of a gel-like material at the droplet surfaces at a particular surfactant-oil-water ratio, which would have occurred as hydrophilic surfactant molecules diffused into the aqueous phase and water molecules diffused into the organic phase. It has been hypothesized that the formation of liquid crystals or bicontinuous microemulsions at the boundary between an aqueous and organic phase may be responsible for the spontaneous formation of oil droplets by this method [30, 40]. However, when emulsions are formed by the spontaneous emulsification method the system is continuously stirred thereby removing any liquid crystals or bicontinuous microemulsions from the droplet surfaces, which would not occur during static droplet shape analysis.

The decrease in droplet size with increasing SOR may be related to the influence of phospholipids on the spontaneous formation of oil droplets at the organic-aqueous phase boundary. A certain surfactant-oil-water ratio probably leads to the formation of a relatively low viscosity liquid crystalline or bicontinuous microemulsion phase that promotes droplet formation through a budding off mechanism as the phospholipid molecules move from the organic phase to the aqueous phase.

We also measured the electrical characteristics (ζ -potential) of oil droplets prepared using different SORs (**Table 15**). All the droplets had a relatively low negative charge under the conditions studied. There was a significant difference in droplet charge for the emulsions made at an SOR of 0.1 and those made at an SOR of 0.5 and 1. The

magnitude of the negative charge on lipid droplets coated with Sunlipon™ 90 (about -0.1 to -5 mV) was much lower than that reported for lipid droplets coated with soybean lecithin (about -60 mV) [161], which can be attributed to the high level of PC in the lecithin used in this study. Interestingly, the ζ -potential results did not correlated to the physical stability of the emulsions, *i.e.*, an increase in droplet charge did not lead to greater stability to droplet aggregation. This suggests that electrostatic repulsion was not the major physicochemical mechanism promoting droplet stability in these systems. Other factors that may have also been important are differences in the sizes of the droplets produced by different phospholipids, as well as differences in their interfacial characteristics, such as thickness, flexibility, and free energy.

Table 15. Zeta Potential of emulsions made with Sunlipon™ 90 at various SORs initially after production and after 7 days of storage at room temperature. Different lower case letters (a, b) mean statistical differences in the zeta potential on a given day (*i.e.*, the effect of SOR). No significant difference was observed between day 0 and day 7.

	SOR 0.1	SOR 0.5	SOR 1
Day 0	-4.68±3.25 ^b	-1.37±0.83 ^a	-0.10±0.66 ^a
Day 7	-2.93±2.03 ^b	-1.69±0.77 ^{ab}	-0.64±0.89 ^a

6.4.3. Influence of surfactant location

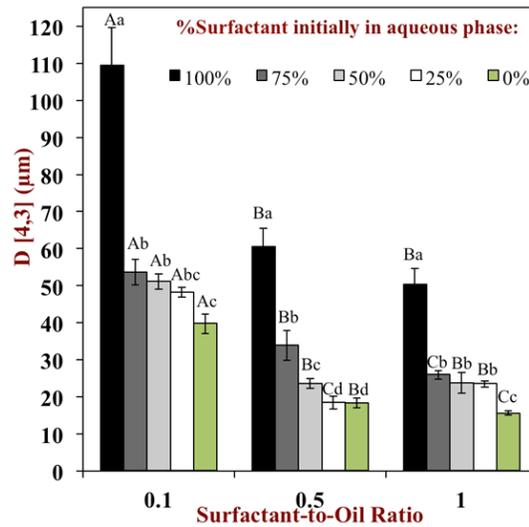
To understand the influence of surfactant location, we varied the initial surfactant location at three surfactant-to-oil ratios (**Figure 37a**). Similar trends were observed at all surfactant concentrations. When 100% of the surfactant was dissolved in the aqueous phase, the particle size was always significantly higher than that measured at other

surfactant distributions. This implies that the formation of relatively small droplets requires the movement of phospholipid molecules from the organic phase into the aqueous phase. Additionally, for SORs of 0.1 and 0.5 the particle size was significantly smaller when 25% or less of the surfactant was dissolved in the aqueous phase. At the highest SOR tested (1.0), the particle size was significantly smaller than all other preparation conditions when none of the surfactant was dissolved in the aqueous phase. Although the oil droplets formed are much larger than those formed by synthetic surfactants, the overall trends observed were similar [15]. Comparing the effects of surfactant level, we saw similar trends for all initial surfactant locations: the droplets formed in emulsions prepared at SOR 0.1 were significantly larger than those prepared at SORs of 0.5 and 1.

These differences could be observed visually as well (**Figure 37b**). When 100% of the surfactant was dissolved in the aqueous phase, a sediment layer formed in the bottom of the tubes and a cream layer formed at the top. However, when less surfactant was dissolved in the aqueous phase (75% and less) only a cream layer was observed. The volume of the cream layer increased with increasing phospholipid concentration.

These results suggest that it is important that the phospholipids are initially located within the organic phase of the system, presumably because a certain surfactant-oil-water composition is formed when they move from the organic to aqueous phases, which promotes the spontaneous formation of small lipid droplets.

a)



b)

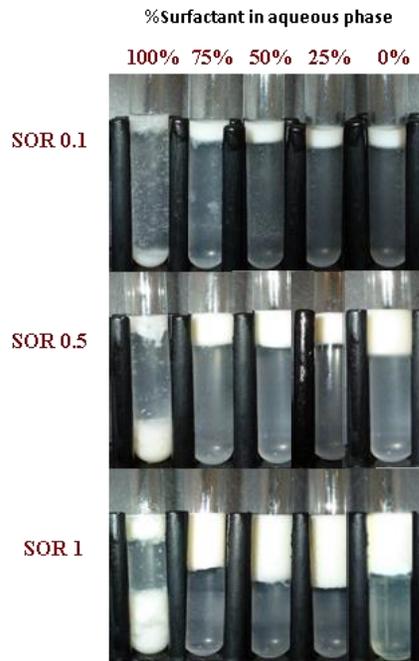


Figure 37. a) Effect of surfactant location on particle size when emulsions were made by spontaneous emulsification at SORs 0.1, 0.5, and 1 using Sunlipon™ 90 and b) visual appearance of emulsions produced by spontaneous emulsification using Sunlipon™ 90 at SORs 0.1, 0.5, and 1. Different capital letters (A, B, C) indicate statistical differences in the particle diameter of a given surfactant percentage (i.e. the effect of SOR). Different lower case letters (a, b, c) mean statistical differences in the particle diameter of a given SOR (i.e., the effect of phospholipid location). Pictures were taken 24 hours after production.

6.4.4. Influence of storage time

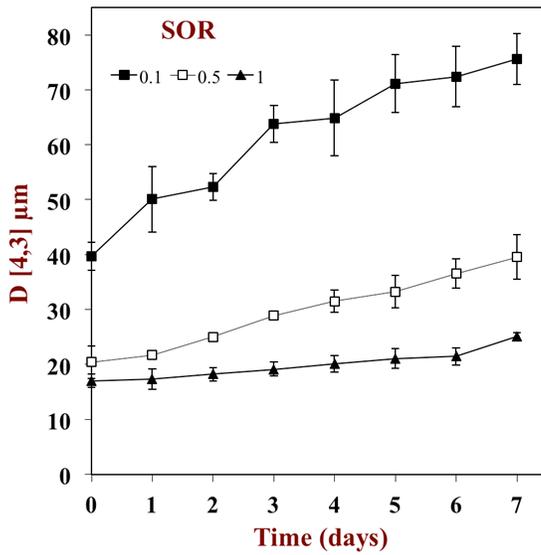
For practical applications, it is important to understand how emulsion stability changes during storage and so we investigated the influence of storage time. All samples were stored at room temperature and the particle size, visual appearance, creaming index, and microstructure were measured periodically (**Figure 38**). Throughout one-week storage, the oil droplets in all three emulsions increased in size, with the rate of increase decreasing with increasing SOR (**Figure 38a**). Visually creaming could be observed in all emulsions after 1-day storage, with the extent of creaming decreasing with increasing SOR (**Figure 38b and Figure 38c**). Rapid creaming is to be expected because of the relatively large particle diameters of the emulsions. Previously researchers have also observed that higher sunflower lecithin levels slowed the creaming process with coalescence being observed in the upper portion at all concentrations tested [154, 160]. For commercial applications, it would be possible to retard creaming and coalescence by increasing the viscosity of the continuous phase using starches, gums, or proteins [153, 154].

The optical microscopy images indicated that emulsion microstructure depended on storage time and phospholipid level (**Figure 38d**). On Day 0, the emulsion produced at an SOR of 0.1 had much larger droplets than those produced at an SOR of 0.5 and 1, which is in agreement with the light scattering results. After 7 days, there appeared to be less change in the microstructure of the emulsions with the highest surfactant level when compared to the initial emulsions, which suggests they were more stable to droplet growth. Interestingly, the larger droplets appeared to be surrounded by many smaller droplets and other small structures (possibly liquid crystals or vesicles containing phospholipids). When analyzing the cream layer, it was observed that the droplets were

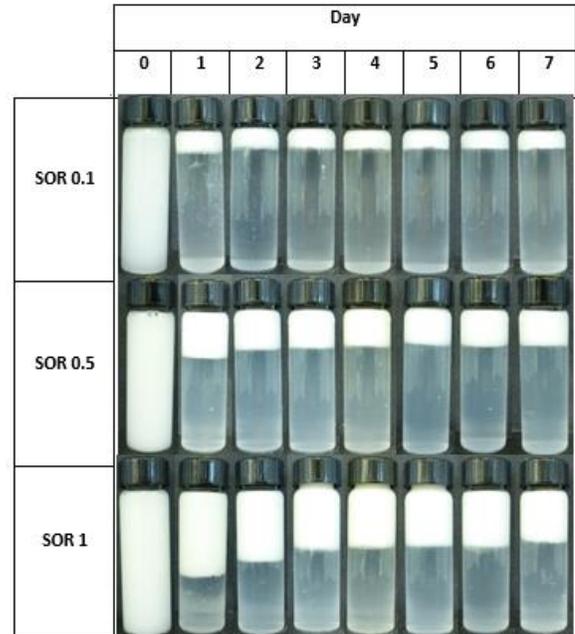
larger and more densely packed than in the overall emulsions (prepared by gently inverting them). This can be attributed to the fact that large droplets tend to cream faster, and that droplets in the cream layer tend to pack closely together, which may facilitate coalescence due to the extended period of contact [162].

The changes in ζ -potential between day 0 and 7 were not significant, but there were differences in the electrical characteristics depending on phospholipid level (**Table 15**). The emulsions with an SOR of 0.1 had the highest negative charge, which would be expected to generate a stronger electrostatic repulsion between the droplets. Nevertheless, these emulsions were the most unstable to phase separation, exhibiting the highest creaming index and percent droplet growth (**Table 16**). These results again suggest that electrostatic repulsion is not the major factor influencing droplet coalescence in the emulsions. It is well known that droplet coalescence increases with increasing droplet size due to the greater contact area between the droplets [162]. Consequently, the emulsions with the lowest phospholipid levels may be the most susceptible to coalescence because they contained the largest droplets. In addition, it is possible that high levels of phospholipids may provide protection against coalescence by forming multilayer structures around the droplets.

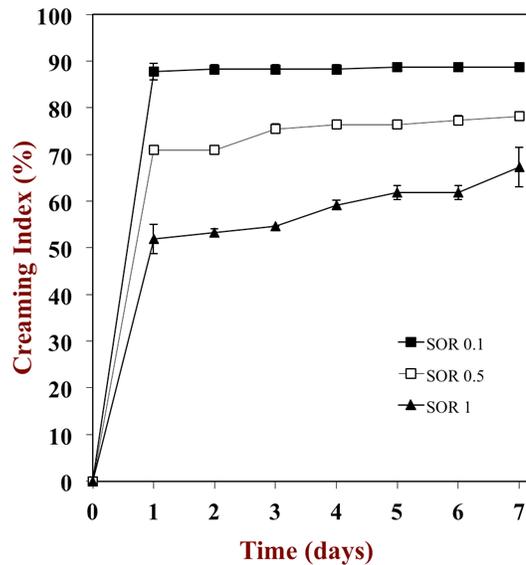
a) Particle size



b) Pictures of shelf-life



c) Creaming index



d) Initial and 1 week storage microscope images (20x) of emulsions.

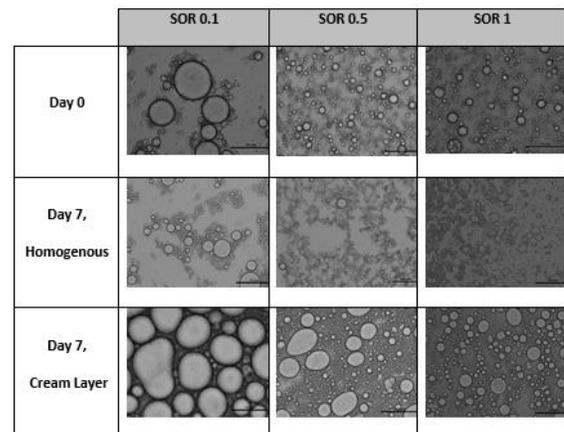


Figure 38. Shelf-life study of emulsions produced by spontaneous emulsification with Sunlipon™ 90 at various SORs.

Table 16. Growth (%) of emulsions made by spontaneous emulsification, homogenization, or sonication with Sunlipon™ 90 at various SORs after one week. Different capital letters (A, B, C) mean statistical differences in the particle diameter of a given surfactant-to-oil ratio (i.e. the effect of preparation method). Different lower case letters (a, b, c) mean statistical differences in the particle diameter of a given preparation method (i.e., the effect of SOR).

	SOR 0.1	SOR 0.5	SOR 1
Spontaneous Emulsification	90.6 ± 3.1 ^{Aa}	86.6 ± 0.9 ^{Aa}	42.1 ± 7.6 ^{Bb}
Homogenization	14.4 ± 0.5 ^{Bc}	76.9 ± 2.0 ^{Bb}	141.2 ± 3.3 ^{Aa}
Sonication	13.5 ± 1.6 ^{Ba}	3.9 ± 2.2 ^{Cb}	-1.6 ± 0.7 ^{Cc}

6.4.5. Influence of surfactant type

A major goal of this research was to determine whether a natural surfactant could be utilized to form emulsions using the low-energy spontaneous emulsification process. Up to this point, mostly synthetic surfactants have been utilized to form emulsions using this approach [15, 30, 33, 40, 46, 51]. Previously we had found that out of a group of synthetic surfactants, Tween® 80 could form small droplets when used at high surfactant-to-oil ratios (SOR) [15]. Therefore, we prepared emulsions using Tween® 80 using the same method as used for the phospholipids and measured the particle size of the emulsions formed (**Figure 39**). We found that at the lower SORs of 0.1 and 0.5, Sunlipon™ 90 produced significantly smaller droplets than emulsions prepared with Tween® 80. However, at the higher SOR of 1 Tween® 80 produced much smaller droplets ($d = 0.4 \mu\text{m}$) than Sunlipon™ 90 ($d = 17 \mu\text{m}$). Within each surfactant, we saw similar results. For Sunlipon™ 90 there was a significant difference between emulsions produced at an SOR of 0.1 and those produced at an SOR of 0.5 or 1. We saw no significant difference between emulsions produced at an SOR of 0.5 or 1 when using

Sunlipon™ 90. We have previously observed this trend of little added benefit with increasing SOR when using Tween® 80 [15]. In these experiments, however, we saw significant differences between all three SORs with an increasing Tween® 80 concentration causing a significant decrease in particle size at all levels tested. We did not reach the surfactant concentration of little added benefit with Tween® 80 like we did with Sunlipon™ 90. One of the major disadvantages with spontaneous emulsification is the need for synthetic surfactants at high levels. If the desire is to produce emulsion droplets with a particle size greater than 20 µm, natural surfactants such as Sunlipon™ 90 may be a better choice.

These results indicate that phospholipids alone are unable to form the very small droplets that can be formed by certain types of small molecule non-ionic surfactants. This may be due to differences in the optimum curvature or flexibility of the monolayers formed by the different surfactants. Small molecule surfactants tend to be better at reducing the interfacial tension and at forming more flexible interfaces than phospholipids, which may account for their ability to form small droplets. Additionally, the use of cosolvents (such as ethanol and glycerol) may be utilized to help form small droplets with phospholipids; however, these emulsions have also been reported to be physically unstable during storage [49]. Future work should focus on optimizing surfactant-oil-water systems with phospholipids to ensure fine droplets can initially be formed that remain stable during subsequent storage.

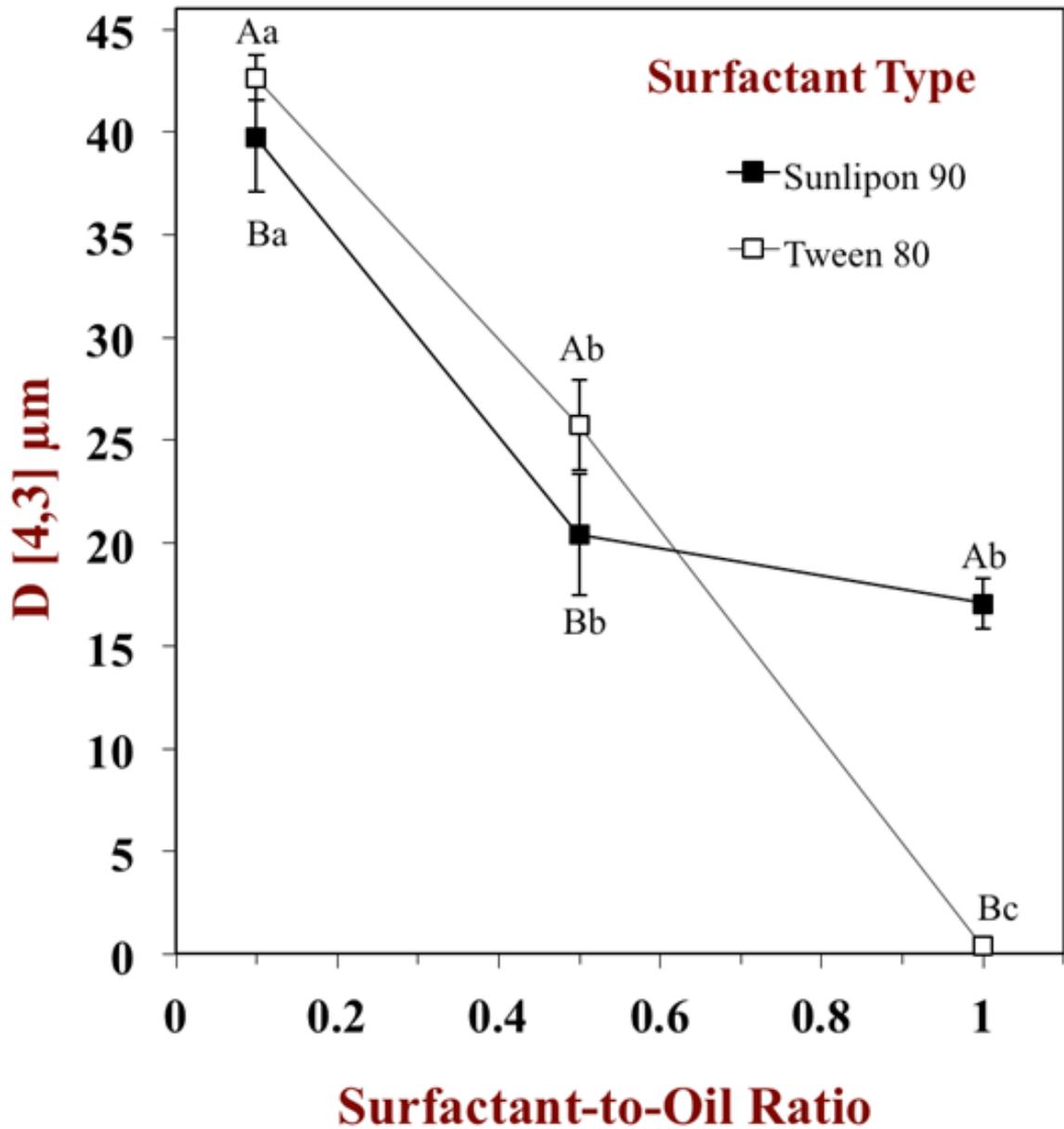


Figure 39. Comparison between emulsions prepared using spontaneous emulsification and a synthetic surfactant (Tween[®] 80) or natural surfactant (Sunlipon[™] 90) at various SORs. Different capital letters (A, B) mean statistical differences in the particle diameter of a given surfactant-to-oil ratio (i.e. the effect of surfactant type). Different lower case letters (a, b, c) mean statistical differences in the particle diameter of surfactant (i.e., the effect of SOR).

6.4.6. Influence of preparation method

While the main objective of this study was to test the use of sunflower phospholipids using the low-energy spontaneous emulsification method, we also compared the same surfactant utilizing high-energy methods. In this section we compared the particle size of emulsions produced using spontaneous emulsification and two commonly used high-energy methods: microfluidization and sonication [143]. In comparing the low-energy and high-energy methods we used preparation conditions commonly utilized for each method: the surfactant is typically dissolved in the oil phase for low-energy methods, but in the water phase for high-energy methods. It should be noted that initial surfactant location may be one factor contributing to the difference between low- and high-energy methods.

We found that the two high-energy methods produced significantly smaller droplets than the spontaneous emulsification method and there was no significant difference between homogenization and sonication at all SORs tested (**Figure 40**). Previous research using synthetic surfactants has also shown that the minimum droplet size formed was independent of preparation method for ultrasonic and microfluidization methods [143]. Our results are consistent with this when using medium chain triglycerides and a natural sunflower phospholipid. Additionally, with spontaneous emulsification we saw no significant difference between an SOR of 0.5 and of 1. However, with the two high-energy methods, a significantly smaller particle size was achieved as we increased the surfactant concentration. With the spontaneous emulsification we observed a level where the addition of surfactant did not further decrease particle size. This level was not reached with the high-energy methods. Lastly, the particle diameter achieved with the higher-energy methods ranged from

approximately 0.2-0.8 μm , which was much smaller than that which could be achieved by the low-energy method. Depending on the application, this particle size may be more appropriate than the emulsions containing relatively large droplets formed using spontaneous emulsification.

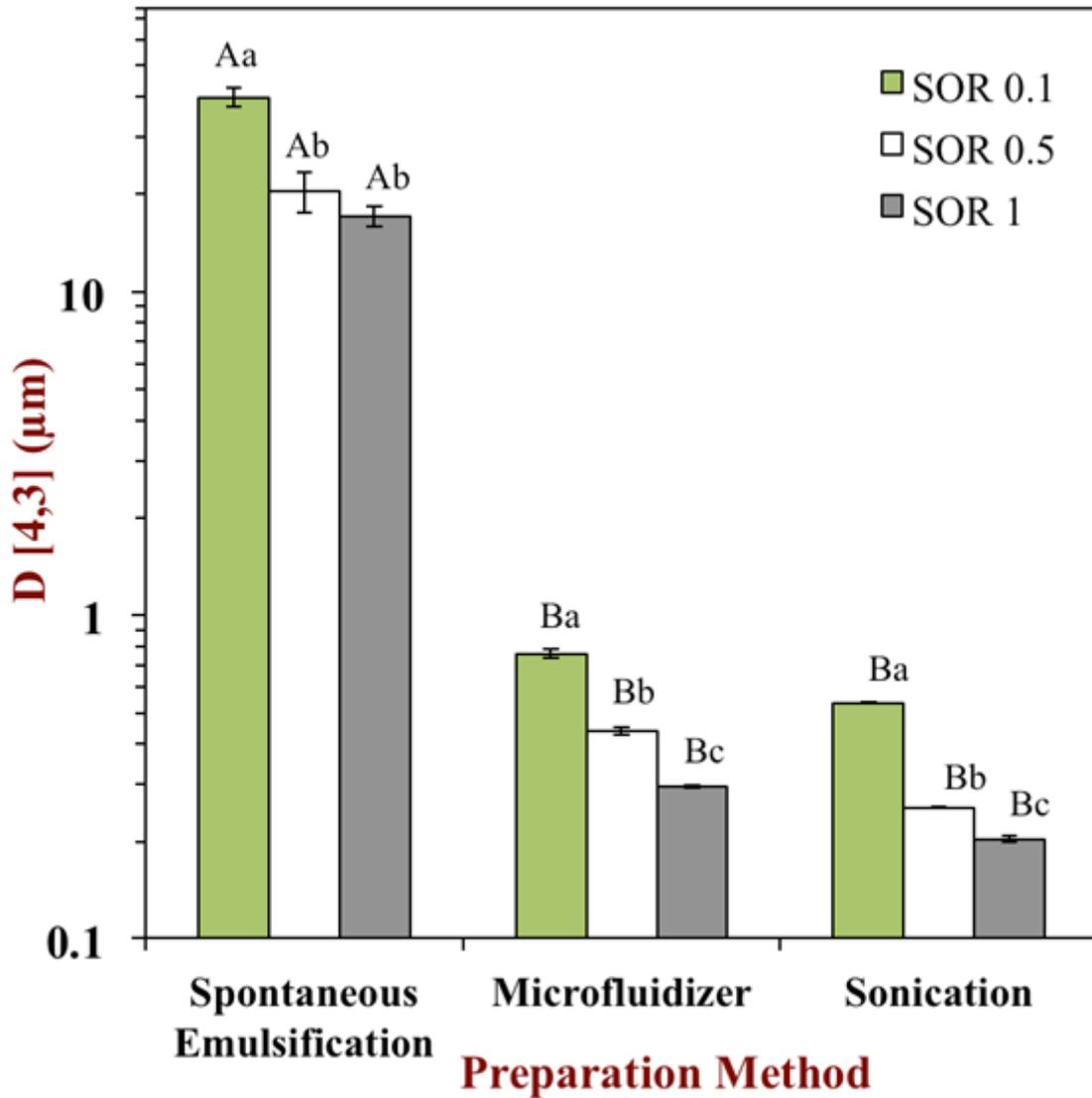


Figure 40. Effect of preparation method on particle size using sunflower phospholipids at various SORs. Different capital letters (A, B, C) mean statistical differences in the particle diameter of a given surfactant-to-oil ratio (i.e. the effect of preparation method). Different lower case letters (a, b, c) mean statistical differences in the particle diameter of a given preparation method (i.e., the effect of SOR).

While all the emulsions produced were milky white in color, the stability of the emulsions produced using the high-energy methods was in general superior (**Table 16**). After a week, the emulsions produced using sonication had grown the least at all SORs tested. At SORs of 0.1 and 0.5, the droplets fabricated using both high-energy methods exhibited significantly less growth than those produced using spontaneous emulsification. At the highest SOR tested, however, microfluidization showed the largest droplet growth while sonication actually saw a slight decrease in droplet size. Spontaneous emulsification and sonication both saw decreases in droplet growth with an increase in surfactant concentration, but the opposite was true for microfluidization. These results indicate that both preparation method and surfactant concentration affect particle formation and growth. Interestingly, the nature of the particles formed within the emulsions prepared using the two different high-energy homogenization methods must have been different. It is likely that not all of the phospholipids were adsorbed to the droplet surfaces at the highest surfactant levels, which may have meant that some phospholipids or other structures were also present. In the microfluidized samples this excess surfactant may have help transferred oil between droplets thus leading to droplet growth through Ostwald ripening.

6.5. Conclusion

In summary, we have shown that oil-in-water emulsions can be produced by spontaneous emulsification utilizing natural sunflower phospholipids, however, the size of the droplets formed was relatively large ($d > 10 \mu\text{m}$). The droplet size produced depended on phospholipid type, surfactant-to-oil ratio, surfactant location, storage time,

surfactant type, and preparation method. Emulsions made with the highest ratio of phosphatidylcholine produced the smallest particle size. The droplet size decreased with increasing surfactant-to-oil ratio, and was smaller when a greater fraction of the phospholipids was initially dispersed within the organic phase rather than the aqueous phase. At SORs of 0.1 and 0.5, the sunflower phospholipid produced smaller droplets than the synthetic surfactant. Conversely, at a SOR of 1, the synthetic surfactant produced much smaller droplets than the phospholipids. Additionally, significantly smaller emulsions could be produced using high-energy methods compared to spontaneous emulsification.

These results suggest that the spontaneous emulsification method may be useful for producing conventional emulsions from natural sunflower phospholipids. Conventional oil-in-water emulsions containing relatively large droplets are widely used in the food industry, *e.g.*, in dressings and sauces [142] and thus their production with natural surfactants and low-energy methods is relevant. However, their relatively large particle dimensions means they are susceptible to breakdown due to creaming or coalescence, which may be inhibited by adding thickening or gelling agents [154]. In addition, the relatively high phospholipid-to-oil levels required to produce emulsions using the spontaneous emulsification method may be unsuitable for commercial applications where high fat contents are required. Nevertheless, this method may be useful in products that only require relatively low lipid droplet concentrations, such as some low-fat beverages, dressings, dips, or sauces.

6.6. Acknowledgements

This material was partly based upon work supported by the Cooperative State Research, Extension, Education Service, USDA, Massachusetts Agricultural Experiment Station (Project No. 831) and USDA, NRI Grants (2011-03539, 2013-03795, 2011-67021, and 2014-67021). This project was also partly funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant numbers 330-130-1435-DSR, 299-130-1435-DSR, 87-130-35-HiCi. Ashtri Sastrosubroto was also incredibly helpful in the completion of the experiments.

CHAPTER 7

ENCAPSULATION OF ω -3 FATTY ACIDS IN NANOEMULSION-BASED DELIVERY SYSTEMS FABRICATED FROM NATURAL EMULSIFIERS: SUNFLOWER PHOSPHOLIPIDS

7.1. Abstract

Nanoemulsions have considerable potential for encapsulating and delivering ω -3 fatty acids, but they are typically fabricated from synthetic surfactants. This study shows that fish oil-in-water nanoemulsions can be formed from sunflower phospholipids, which have advantages for food applications because they have low allergenicity and do not come from genetically modified organisms. Nanoemulsions containing small droplets ($d < 150$ nm) could be produced using microfluidization by optimizing phospholipid type and concentration, with the smallest droplets being formed at high phosphatidylcholine levels and at surfactant-to-oil ratios exceeding unity. The stability of the emulsions was mainly attributed to electrostatic repulsion, with droplet aggregation occurring at low pH values (low charge magnitude) and at high ionic strengths (electrostatic screening). These results suggest that sunflower phospholipids may be a viable natural emulsifier to deliver ω -3 fatty acids into food and beverage products.

7.2. Introduction

Omega-3 fatty acids (ω -3s) have a number of potential health benefits when consumed at sufficient levels including those related to neurodevelopment and vascular health [163-165]. However, many American adults are not currently consuming

sufficiently high levels of ω -3s to gain these beneficial effects [166]. Therefore, there is interest in developing functional foods and beverages enriched with ω -3s [64, 167, 168]. Fish oil is of particular interest as an ω -3 source because it contains relatively high levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are the most important ω -3 fatty acids for human health [169].

However, ω -3s cannot simply be added into many food products due to issues such as low water-solubility, chemical instability, and low bioavailability [170-172]. Therefore, there is a pressing need for effective delivery systems to overcome these challenges so that these bioactive compounds can be incorporated into a wider range of functional food products [142, 173]. Oil-in-water nanoemulsions, which consist of small ($d < 200$ nm) oil droplets dispersed in an aqueous medium, are particularly suitable templates for the development of delivery systems because of their good water-dispersibility, high optical clarity, enhanced physical stability, and improved bioavailability [6, 10, 76]. Nanoemulsions can be prepared using either low-energy or high-energy methods, which each have their own advantages and disadvantages. Low-energy methods rely on the spontaneous formation of small oil droplets in certain surfactant-oil-water systems when their composition or environmental conditions are altered in a specific manner [3]. The main advantages of these methods are that they are simple to implement, and that they do not require any expensive specialized equipment [15, 76]. However, they typically require relatively high surfactant-to-oil ratios, and currently the only surfactants that have proved effective at producing small droplets are synthetic ones, such as Tweens and Spans [39, 174]. This approach is therefore

unsuitable for many types of food and beverage products due to the desire to have “clean labels” that are free of synthetic ingredients [149, 150].

High-energy methods of producing nanoemulsions, such as high-pressure valve homogenizers (HPVH), microfluidizers, and sonicators, rely on specialized equipment to disrupt and intermingle the oil and water phases thereby forming small oil droplets [6, 98]. Within the food industry, high-energy methods are currently the most commonly used because they are already well-established, capable of large-scale production, and can produce emulsions and nanoemulsions from a range of different components [76]. In particular, they can form nanoemulsions from both synthetic and natural emulsifiers, such as surfactants, phospholipids, proteins, and polysaccharides. HPVHs are presently the most widely utilized homogenization technique for producing emulsions in the food industry. However, microfluidizers have been shown to be more efficient at producing nanoemulsions containing very fine droplets than HPVHs [148, 175], and are therefore gaining increasing application within the food industry. Microfluidizers are able to produce small droplets by using specially designed channels to split an inputted coarse emulsion into two streams that are then directed towards each other at high velocity [176]. The intense disruptive forces generated within the interaction chamber are highly efficient at promoting droplet fragmentation and nanoemulsion formation [76]. Due to its advantages over other high-energy approaches, we have utilized a microfluidizer to form the ω -3-enriched nanoemulsions prepared in this study.

There are a variety of natural emulsifiers that can be utilized to form emulsions and nanoemulsions including phospholipids, proteins and polysaccharides [13, 153, 177]. In the current study, we focus on the utilization of phospholipids to form ω -3-enriched

nanoemulsions. Phospholipids are amphiphilic molecules because they have a hydrophilic head group and two hydrophobic tails, and so they can adsorb to oil-water interfaces and stabilize emulsions and nanoemulsions [14, 154]. However, there are a number of concerns associated with the application of phospholipids as food ingredients, such as the fact that they may come from sources that contain allergens, *e.g.*, eggs [156], or from sources that are genetically modified, *e.g.*, soybeans [154]. For these reasons there has been considerable interest in investigating the utilization of sunflower phospholipids, which are not a common source of allergens [155, 156] and which are not sourced from a genetically modified organism (GMO) [152, 158].

The main objective of this research was therefore to determine whether stable ω -3 nanoemulsions containing small droplets could be fabricated using microfluidization and sunflower phospholipids. In addition, the role of electrostatic interactions on the stability of the phospholipid-coated oil droplets was established by measuring the influence of pH and ionic strength on their electrical charge and aggregation. The knowledge gained from this study could help identify functional food products where ω -3-enriched nanoemulsions made with sunflower phospholipids could be successfully applied.

7.3. Materials and methods

7.3.1. Materials

Four different natural phospholipid surfactants derived from sunflower oil were kindly donated by Perimondo (New York, NY) (**Table 13** and **Table 17**). Fish oil was kindly donated by DSM Nutritional Products Ltd (Ropufa 30 n-3 food oil, Basel, Switzerland). The aqueous phase for all emulsions was prepared using a sodium

phosphate buffer solution (5 mM; pH 2-7). Distilled and deionized water (Milli-Q®) was used to prepare all solutions and emulsions.

Table 17. Additional properties of the sunflower phospholipids used in this study.

	Sunlipon™ 50	Sunlipon™ 65	Sunlipon™ 75	Sunlipon™ 90
Physical Appearance	Brown Paste	Brown Paste	White Powder	Amber Paste
Γ_{sat} (mg/m ²)	2.04	2.27	3.67	10.0
D _{min} (µm)	0.124	0.138	0.356	0.137

7.3.2. Methods

7.3.2.1. Emulsion preparation

Initially, an aqueous emulsifier solution was prepared by dispersing the phospholipids in the aqueous phase and stirring overnight at room temperature to ensure complete dissolution. Coarse emulsions were then prepared by blending organic (fish oil) and aqueous phases (buffer and phospholipids) together using a high-speed blender (Bamix, Switzerland) for 2 minutes at room temperature. The coarse emulsions were then passed through a microfluidizer (Microfluidics M110L, Newton, MA, USA) for 3 cycles at 12,000 psi to produce fine emulsions or nanoemulsions.

7.3.2.2. Variables tested

Five main variables were tested: phospholipid composition, surfactant-to-oil ratio, oil presence, pH stability, and salt stability.

7.3.2.2.1. Phospholipid composition

Four different sunflower phospholipids were tested (Sunlipon™ 50, 65, 75, and 90), which mainly differ in percentage of phosphatidylcholine (PC) they contained (**Table 13**).

7.3.2.2.2. Surfactant-to-oil ratio

The influence of surfactant concentration was investigated by varying the surfactant-to-oil ratio (SOR). The total oil content in the final systems was held constant at 5%, while the SOR was varied by altering the amounts of surfactant and buffer in the final system:

$$\text{SOR} = \frac{m_s}{m_o} \quad (7.1)$$

$$m_b = 100 - m_o - m_s \quad (7.2)$$

Here, m_s , m_o and m_b are the mass percentages of surfactant, oil and buffer in the overall system, respectively. SORs tested included 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2.

7.3.2.2.3. Oil presence

To better understand the difference between liposome and emulsion formation, systems were made up with and without fish oil at a phospholipid concentration of 1.25%. In systems without oil, the aqueous phases (buffer and Sunlipon™) were still stirred overnight and then mixed using a high-speed blender (Bamix, Switzerland) for 2 minutes at room temperature prior to microfluidization. Emulsions containing 5% fish oil were prepared at a similar phospholipid level as described in Section 7.3.2.1.

7.3.2.2.4. Effect of pH on emulsion stability

Emulsions were prepared at pH 7 and then diluted 10-fold using buffer solutions with different pH values (pH 2 to 7). Samples were adjusted to ensure that they were at the specified pH values and stored for 24 hours prior to measurements.

7.3.2.2.5. Effect of salt on emulsion stability

Emulsions were prepared at pH 7 and then diluted 10-fold using solutions containing a range of salt levels to achieve final values of 0 to 500 mM NaCl. Samples were stored for 24 hours prior to measurements.

7.3.2.3. Emulsion characterization

Samples were analyzed using a variety of techniques including particle size analysis, zeta potential analysis, visual observation, and optical microscopy

7.3.2.3.1. Particle size analysis

The particle size distribution and mean particle diameter ($D[3,2]$ or $D[4,3]$) were measured using a commercial static light scattering (SLS) instrument (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in double distilled water prior to analysis to avoid multiple scattering effects. The diameter is displayed primarily as the surface-weight diameter ($D[3,2]$) except for the effect of salt on physiochemical stability where the mean volume-weighted diameter ($D[4,3]$) is also shown because the mean-volume weighted diameter is more sensitive to particle aggregation [158, 159].

7.3.2.3.2. ζ -potential analysis

The electrical charge (ζ -potential) on the droplets was determined using particle electrophoresis (Zetasizer Nano ZS-90, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were diluted in buffer solutions (same pH as sample) prior to measurement to avoid multiple scattering effects.

7.3.2.3.3. Visual observation

Samples were observed visually and recorded using a digital camera (Lumix ZS8, Panasonic, Osaka, Japan). All pictures were taken against a black background in a photo box using ambient light.

7.3.2.3.4. *Microscopy*

An optical microscope (C1 80i Digital Eclipse, Nikon, Tokyo, Japan) with a 20× objective lens and 10× eyepiece was used to investigate the microstructure of the samples.

7.3.2.4. Experimental design

All measurements were performed on two freshly prepared samples in triplicate. The mean and standard deviations were calculated from this data. Statistical analysis was performed through subsection of the data to analysis of variance (ANOVA) using commercial statistics software (Minitab 16.2.4, Minitab Inc., State College, PA). Means were subject to Tukey's test and a *P*-value of <0.05 was considered statistically significant.

7.4. Results and discussion

7.4.1. Influence of phospholipid composition and surfactant-to-oil ratio

The influence of phospholipid type and concentration on the properties of emulsions formed by microfluidization was investigated (**Figure 41**). For Sunlipon 50, 65 and 90, a similar trend of decreasing mean particle size with increasing surfactant concentration was observed (**Figure 41a**). This trend can mainly be attributed to the fact that there were more surfactant molecules available to cover the oil-water interfaces created during homogenization, which enabled smaller droplets (larger surface area) to be formed [178]. In addition, a higher surfactant concentration typically leads to faster coverage of the oil droplet surfaces within a homogenizer, thereby suppressing the tendency for coalescence to occur [179]. For Sunlipon 75, we found that the mean particle diameter initially decreased with increasing surfactant concentration (up to SOR

0.1), but then it increased steeply and the droplets formed were always large at high surfactant levels (**Figure 41a**). Visual observation of these samples indicated that they were highly viscous at high SOR values, *i.e.*, the Sunlipon 75 emulsions would not flow to the bottom of the test tubes used to contain them (**Figure 41c**). It is therefore possible that the high viscosity of these samples prevented effective droplet disruption within the microfluidizer leading to the generation of large droplets. It is not clear why this system had such a higher viscosity than the other types of phospholipids studied, but it may be due to differences in its phase behavior. Previous studies using sunflower oil (30 wt%) and sunflower lecithin (0.1-2%) also reported a decrease in particle diameter with an increase in lecithin concentration using homogenization methods [152]. However, the surfactant-to-oil ratio used in that study was much lower ($SOR \approx 0.07$) and so the minimum droplet diameter formed was much larger ($d[3,2] \approx 20 \mu\text{m}$). For many applications of ω -3 fatty acids in functional foods and beverages it is important that the particle size is relatively low to protect against gravitational separation and ensure a high bioavailability [64]. Thus, it may be necessary to use relatively high levels of phospholipids to achieve these small particle sizes.

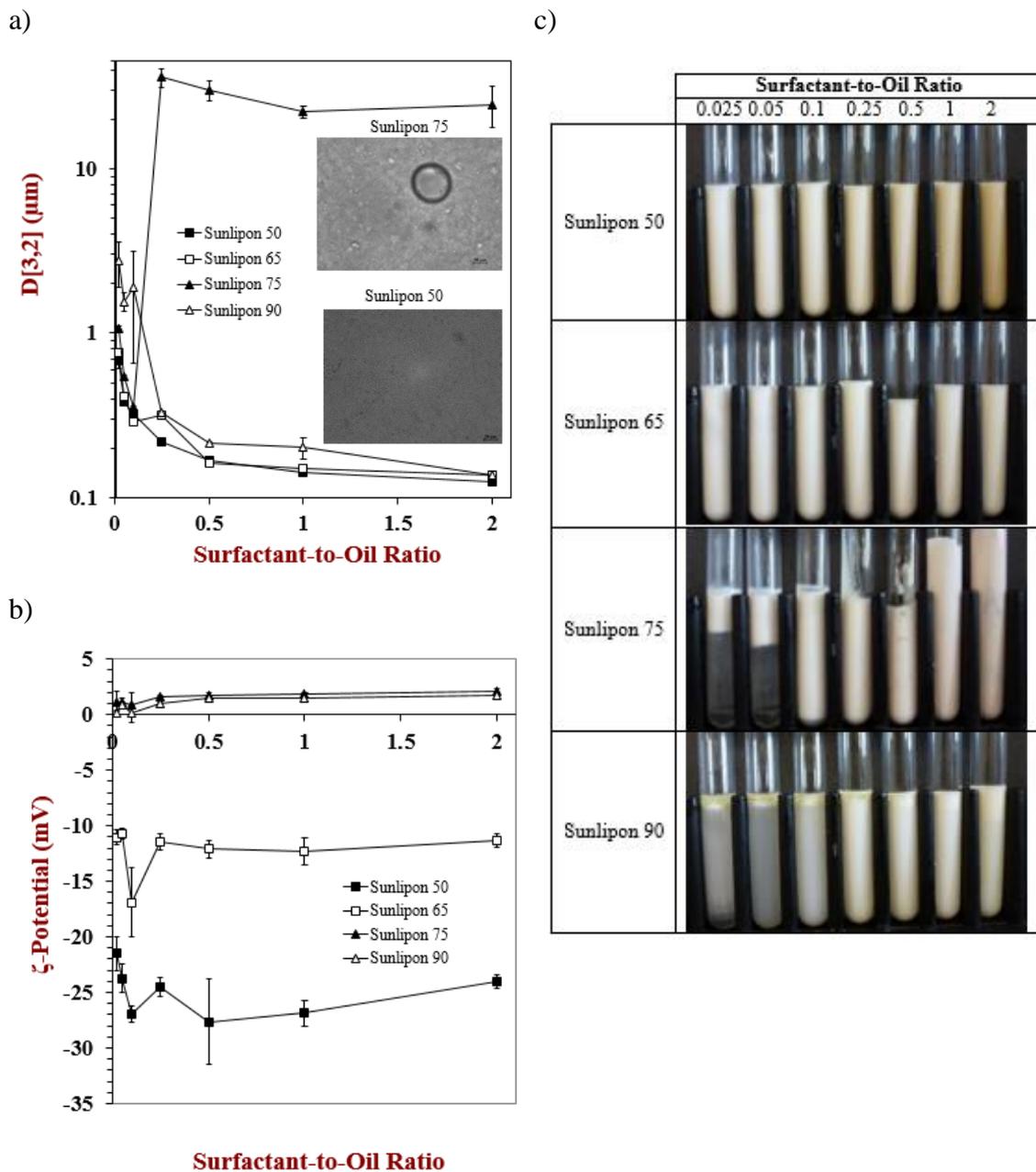


Figure 41. Effect of SOR and phospholipid composition on a) particle size, b) zeta potential, and c) physical appearance of emulsions. The microstructures of emulsions formed with Sunlipon 50 and 75 are shown as insets in figure 1a (SOR = 2).

The major components in sunflower phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA) [154]. Sunlipon 50, 65, 75 and 90 mainly differed in the amount of PC they contained, with the manufacturers designation number roughly corresponding to the weight percentage of PC present (**Table 13**). Emulsifier performance has previously been linked to phospholipid composition, with studies showing that PC is capable of forming well-ordered mono-layers or bi-layers in a lamellar structure around fat droplets, which is proposed to facilitate emulsion formation and enhance emulsion stability [153]. For sunflower oil-in-water emulsions, it was reported that the highest emulsion stability occurred when there was a high ratio of PC to PE [158]. Other studies have also shown that lecithin ingredients enriched with PC have the best emulsifying properties and are the most effective at stabilizing lipid droplets [152]. Therefore, based on our starting materials, we would have predicted that Sunlipon 90 would have formed the smallest droplets, followed by Sunlipon 75, 65 and 50. However, we found no significant difference in the minimum droplet diameter obtained using Sunlipon 50, 65, and 90, and a significantly larger droplet size with Sunlipon 75 (at SOR = 2). Thus, our results do not support the hypothesis that higher PC levels lead to better emulsion formation or stability. It is therefore clear that other factors are also important in determining the performance of the different sunflower phospholipid emulsifiers. For example, the electrical characteristics of the phospholipid head groups will impact the formation of the interfacial layers around the fat droplets, as well as their aggregation stability. In addition, the rheology of the emulsions formed by the phospholipids will influence the efficiency of droplet disruption within a microfluidizer. Finally, phospholipid ingredients

may contain different minor components or impurities that impact their performance. The darker color of Sunlipon 50, 65, and 90 suggests that they may contain Maillard reaction products generated during their manufacture, or colored impurities such as carotenoids, melanoids and porphyrins [151], which could influence their emulsifying ability. It should also be noted that the Sunlipon 50, 65, and 90 ingredients were pastes, whereas the Sunlipon 75 ingredient was a white powder (**Table 17**), which suggests that they have a tendency to organize into different kinds of structures.

When comparing the ability of emulsifiers to form emulsions it is useful to calculate their surface loads, *i.e.*, the amount of emulsifier required to cover a given surface area [180]. The surface load (Γ) of an emulsifier is related to the mean droplet diameter (d_{32}), disperse phase volume fraction (ϕ), and concentration of adsorbed emulsifier (C_s): $\Gamma = d_{32}C_s/6\phi$ [178]. Consequently, the surface load can be calculated from the slope of a plot of d_{32} versus $1/C_s$: $\Gamma = \text{slope}/6\phi$. This approach assumes that the droplet size is limited by the amount of emulsifier present, rather than by the disruptive forces generated by the homogenizer, and therefore only the data at relatively low emulsifier concentrations is used in the calculations. The surface loads of the Sunlipon 50, 65, 75, and 90 calculated using this approach were 2.04, 2.27, 3.67 and 10.0 mg m⁻² (**Table 17**). These calculations indicate that smaller amounts of Sunlipon 50 and 65 are required to cover a given droplet surface area than for Sunlipon 75 or 90. A possible explanation for this effect is that Sunlipon 75 and 90 formed multiple layers of phospholipids at the droplet surfaces, whereas the other two phospholipids only formed monolayers. Indeed, the calculated surface loads for Sunlipon 50 and 65 are close to the values reported for small molecule surfactants that typically form monolayers [180]. An

alternative explanation is that there an appreciable fraction of the phospholipids were not adsorbed to the droplet surfaces for Sunlipon 75 and 90, and instead formed liposomes or other structures in the aqueous phase surrounding the fat droplets. In future studies it would be useful to examine the microstructure of the emulsions using electron microscopy or other high-resolution methods to establish the precise nature of the structures formed.

The ζ -potential measurements indicated that phospholipid type had a pronounced influence on the electrical characteristics of the droplets (**Figure 41b**). For example, the droplets in emulsions made with Sunlipon 50 had a high negative charge, those made with Sunlipon 65 had a modest negative charge, and those made with Sunlipon 75 and 90 were slightly positive. For each phospholipid type, the ζ -potential remained relatively unchanged for all SOR levels tested. The relatively low cationic charge on the droplets coated by Sunlipon 75 and 90 can be attributed to the fact that they contained relatively high levels of PC, which has a zwitterionic head group that does not carry much net charge at neutral pH. Conversely, the anionic nature of the droplets coated by Sunlipon 50 and 90 may be due to the presence of other phospholipids (**Table 13**) that do have a net negative charge at this pH [181].

Overall, our results indicate that there was not a direct relationship between the size of the droplets formed by homogenization and the electrical characteristics of the phospholipids. This conclusion is based on the fact that droplets formed by Sunlipon 50, 65, and 90 had similar minimum droplet diameters but different particle charges, and that Sunlipon 75 and 90 had similar electrical charges but very different mean particle diameters (**Table 17**).

Visually, the emulsions containing droplets coated by Sunlipon 50 and 65 appeared stable to phase separation across the entire surfactant range studied (**Figure 41c**). For Sunlipon 50 a slight brown color could be observed at the highest surfactant concentrations, while Sunlipon 65 always appeared milky white. Sunlipon 75 was unstable to phase separation at the lowest SORs and was highly viscous at the highest SORs studied. For Sunlipon 90 a layer of free oil (“oiling off”) was observed at the top of the emulsions after storage. This suggests that some droplet coalescence may have occurred due to the relatively low charge on the droplets in this system allowing them to come into close proximity.

7.4.2. Influence of oil presence

In the absence of oils, phospholipids can form colloidal structures when they are dispersed in aqueous solutions, such as vesicles and liquid crystals [182, 183]. These colloidal structures may influence the formation, stability, and properties of the emulsions prepared from phospholipids in the presence of oils. We therefore examined the properties of phospholipid dispersions in the absence of oils (presumably liposomes), and compared them to the properties measured in the presence of oils (presumably emulsions). Interestingly, the general trends in particle size and charge were fairly similar for the dispersions prepared in the absence and presence of oil Sunlipon 50, 65 and 90 formed relatively small particles, whereas Sunlipon 75 formed relatively large ones (**Figure 42a**). Sunlipon 50 and 65 formed negatively charged particles, whereas Sunlipon 75 and 90 formed positively charged ones (**Figure 42b**). The similar electrical characteristics of the two systems can be attributed to the fact that both liposomes and fat droplets are coated by a layer of phospholipids. Visually, there were distinct differences between the overall appearance of the dispersions with and without oil, which depended

on phospholipid type (**Figure 42c**). The emulsions (with oil) were all optically opaque and milky white in appearance, whereas the liposomes (without oil) varied from transparent (Sunlipon 50 and 65) to cloudy (Sunlipon 75). These differences in optical properties can be attributed to differences in the light scattering characteristics of the colloidal dispersions. In general, the light scattering behavior of a colloidal dispersion depends on particle size, concentration, refractive index, and spatial distribution [2]. The milky white appearance of the emulsions can therefore be attributed to the fact that the fat droplets had an appreciably different refractive index than the surrounding water phase, and that they contained particles with dimensions similar to the wavelength of light. In contrast, the smaller degree of light scattering by the liposomes can be attributed to the fact that the phospholipid bilayers had dimensions that were much smaller than the wavelength of light. In this case, it is thickness of the bilayers, rather than the overall particle size, that mainly governs their light scattering behavior. The fact that the liposome suspensions formed using Sunlipon 75 had a higher turbidity than for the other phospholipids may be because the phospholipid layers were packed closer together, either due to aggregation or multilayer formation. The ζ -potential for all systems without oil was significantly less negative (Sunlipon 50 and 65) or more positive (Sunlipon 75 and 90) than those with oil (**Figure 42b**), which implies that the fish oil contributed some negative charge to the droplets. This could have been due to hydrolysis of triacylglycerols into free fatty acids with anionic carboxylic acid head groups that accumulated at the fat droplet surfaces.

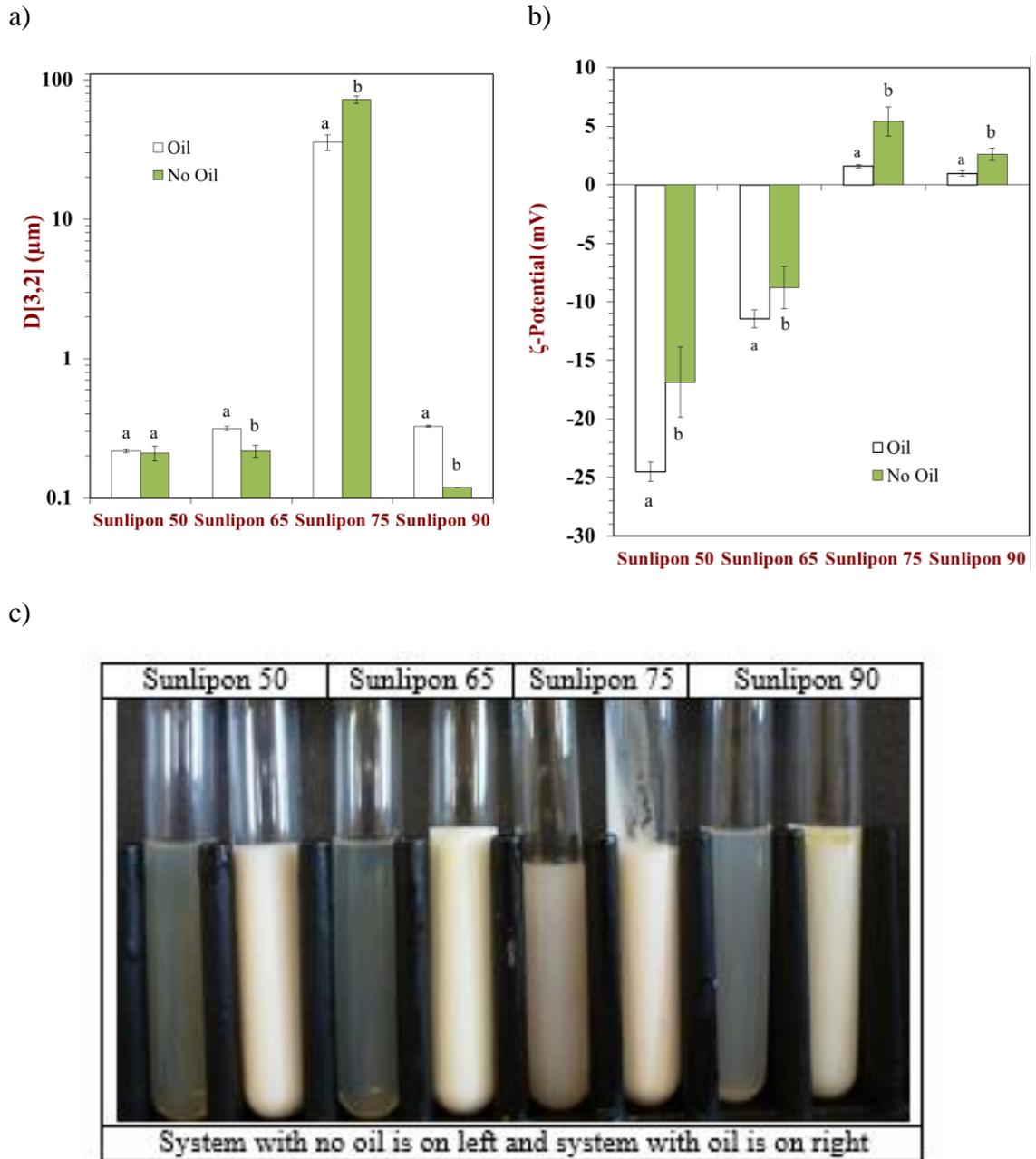
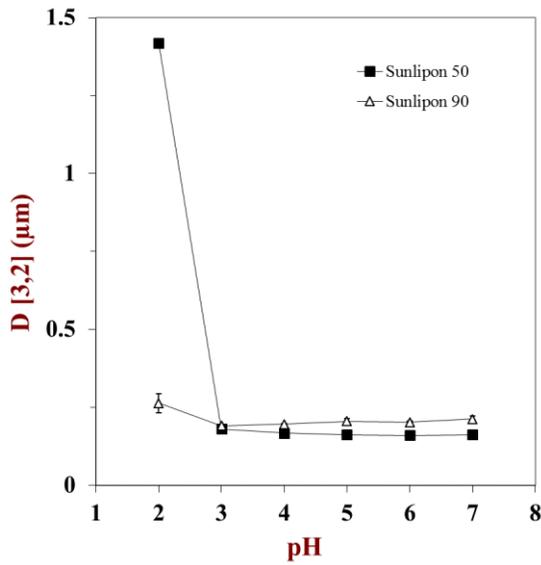


Figure 42. Exploration of liposome versus emulsion formation by looking at the effect of the presence of oil on the a) particle size, b) zeta potential, and c) physical appearance of the systems. Different lower case letters (a, b) mean statistical differences in the particle diameter or zeta potential of a given surfactant type (i.e., the effect of oil versus no oil).

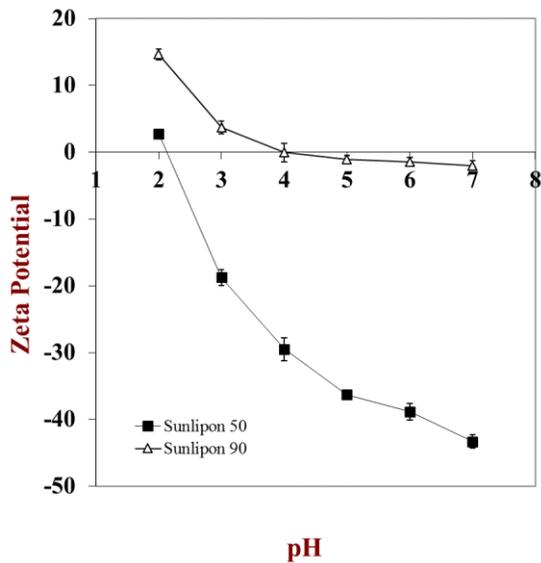
7.4.3. Effect of pH on physicochemical stability

The goal of this research was to create nanoemulsion-based delivery systems for ω -3 fatty acids that could be incorporated into a variety of different functional food products ranging from acidic soft drinks (\approx pH 2) to neutral nutritional beverages (\approx pH 7). Therefore, the influence of pH on the stability of nanoemulsions made using sunflower phospholipids was investigated. In this series of experiments, we compared the performance of phospholipids containing around 50% PC (Sunlipon 50) and 90% PC (Sunlipon 90) to provide some insight into the role of phospholipid composition. For Sunlipon 50, the mean particle diameter remained relatively low from pH 7 to 3, but increased at pH 2 (**Figure 43a**). Correspondingly, these emulsions remained visibly stable to gravitational separation from pH 7 to 3, but creamed at pH 2 as demonstrated by some clearing in the bottom of the test tubes (**Figure 43b**). The creaming observed at pH 2 can be attributed to the increase in particle size, since the creaming velocity is known to increase with particle size [6]. The physicochemical origin of pH-stability of this system can be attributed to changes in the electrical characteristics of the droplets with pH (**Figure 43b**). The droplets coated by Sunlipon 50 had a relatively high negative charge from pH 7 to 3 (\approx -43 to -19 mV), which would generate a strong electrostatic repulsion between them, thereby inhibiting aggregation. Conversely, the droplets had a relatively low positive charge at pH 3 (\approx +3 mV), which would not be sufficient to inhibit droplet aggregation.

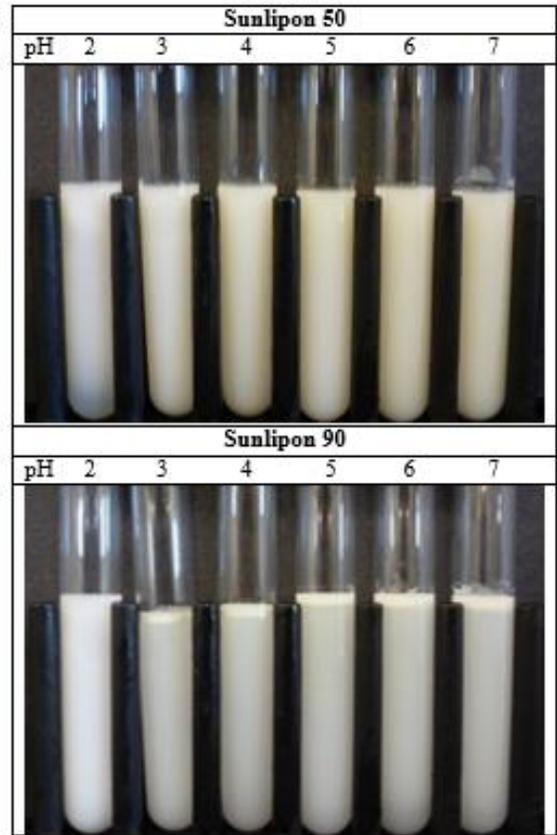
a) particle size



b) zeta potential



c) physical appearance



d) microstructure at pH 7

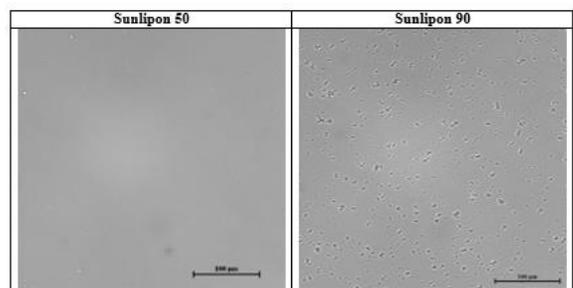


Figure 43. Effect of pH on the stability of emulsion systems formed using Sunlipon 50 and Sunlipon 90 at an SOR of 1 after 24 hours. The microstructure was investigated at pH 7.

For Sunlipon 90, the mean droplet diameter remained relatively low across the entire pH range studied (**Figure 43a**), which suggested that the individual emulsion droplets were relatively small. However, these emulsions were highly unstable to creaming across the whole pH range, with an opaque creamed layer being observed at the top of the samples (**Figure 43c**). Interestingly, there appeared to be less creaming in the emulsions stored at pH 2, since the lower serum layer was more opaque than for the other pH values. The fact that creaming was observed in these emulsions suggests that there was some aggregation of the oil droplets, which was confirmed by optical microscopy at neutral pH (**Figure 43d**). The emulsions formed from Sunlipon 90 were seen to contain large aggregates, whereas those containing Sunlipon 50 were not. The fact that the particle size measured by light scattering was relatively small, even though droplet aggregation occurred, suggests that these emulsions were flocculated. The origin of droplet flocculation can again be attributed to the influence of electrostatic interactions. The ζ -potential of the droplets in the emulsions stabilized by Sunlipon 90 remained relatively low across the entire pH range, with the exception of pH 2 where there was a modest positive charge (+15 mV) (**Figure 43b**). The relatively low droplet charge from pH 3 to 7 would account for the fact that the droplets were highly susceptible to flocculation in this pH range, because there would only be a very weak electrostatic repulsion between them. The modest positive charge at pH 2 may have been responsible for the fact that these emulsions were more stable to creaming than at other pH values.

7.4.4. Effect of salt on physiochemical stability

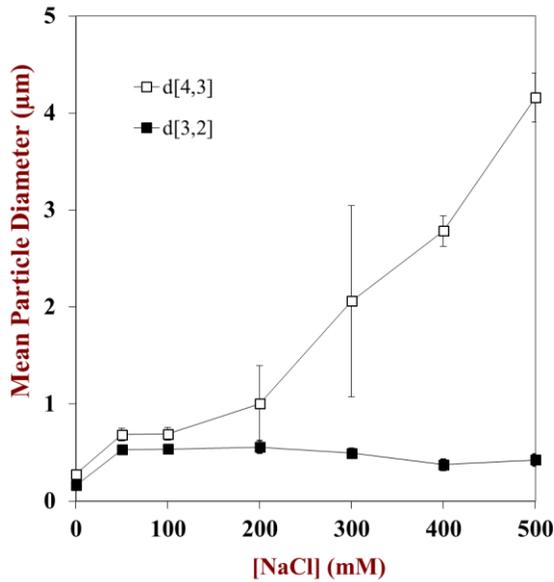
Commercial foods and beverages differ in the ionic compositions of their aqueous phases, with beverages like mineral water having a low ionic strength and foods like soups and sauces having high ionic strengths. The influence of ionic strength on the

stability of the ω -3 enriched nanoemulsions was therefore examined for the Sunlipon 50 emulsions. The Sunlipon 90 emulsions were not included in this series of experiments since they were already unstable to flocculation in the absence of salt (Section 7.4.3).

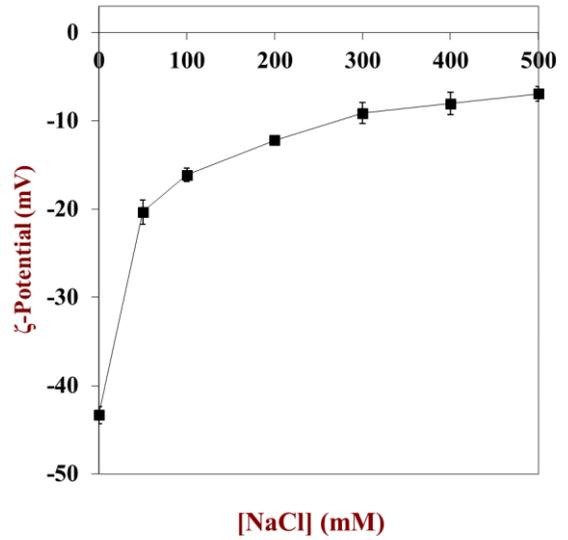
The influence of salt addition (0 to 500 mM NaCl) on the mean particle diameter, ζ -potential, physical appearance, and microstructure of the nanoemulsions was measured (**Figure 44**). In this case, the mean particle diameter was represented by both $d[3,2]$ and $d[4,3]$ to highlight the presence of a population of large aggregates observed in the particle size distribution data. There was clear evidence of an increase in particle size with increasing salt concentration (**Figure 44a**), which suggests that some droplet aggregation occurred when the ionic strength was increased. This effect was confirmed by optical microscopy images of the samples, which showed that they were highly susceptible to droplet coalescence at higher salt concentrations, *i.e.*, there was an increase in the size of the individual droplets (**Figure 44d**). The origin of droplet aggregation upon addition of salt can be attributed to a reduction in the electrostatic repulsion operating between oil droplets with increasing ionic strength [6]. Indeed, the addition of salt to the emulsions caused an appreciable decrease in the magnitude of the ζ -potential (**Figure 44b**), which can be attributed to electrostatic screening effects. Surprisingly, there was little change in the visual appearance of the emulsions containing different salt levels (**Figure 44c**). One would have expected larger oil droplets to move more rapidly to the top of the emulsions. The fact that we did not observe creaming may have been because the aggregated droplets formed a three-dimensional network that inhibited their movement, or because the presence of liposomes in the aqueous phase inhibited their movement. Our results are in agreement with earlier studies, which have also shown that

oil-in-water emulsions stabilized by phospholipids are unstable in the presence of electrolytes [184].

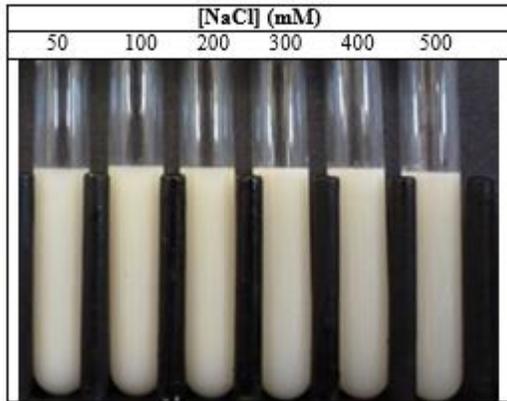
a) particle size



b) zeta potential



c) physical appearance



d) microstructure

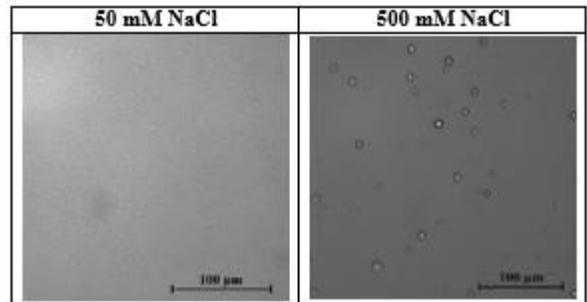


Figure 44. Effect of salt on the stability of emulsion systems formed using Sunlipon 50 at an SOR of 1 and pH of 7 after 24 hours. Particle size is represented as d[3,2] and d[4,3] since the d[4,3] better represented the large particles that were formed.

7.5. Conclusions

We have shown that ω -3-enriched nanoemulsions can be produced by microfluidization using certain kinds of natural sunflower phospholipids. The initial size of the droplets produced depended on phospholipid type and surfactant-to-oil ratio. There appeared to be no simple correlation between the phosphatidylcholine content of the phospholipid ingredients, and their ability to form small droplets. Emulsions made with the highest overall phospholipid concentration produced the smallest droplet sizes. The emulsions formed were primarily stabilized by electrostatic repulsion, and were therefore susceptible to aggregation under conditions where the droplets had low net charges or where the aqueous phase had a high ionic strength. These results suggest that sunflower phospholipids can be used as natural emulsifiers to form ω -3 nanoemulsion-based delivery systems suitable for application in certain types of foods and beverages. However, it is important to ensure that the pH and ionic strength of a particular product do not promote droplet aggregation by reducing electrostatic repulsion. In future studies, it would be interesting to investigate the chemical stability of these systems, as oils rich in polyunsaturated lipids are known to be highly unstable to oxidation.

7.6. Acknowledgements

This material was partly based upon work supported by the Cooperative State Research, Extension, Education Service, USDA, Massachusetts Agricultural Experiment Station (Project No. 831) and USDA, NRI Grants (2011-03539, 2013-03795, 2011-67021, and 2014-67021). This project was also partly funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant number 299-

130-1435-DSR. The authors, therefore, acknowledge with thanks DSR for technical and financial support. Ashtri Sastrosubroto was also incredibly helpful in the completion of the experiments.

CHAPTER 8

CONCLUSION

Nanoemulsions hold a lot of potential to incorporate lipophilic bioactive compounds into aqueous based food and beverage products. Their small size leads them to be optically translucent as well as have greater stability against gravitational separation compared to conventional emulsions. Low-energy methods are of particular interest for nanoemulsion production because of their ease of implementation and the lack of a requirement for expensive equipment. In this dissertation the factors affecting nanoemulsion formation by isothermal low-energy methods were investigated in model non-food and food systems. This work helped us to identify the major factors impacting the formation and stability of nanoemulsions by this method, including surfactant type, surfactant-to-oil ratio, oil type, temperature, and preparation conditions. We also demonstrated the practical utility of nanoemulsions formed by spontaneous emulsification by incorporating them into a filled hydrogel system. Finally, we showed that natural emulsifiers (sunflower phospholipids) could be used to form delivery systems using spontaneous emulsification or microfluidization. These findings suggest that low-energy methods are a viable option for nanoemulsion formation and incorporation into real food products, but if a natural emulsifier is required then high-energy methods may be a better choice.

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