# Preparation of Nano- and Microemulsions using Phase Inversion and Emulsion Titration Methods

MASSEY UNIVERSITY

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**Yue Wang**

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# Abstract

The formation of nano- and microemulsions with droplet size smaller than 100 nm in diameter and stabilised by non-ionic surfactants was investigated by using two different methods, emulsion phase inversion and emulsion titration. A series of ternary systems consisting of three components (lemon oil, Tween 20 or 80 and water) were prepared at different ratios via gentle agitation by the phase inversion composition method involving the spontaneous formation of microemulsion. The phase behaviour and nano- and microemulsion formation of the ternary mixtures prepared were characterised by visual observation for their phase separation and optical clarity (e.g. transparency and opacity). The samples were also analysed for their particle size and size distribution, viscosity, conductivity and birefringence. As a consequence, phase diagrams based on two different types of small molecule surfactants (Tween 20 or Tween 80) were constructed which define the ratios of three components in the composition of the ternary mixtures that allow the formation of oil-in-water (o/w) or water-in-oil (w/o) nano- and microemulsions. Overall, the o/w microemulsions were found to form at a small region of the ternary phase diagrams with a relatively large ratio of water, compared to w/o nanoemulsion, along dilution lines 1 and 2. On the other hand, w/o microemulsions were determined at the corner of surfactant-rich region along dilution lines 1, 2, 3 and 4 in the ternary phase diagrams. Between the two ternary phase diagrams based on Tween 20 and 80, there were some differences in their composition regions responsible for the formation of nano- and microemulsions as well as for other types of phases formed, including bi- and multiphase, liquid crystals, gel and coarse emulsions. In this study, nano- and microemulsions were also produced by a method called 'titration method' involving a two-step process; i) preparation of non-ionic small molecule surfactant-stabilised o/w emulsions by high pressure homogenisation and ii) titration of the o/w emulsions into non-ionic surfactant micelle solutions. Types and concentrations of surfactants (Tween 20, 40, 60 and 80) and oils (sunflower oil, lemon oil, tributyrin, isopropyl myristate and Imwitor 308) were investigated for their influence on the solubilisation of oil molecules from emulsion droplets into surfactant micelles, thus the formation of nano- and microemulsion. The results showed that Tween 60 and Tween 80 had the better capacity of oil droplet solubilisation compared to Tween 20 and Tween 40. The system containing a higher concentration of 2 wt% Tween 80 micelles had the larger capacity of droplet solubilisation than the other systems containing 0.5 wt% or 1 wt% Tween 80 micelles. In terms of the types of oil used, microemulsions could be fabricated using lemon oil, tributyrin, isopropyl myristate and Imwitor 308, whereas it could not be formed by using sunflower oil due to its high viscosity. Among the oils with relatively low viscosities, the order of the maximum amount of oil incorporated in 1 wt% Tween 80 micelles was Imwitor 308 > lemon oil > isopropyl myristate > tributyrin. This implies the lower viscosity oil has a higher rate of solubilisation in non-ionic surfactant micelles. The effects of pH, salt concentration and heat treatment on the stability of microemulsions were also determined. The results found that the nano- and microemulsion systems prepared by the emulsion titration method were relative stable to pH and ionic strength but sensitive to thermal treatment. This study provides useful information for the rational design of transparent nano- and microemulsions as delivery systems potentially for bioactive compounds for applications in food, beverage and non-food areas.

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# Chapter 1
# Introduction

Oil-in-water (o/w) nano- and microemulsions can be defined as emulsions containing oil droplets in the range of 50-100 nm in diameter (McClements 2010; Tadros, Izquierdo, Esquena, & Solans 2004) and 5-50 nm (McClements 2010), respectively. There is a growing interest in the development of nano- and microemulsions to encapsulate, protect, and deliver lipophilic compounds, such as nutraceuticals, drugs, flavours, antioxidants and antimicrobial agents, for their application in the food, pharmaceutical, agrochemical and other industries (Acosta 2009; Lee & McClements 2010; McClements 2011; McClements & Rao 2011; Ziani, Fang, & McClements 2012b). There are some advanges of nano- and microemulsions over conventaionl emulsions that have relatively large particle size. The emulsion systems can increase the bioavailability of lipophilic bioactive lipophilic substances encapsulated within them due to their small particle size and high surface-to-volume ratio (Acosta 2009; Chen, Khemtong, Yang, Chang, & Gao 2011; Lee & McClements 2010; McClements 2012). The stability of emulsion droplets to aggregation and gravitational separation is higher in nano- and microemulsions than conventional emulsion (Lee & McClements 2010; McClements 2005). Nano- and microemulsions are also tend to be optically clear in their appearance or only slightly turbid as the scattering of light induced by their small droplets is weak (Mason et al. 2006; McClements 2012; Velikov & Pelan 2008; Wooster, Golding, & Sanguansri 2008). Therefoere, nano- and microemulsions are considered as a delivery system suitable for dispersion and incorporation of lipophilic bioactive agents into transparent aqueous-based products (e.g. water, beverages, clear gel, etc) (Lee & McClements 2010).

The methods used in making emulsions can be divided into high energy and low energy methods. High energy methods use mechanical devices (e.g. high-pressure homogenizer, microfluidizer, ultrasonicator, etc) to break down oil into small droplets through their intense disruptive forces (Lee, Choi, Li, Decker, & McClements 2011; Lee & McClements 2010). The size of emulsion with droplets less than 100 nm is not readily achieved by high-energy methods alone (Wooster et al. 2008). Microemulsions are produced through simply mixing oil, water and surfactant together by using low enery methods which do not require a high mechanical force and rely on the spontaneous formation of small droplets by chagining the solution composition or environmental conditions (e.g. temperature, pH and ionic strength) (McClements 2012). These include phase inversion methods, such as phase inversion temperature (PIT), phase inversion composition (PIC) and emulsion inversion point (EIP).

In recent years, nanoemulsions have been shown to be able to be produced by a combined method of high-pressure homogenization and solvent evaporation (Chu, Ichikawa, Kanafusa, & Nakajima 2007, 2008; Lee et al. 2011; Lee & McClements 2010; Tan & Nakajima 2005). Another method for the formation of nano- and microemulsions is a titration (dilution) method which involves a mass transport and solubilisation of non-polar molecules from emulsion droplets into surfactant micelles when dispersing a pre-formed emulsion into surfactant micellar solutions (Rao & McClements 2012; Weiss, Canceliere, & McClements 2000; Ziani, Fang, & McClements 2012a; Ziani et al. 2012b). The formation, properties and stability of nano- and microemulsions are affected by a number of factors, such as type and concentration of emuslifers and oils, ratio of oil and water, pH, ionic strength and temperatue. There are som disadvantages and limitations of the methods used in the preparation of nano- and microemulsions, including requirements of only certain types of emuslifiers and oils and a large amount of surfactant, use of organic solvents and low capacity of oil solubilisation (Lawrence & Rees 2000; Malcolmson & Lawrence 1995; Zhong et al. 2009).

The overall aim of this research was to explore a new emerging research area of nano- and microemulsions for the potential food applications by using phase inversion method and emulsion titration method. The specific objectives of the study were as follows:

By using the phase inversion method

- To investigate the phase behaviours of ternary systems consisting of lemon oil, Tween 20 (or Tween 80) and water at different ratios.
- To constrctur ternary phase diagrams that indicate and identify the optimum composition of ternary systems allowing the formation of microemulsions.

By using the emulsion titration method

- To understand the effect of type and concentration of surfactants (Tween 20, 40, 60 and 80) and oils (sunflower oil, lemon oil, tributyrin, isopropyl myristate and Imwitor 308) on the formation of o/w nano- and microemulsion system.
- To investigate the effect of external factors (pH, ionic strength and thermal treatment) on the stability and properties of nano- and microemulsions.

# Chapter 2
# Literature Review

## 2.1 Introduction

An emulsion is defined as a colloidal dispersion system, usually water and oil, in which one liquid is dispersed as small droplets in another liquid. The original purpose of food emulsion was to deliver lipids in water in order to improve their dispersibility in aqueous phase (water) and their digestion in the human body (Robins 2000). There are two common types of emulsions, such as oil-in-water (o/w) emulsion and water-in-oil (w/o) emulsion (Figure 2.1). Between these two types, o/w emulsion is the most common type of an emulsion system found in foods in which oil droplets are dispersed in an aqueous medium. This literature review focuses on providing the background literature information for o/w emulsion systems. Many natural and processed food products are o/w emulsions, including milk, cream, ice cream, salad dressings, mayonnaise, soups, sauces, and infant formulas (Dickinson 1997; McClements 2005). The size of oil droplets varies, depending on type and concentration of materials (oils and emulsifiers) and emulsification methods used in the preparation of emulsions which have significant influence on the properties and stability of emulsion systems.

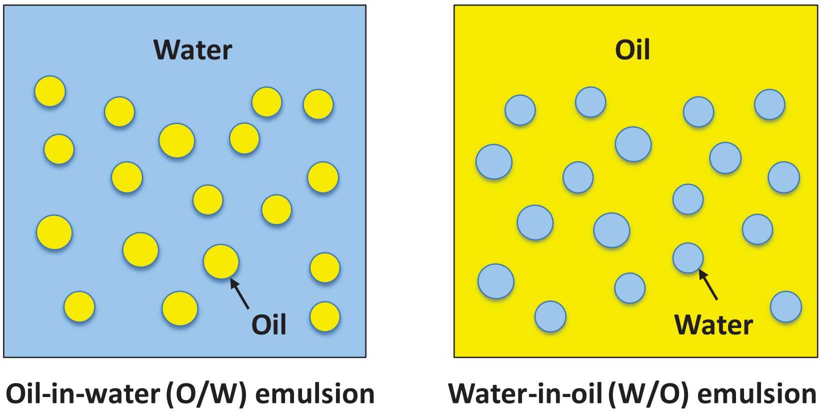


**Figure 2.1** Two types of emulsions, oil-in-water emulsion and water-in-oil emulsion.

Conventionally, most food emulsions are opaque in their appearance due to the scattering of light by the emulsion droplets, which are relatively big in comparison to the nanoscale particles of nano- and microemulsions that only scatter light weakly, thus tending to be transparent or translucent. According to Wooster, Golding, and Sanguansri

(2008), in order to produce a stable transparent emulsion the entire droplet size distribution should have a diameter smaller than 100 nm. The term "transparent emulsion" is thus usually used to refer to nano- and microemulsion systems unlike conventional emulsions are optically turbid. These nanoscale emulsion systems are currently of interest to food scientists because of their potential as delivery vehicles of lipophilic bioactive compounds for application in functional foods.

Nano- and microemulsions containing very small droplets have a number of advantages. They have better stability to gravitational separation and aggregation of particles because of the strong Brownian motion associated with their small particle size (McClements 2012; McClements & Rao 2011; Tadros, Izquierdo, Esquena, & Solans 2004). Bioavailability of certain types of bioactive lipophilic components can be increased greatly when the radius of droplets is below about 100 nm (Acosta 2009; Chen, Khemtong, Yang, Chang, & Gao 2011; McClements 2012). Since the dimensions of nano- and microemulsion oil droplets are much smaller than the wavelength of visible light, they are also suitable for incorporation into products that need to be optically clear or slightly turbid (Mason, Wilking, Meleson, Chang, & Graves 2006; Wooster et al. 2008).

## 2.2 Classification of emulsions

Emulsions can be broadly classified into conventional emulsions, nanoemulsions and microemulsions according to their particle size and properties although the classification of emulsion is not well defined clearly in the literature. This is because the terms are often used interchangeably in the literature with no clear distinction. The term "micro-" usually means $10^{-6}$, while the term "nano-" means $10^{-9}$, which implies that the particles in microemulsions are larger than those of nanoemulsions. However, the particles in a nanoemulsion are defined as larger than those in a microemulsion. According to McClements (2012), the historical development of the field of colloid science has caused this confusing terminology.

### 2.2.1 Conventional emulsion

Emulsion containing droplets with mean diameters in the range of 0.1 μm-100 μm are referred to as conventional emulsions (Lee & McClements 2010; McClements 2010; Robins 2000). Conventional emulsions are thermodynamically unstable and are optically turbid or opaque because the droplets have a size similar to or larger than the wavelength of visible light, thus scattering light more strongly.

### 2.2.2 Nanoemulsion

A nanoemulsion can also be considered as a conventional emulsion but it contains droplets smaller than 100 nm (McClements 2010; Tadros et al. 2004). Nanoemulsion is more stable against droplet aggregation and creaming than conventional emulsion due to its larger surface area to volume ratio of the oil droplets (McClements 2005). Nanoemulsions can improve the solubility, bioavailability and absorption of lipophilic compounds incorporated in their oil droplets (Krause & Muller 2001; Sanguansri & Augustin 2006). As described above, nanoemulsions also tend to be optically transparent because their droplet dimensions are smaller than the wavelength of visible light, thereby less light scattering (Lee & McClements 2010). Nanoemulsions have a significant potential for its use in food application (Lee & McClements 2010; McClements 2010). However, nanoemulsions are still thermodynamically unstable systems like conventional emulsions because the separated two phases of oil and water have a lower free energy state (McClements 2010).

### 2.2.3 Microemulsion

Microemulsion is defined as an emulsion system that contains oil droplets with diameters in the range of 5 nm – 50 nm (McClements 2010). Due to their particle size being much smaller than the wavelength of visible light, microemulsions are visually clear and optically transparent. Unlike nanoemulsions and conventional emulsions, microemulsions are characterised as a thermodynamically stable system because their free energy of the system is lower than the separated oil and water phases (McClements 2010). The formation of microemulsions containing very fine droplet sizes is not readily achievable by using high energy emulsification methods that are conventionally used in the preparation of emulsion (McClements 2010). The preparation of microemulsions is generally carried out by using ‘low energy methods’ and has some limitations with only certain types of emulsifiers (e.g. non-ionic small molecule surfactants at high concentrations) and oils (e.g. non-triglyceride oils such as hydrocarbon-based oils and mineral oils with very low viscosity).

## 2.3 Emulsion Ingredients

### 2.3.1 Aqueous Phase

Water is one of the main components of o/w emulsion, forming an aqueous continuous phase, which also contains other materials, such as emulsifiers, proteins, carbohydrates and minerals. The presence of these components change the refractive index, phase behaviour, pH value, ionic strength, and rheology of the aqueous phase, according to their types and concentrations, thus affecting the formation, stability, and physicochemical properties of emulsions (McClements & Rao 2011).

### 2.3.2 Oil Phase

A variety of non-polar lipids are used or incorporated to form an oil phase of o/w emulsions, including triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, flavour oils, essential oils, mineral oils, fat substitutes, waxes, oil-soluble vitamins, and lipophilic nutraceuticals (McClements & Rao 2011). In the food industry, typically, triacylglycerol oils, such as corn, soybean, sunflower, olive and fish oil, are used to formulate emulsions because of their low cost, abundance and functional or nutritional attributes (McClements & Rao 2011). These oils have low polarity, high interfacial tension and high viscosity due to their large molecular weight with long-chain hydrocarbon fatty acids. Therefore, it is difficult for them to be used in producing nano- or microemulsions by using high energy (e.g. high-pressure homogenisation) and low energy (e.g. phase inversion) emulsification methods (Flanagan & Singh 2006; McClements & Rao 2011; Witthayapanyanon, Acosta, Harwell, & Sabatini 2006; Wooster et al. 2008) as they are not readily broken down into very fine droplets in size smaller than 100 nm.

Research studies have shown that some types of short-chain triacylglycerol oils or smaller molecules of non-triglyceride oils could be used to produce nano- or microemulsions (Flanagan & Singh 2006; McClements & Rao 2011). For example, flavour essential oils (e.g. limonene) with high polarity, low interfacial tension, and low viscosity have been used to prepare microemulsions by using high-pressure homogenisation or phase-inversion temperature method (Fanun 2009; Flanagan & Singh 2006; McClements & Rao 2011; Rao & McClements 2011a, 2011b, 2012c; Zhao et al. 2010). However, emulsions prepared with flavour essentials oils have been shown to be less physically stable than some emulsions made from medium- or long-chain triacylglycerol oils. The reason is due to the effect of Ostwald ripening associated with the relatively high polarity (water solubility) and low viscosity of oil phase, resulting in faster droplet coalescence (Flanagan & Singh 2006).

In this literature review, several types of oils, such as sunflower oil, lemon oil, isopropyl myristate (IPM), tributyrin and Imwitor 308, are described below, including some differences in their physicochemical properties (Table 2.1). These oils were used in this study to investigate their influence on the formation and stability of nano- and/or microemulsions. Also, β-carotene is described in this literature review as it can be incorporated into the oil phase of nano- and microemulsions as one of the potential lipophilic bioactive compounds.

**Table 2.1** Some physicochemical properties of sunflower oil, lemon oil, isopropyl myristate (IPM), tributyrin and Imwitor 308 and their comparison with water.

| Oil | Density (g/ml) | Melting points (°C) | Refractive Index | Viscosity (mPas, 20°C) | Iodine values |
|---|---|---|---|---|---|
| Sunflower oil | 0.918-0.923 | -15 | 1.474-1.476 | 50 | 118-141 |
| Lemon oil | 0.849-0.855 | 7 | 1.464-1.467 | 1.41-1.85 | 99.25 |
| Isopropyl myristate | 0.853 | -3 | 1.434-1.437 | 5-6 | 1 |
| Tributyrin | 1.032 | -75 | 1.435 | 10 | |
| Imwitor 308 | | 30-34 | 1.450 | 3.3 | 1 |
| Water | 1 | 0 | 1.33 | 1.002 | |

Information adapted and summarised from various sources (Bailey & Shahidi 2005; Homburger & Boger 1968; Liebert 1982).

#### *2.3.2.1 Sunflower oil*

Sunflower oil is a non-volatile vegetable oil composed of triglycerides, which is extracted from sunflower seeds. The fatty acid composition of sunflower oil is made up about 74% unsaturated fatty acids, mainly linoleic acid ($C_{18:2}$) and oleic acid ($C_{18:1}$), and 8-15% saturated fatty acids with palmitic oil ($C_{16}$) and stearic acid ($C_{18}$) as predominant saturated fatty acids (Grompone 2005; Raß, Schein, & Matthäus 2008). Like most other triglyceride vegetable oils, sunflower oil is higher in viscosity than other types of lipid oils, such as lemon oil, IPM, tributyrin and Imwitor 308. Due to its light flavour, light golden-yellow colour, and relatively high oxidative stability, sunflower oil is commonly used for domestic and industrial applications. For example, it is used as cooking oil or in the manufacture of mayonnaise (Given Jr 2009).

#### *2.3.2.2 Lemon oil*

Lemon oil is one of the most common flavour oils used to prepare soft drinks (Given Jr 2009; Ziani et al. 2012a). It is a low viscosity, clear liquid oil with pale-yellow or greenish-yellow colour due to the presence of some carotenoid pigments. Lemon oil is usually extracted from lemon peel by cold pressing (Ziani et al. 2012b) and contains a wide variety of lipophilic components, such as limonene, citral, and gamma-terpinene, which have different molecular characteristics (Ziani et al. 2012a). The lemon oil extracted from peel is separated from the peel and juice by using centrifugation.

Limonene is the main constituent of lemon oils, which is chemically a cyclic terpene containing isoprene units (Figure 2.2) and possesses a strong smell of orange (Fahlbusch et al. 2003). It is a relatively stable terpene and can be distilled without decomposition, however, it oxidises easily in moist air (Karlberg, Magnusson, & Nilsson 1992; Pakdel, Pantea, & Roy 2001). Citral is a mixture of two terpenoids, which are double bond isomers, namely geranial (or citral A) and neral (or citral B) as shown in Figure 2.2. Geranial has a strong lemon odour, while neral has less intense but sweeter odours (Burdock 2004). Due to volatile constituents with characteristic aroma profiles, lemon oils are widely used in the food and beverage industries as flavouring agents (Rao & McClements 2012b). Lemon oil is also known to have antioxidant, antitumor and radical-scavenging activities, which may help prevent free radical-induced various chronic diseases (Shahidi & Zhong 2005).

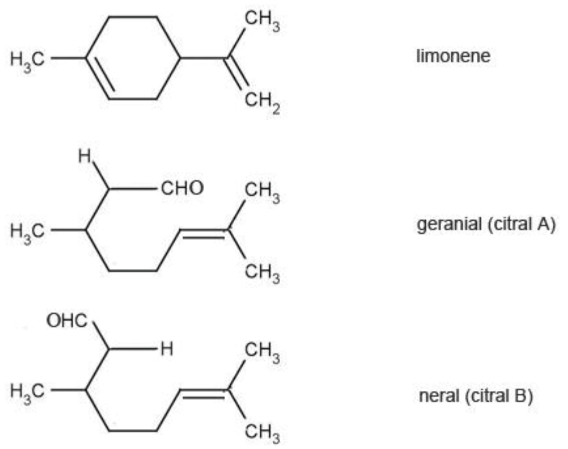


**Figure 2.2** Chemical structures of some major compounds found in lemon oils.

Commercially, lemon oils are available in different forms based on their concentration. Single-fold (1×) lemon oil is that directly extracted from citrus peel by cold pressing (Misharina, Terenina, Krikunova, & Medvedeva 2010), whereas higher fold lemon oils have undergone a further treatment by a process, usually vacuum distillation (Gamarra, Sakanaka, Tambourgi, & Cabral 2006). Because of differences in biological origins, isolation method and purification processing, the chemical composition of lemon oils can differ appreciably. For example, higher fold lemon oil typically has a higher concentration of specific flavouring components (such as citral) but a lower concentration of other components (such as limonene) (Gamarra et al. 2006; Rao & McClements 2012b). In a study by Rao and McClements (2012b), the viscosity, refractive index, density, and interfacial tension of lemon oils were shown to increase as the oil fold increased from 1× to 10× which was attributed to reduction in the limonene content of the lemon oils with increasing degree of distillation

### *2.3.2.3 Isopropyl myristate*

Isopropyl myristate is the ester of isopropyl alcohol and myristic acid, and its formula is $CH_3(CH_2)_{12}COOCH(CH_3)_2$, as shown in Figure 2.3. It is a colourless, almost odourless, oily liquid with a bland taste. It is insoluble in water, but soluble in liquid hydrocarbons and vegetable oils (Liebert 1982; Windholz 1976). It is widely used as a solubilising, spreading, and flavouring agent in cosmetics, pharmaceutical and food industries. According to the Flavour and Extract Manufacturers Association (FEMA), isopropyl myristate is generally recognized as safe (GRAS) under conditions of intended use in food flavourings that may be added to foodstuffs. Particularly, its use is limited as a flavouring agent in baked goods at 50 ppm, frozen dairy at 30 ppm, soft candy at 50 ppm, gelatines and puddings at 50 ppm and non-alcoholic beverages at 30 ppm. However, the Council of Europe only approved at a level of 5 ppm intended for use in food flavourings (Liebert 1982).

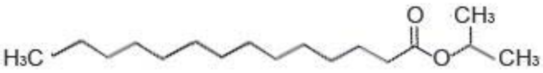


**Figure 2.3** Chemical structure of isopropyl myristate.

### *2.3.2.4 Tributyrin*

Tributyrin is a triglyceride oil containing butyric acid (which is a short chain fatty acid) esterified to glycerol molecules (Conley et al. 1998; Edelman et al. 2003; Kang, Lee, Lee, & Lim 2011; Su, Zhang, & Ho 2004). The chemical structure of tributyrin is shown in Figure 2.4. It is a white, almost odourless, bitter oily liquid with a bland taste. It is insoluble in water but miscible with ethanol. It can be used as an ingredient in margarine. It is worth to note that its density (1.032 g/ml) is higher than that of water (Table 2.1).

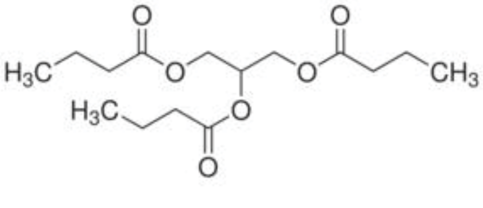


**Figure 2.4** Chemical structure of tributyrin.

### *2.3.2.5 Imwitor 308*

Imwitor 308 as shown in Figure 2.5 is a white, solid, crystalline mass with a faint fatty odour and a very bitter, scratchy taste. It is produced by esterification of glycerol and caprylic acid. It is readily soluble in lipophilic agents such as vegetable oils and in polar solvents such as ethanol. It is insoluble but dispersible in excess of water. According to the code of Federal Regulation of FDA (21 CFR 184.1505), Imwitor 308 is generally recognized as safe (GRAS), and it is known to be a multifunctional lipid compound, acting as co-emulsifier, solubilising agent, dispersion adjuvant, plasticizer, lubricant, consistency regulator, penetration enhancer, refatting agent, active ingredient carrier, and absorption promoter.

**Figure 2.5** Chemical structures of glyceryl caprylate (Imwitor 308).

### *2.3.2.6 Beta-carotene*

Carotenoids are present in a wide variety of foods, such as vegetables, fruits, fishes and some seafood (Ribeiro, Chu, Ichikawa, & Nakajima 2008). They impart a range of red, orange and yellow colours to foods, and they also have antioxidant properties due to their chemical structure consisting of a long chain made of conjugated double bonds, hydroxyl groups, keto groups and/or cyclohexane rings (Bhagavan & Nair 1992; Delgado-Vargas, Jiménez, & Paredes-López 2000; Emerton 2008). The chemical structure of beta-carotene ($C_{40}H_{56}$) shown in Figure 2.6 is one of the major dietary carotenoids (Johnson 2002). Major sources of β-carotene are carrots and sweet potato (Boon, McClements, Weiss, & Decker 2010; Maiani et al. 2009; Namitha & Negi 2010; Ribeiro et al. 2008).

Carotenoids can be converted into retinol (vitamin A) in human body, which plays an important role in eye health and immune system. Research studies have suggested that carotenoids also have a protective function in reducing the risk of cancer and coronary heart disease, and prevent some degenerative diseases due to their antioxidant activities (Boon et al. 2010; Delgado-Vargas et al. 2000; Maiani et al. 2009; Ribeiro et al. 2008). Because of numerous health benefits and their function as natural colorants, carotenoids are widely used in food and pharmaceutical industries. However, they are very sensitive to oxidative degradation when they are exposed to high temperature, light and oxygen

(Aissa et al. 2012; Barbosa, Borsarelli, & Mercadante 2005; Miguel, Martín, Mattea, & Cocero 2008). Carotenoids are nearly insoluble in water and even their solubility in oil and low-polar solvent is low. In order to increase their solubility and bioavailability, appropriate dispersion and delivery systems are required for their incorporation in aqueous foods.

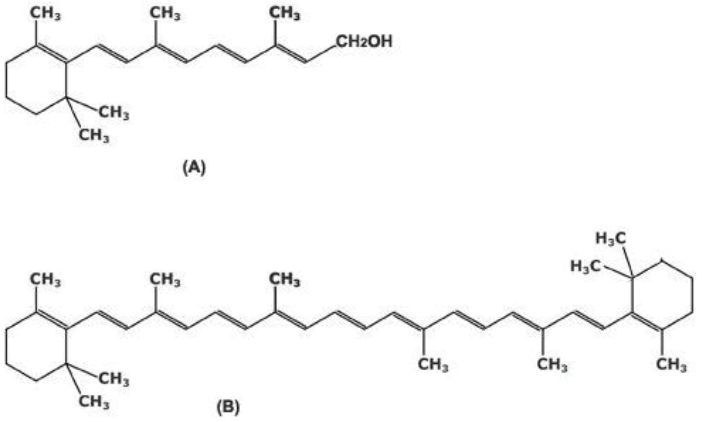


**Figure 2.6** Chemical structure of (A) retinol (vitamin A) and (B) β-carotene.

### 2.3.3 Emulsifiers

Emulsifier is also called surface-active agent or surfactant (Symon & William 2001). Emulsifier reduces interfacial tension and Laplace pressure between oil and water phases, hence the stress acting on breaking up the structure of oil droplets is reduced (Tadros et al. 2004). Various emulsifiers are available that are different in their chemical structures and properties. Emulsifiers can be broadly classified into small molecule surfactants, protein and polysaccharide emulsifiers.

#### *2.3.3.1 Small molecule surfactants*

Small molecule emulsifiers, also called small molecule surfactants, are amphiphilic in nature since they possess both hydrophilic and hydrophobic groups. Figure 2.7 shows a schematic diagram of the chemical structure of small molecule surfactants. Small molecule surfactants are typically represented by two moieties of polar head and non-polar tail groups. Small molecule surfactants usually increase the kinetic stability of o/w emulsions. They adsorb to the surface of oil droplets during homogenization and prevent the oil droplets from coming close to each other. (Lawrence & Rees 2000; Weiss et al. 1997).

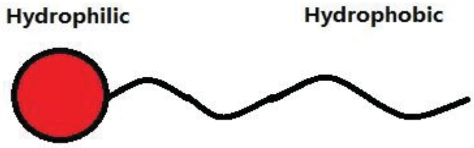


**Figure 2.7** A schematic structure of small molecule surfactants.
Adapted from Sirshendu and Sourav (2012).

Typically, the head of small molecule surfactants is made up of a long hydrocarbon chain or an aromatic ring that contains some functional polar group attached to it, whereas the tail consists of an unsaturated high molecular weight fatty acid or its derivatives (Lawrence & Rees 2000; Sirshendu & Sourav 2012; Weiss et al. 1997). The position of polar groups, the degree of chain length and branching of hydrophobic tail are all important parameters responsible for differences in the physicochemical properties between surfactant molecules (Sirshendu and Sourav (2012). For example, an increase in the length of the lipophilic tail leads to the following consequences: (1) the solubility of the surfactant in organic solvents increases but its aqueous solubility decreases, (2) the surfactant molecules at the interface between oil and water become packed more densely, and (3) the tendency of surfactant to form micelles or liquid crystal increases. The term “hydrophile-lipophile balance (HLB)” is often used to represent the ratio of hydrophilic and hydrophobic moieties of surfactant molecules (Lawrence & Rees 2000). The HLB value is thus one of the important parameters used in selecting an appropriate type of surfactant to formulate emulsion systems.

Small molecule surfactants have the following three characteristics: (1) their molecular structures are composed of polar and non-polar groups, (2) they possess surface activity, and (3) they can form self-assembled aggregates (e.g. micelles, vesicles, liquid crystalline, etc.) in liquids, as shown in Figure 2.8 (Sirshendu & Sourav 2012). According to Hiemenz (1986), small molecule surfactants exist as either individual monomeric units or an aggregated form in solution. The ratio of surfactant monomers to micelles present in solution depends on the concentration of surfactant molecules in an aqueous medium, which means that below the critical micelle concentration (CMC), the surfactant molecules exist only as monomers and do not form micelles. Once the surfactants reach their CMC in water or organic solvents (e.g. alkanes), the surfactants aggregate and form  micelles of various shapes (McClements 2005).

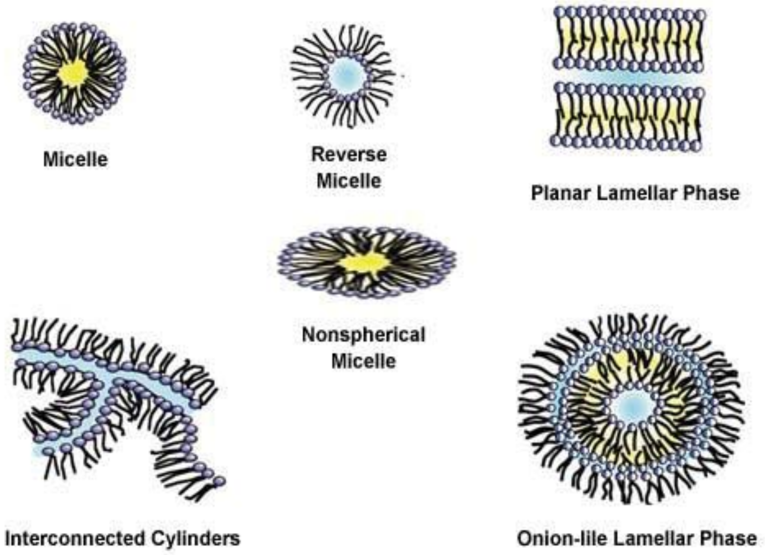


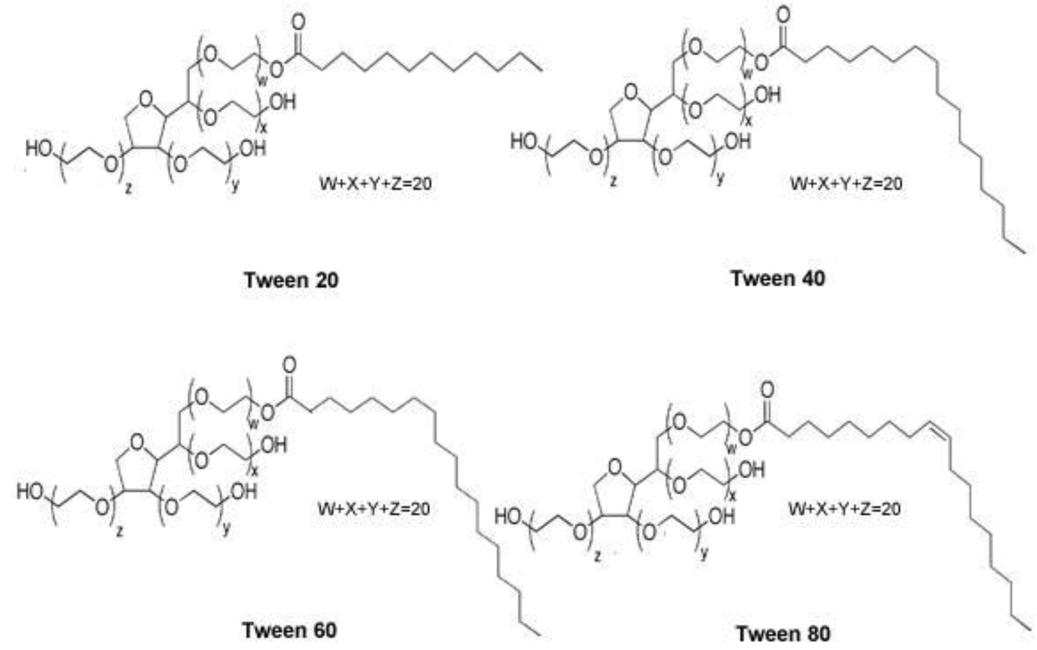
**Figure 2.8** Some typical structures formed due to the self-association of surfactant molecules.
Adapted from McClements (2005) and Pileni (2003).

The formation of micelles is driven by the interplay of various intra- and intermolecular forces acting on surfactant molecules as well as entropy (Lawrence & Rees 2000). A variety of thermodynamically stable micelle structures can be formed spontaneously by small surfactant molecules, depending on their concentrations and environmental conditions (McClements 2005). The micellar structures are held together by physicochemical interactions that are relatively weak and therefore they have highly dynamic and flexible structures (Israelachvili 1992). The size and shape of micelles thus keep continually fluctuating, and individual surfactant molecules exchange in their state between the micelle and the surrounding liquid. The relative weakness of the forces holding micelles in dispersions also means that their structures are particularly sensitive to changes in environmental conditions, such as temperature, pH, ionic strength, and ion type (Israelachvili 1992; McClements 2005).

Some hydrophobic substances can also be solubilised into micelles under appropriate conditions (McClements & Dungan 1995). The ability of micelles to solubilise and transport non-polar molecules through the aqueous phase has a significant effect on the physicochemical properties of emulsions (Rao & McClements 2012a; Weiss, Canceliere, & McClements 2000; Weiss et al. 1997; Ziani et al. 2012a, 2012b).

Small molecule surfactants are generally divided into four different types, non-ionic, anionic, cationic, and zwitterionic, according to their electrical charges (Flanagan & Singh 2006; McClements & Rao 2011). Nonionic surfactants do not carry any electrical charge in aqueous solution, which make them universally compatible in any solution due to their low interaction with other charged chemical components (Sirshendu & Sourav 2012). As a consequence, emulsions stabilised by nonionic emulsifiers are less sensitive to pH and ionic strength. This means that the stability of droplets against aggregation is not driven by electrostatic repulsive force. Instead, their stability against droplet aggregation and coalescence is governed by short range repulsive forces, such as steric repulsion, hydration and thermal fluctuation interactions (McClements 2005). However, nonionic surfactants are sensitive to changes in temperature. For example, when the solution temperature is increased, the solubility of surfactant in aqueous solution is decreased (Lawrence & Rees 2000; Sirshendu & Sourav 2012).

Nonionic small molecule surfactants have been widely used in the food and pharmaceutical industries, including sugar ester (e.g. sucrose monopalmitate), polyoxyethylene ether (POE) (e.g. Brij 96), sorbitan esters (e.g. Tweens and Spans), due to their low toxicity or irritation potential (Henry, Fryer, Frith, & Norton 2009; Liu, Sun, Li, Liu, & Xu 2006; Tenjarla 1999). In case of sorbitan esters, sorbitol, which is a sugar alcohol made by hydrogenation of glucose, is esterified with fatty acids to form them. Sorbitan esters are lipophilic emulsifying agents and they are commercially produced and sold under the trade name “Span” (Garti 2002). Another type of sorbitan esters called ethoxylated sorbitan esters are produced by the reaction of sorbitan fatty acid ester with ethylene oxide. These surfactants are hydrophilic and sold under the trade name “Tween" (Garti 2002). Some examples of chemical structures and properties for several Tween surfactants are shown in Figure 2.9 and Table 2.2.



Tween 20

Tween 40

Tween 60

Tween 80

**Figure 2.9** Chemical structures of small molecule surfactants (Tween 20, 40, 60 and 80).
Adapted from Sigma-Aldrich (2013).

**Table 2.2** Some chemical and physical properties of Tween 20, 40, 60 and 80[a].

| Surfactant | Molecular formula | HLB | CMC[b] (mM) | Density (g/ml) | Refractive index | Viscosity[c] (mPa.s) | ADI[d] (mg/kg) |
|---|---|---|---|---|---|---|---|
| Tween 20 | $C_{58}H_{114}O_{26}$ | 16.7 | 0.06 | 1.09 | 1.468 | 250-450 | 25 |
| Tween 40 | $C_{62}H_{122}O_{26}$ | 15.6 | 0.027 | 1.08 | 1.470 | 400-650 | 25 |
| Tween 60 | $C_{64}H_{126}O_{26}$ | 14.9 | 0.022 | 1.04 | 1.474 | 75-175 | 25 |
| Tween 80 | $C_{64}H_{124}O_{26}$ | 15.4 | 0.012 | 1.08 | 1.472 | 375-480 | 25 |

[a] Sources: (Ćirin, Poša, Krstonošić, & Milanović 2012; European Commission 2001; Griffin 1946; Japanese Food Safety Commission 2007; Sigma-Aldrich 2013).
[b] CMC: Critical micelle concentration in water at 20-25 °C.
[c] Viscosity of Tween 20, 40 and 80 at 25 °C and Tween 60 at 50 °C.
[d] ADI: Acceptable daily intake (mg/kg body weight).

Anionic surfactants, when put in aqueous medium, produce negatively charged heads due to the presence of nucleophilic groups, whereas cationic surfactants are positively charged due to the presence of electrophilic groups (Sirshendu & Sourav 2012). These surfactants are sensitive to pH and electrolyte concentration. When emulsions are prepared and stabilized by ionic emulsifiers, the emulsion droplets carry the surface charges and their stability is maintained by electrostatic repulsion. However, electrostatic repulsion is significantly affected by the ionic strength of the water phase where droplets are dispersed in. When the ionic strength is increased in the water phase, the emulsion droplets lose their repulsive forces and can come close to each other. This promotes the instability mechanisms of droplets, such as droplet flocculation and coalescence. When compared to their nonionic counterparts, ionic surfactants typically have higher critical micelle concentrations (CMC), but they have little or no sensitivity to temperature changes (Lawrence & Rees 2000).

Zwitterionic surfactants contain two or more ionizable groups with opposite charges on the same molecule (McClements & Rao 2011), therefore their properties depend on the pH of a solution. In acidic solution ($pH < 7$), the surfactant molecule obtains a positive charge, whereas in alkaline solution ($pH > 7$), the molecule acquires a negative charge. However, there is an isoelectric pH where the molecule has equal positive and negative groups (Sirshendu & Sourav 2012). For example, lecithin, which is a type of phospholipids possessing an emulsifying property, is a type of zwitterionic surfactant. Zwitterionic surfactants may be more effective when used in combination with co-surfactants, such as anionic or nonionic surfactants, than used as a single emulsifier (Hoeller, Sperger, & Valenta 2009; Sirshendu & Sourav 2012).

#### *2.3.3.2 Protein emulsifiers*

Some proteins (e.g. milk proteins) are commonly used as emulsifying agents in the food industry because of their amphiphilic characteristics and surface binding properties (Jones & McClements 2010). They stabilize emulsion droplets by forming a viscoelastic layer at the oil and water interface (Wilde, Mackie, Husband, Gunning, & Morris 2004). Proteins tend to have a particular molecular orientation at the interface, with the polar portions facing the water phase and the non-polar, hydrophobic portions facing the oil phase (McClements 2005). They stabilize emulsions with a combination of electrostatic repulsion and steric hindrance, and the outer segments of adsorbed protein molecules generate an osmotic pressure gradient. Therefore, these mechanisms prevent droplets from coming together (McClements 2005). However, there is only a weak electrostatic repulsion between the emulsion droplets stabilized by protein molecules when a pH value is close to the isoelectric point of protein molecules or an ionic strength is high (Jones & McClements 2010).

#### *2.3.3.3 Polysaccharide emulsifiers*

Polysaccharides are polymers of monosaccharides that possess varying molecular weights, conformations, branching, electrical characteristics, flexibility and hydrophobicity (Rinaudo 2008). Some polysaccharides have their structures with unique surface-active properties as emulsifying agents (Roger, Eric, & Junmin 2004). For example, gum arabic has been used for emulsifying and stabilizing beverage emulsions (Reiner, Reineccius, & Peppard 2010). Polysaccharides can also be classified, based on their electrical charges, into anionic, cationic and nonionic, which depends on the nature of their functional groups. Compared to proteins, the monomer composition of polysaccharides is more uniform, granting particular physicochemical and functional properties, such as solubility, binding properties, viscosity enhancement, gelation and surface activity.

In general, microemulsions can be prepared only with small molecule surfactants. Protein and polysaccharide emulsifiers are not typically suitable for their use in preparing microemulsions unlike nanoemulsions and conventional emulsions that can be prepared with all of them (e.g. protein and polysaccharide emulsifiers and small molecule surfactants). This is because small molecule surfactants are only able to generate ultralow interfacial tensions at particular monolayer curvatures (McClements 2012; McClements & Rao 2011).

### 2.3.4 Cosurfactants/cosolvents

Sometimes, cosurfactant (such as short- and medium-chain alcohols) or cosolvent (such as polyols like propylene glycol, glycerol, and sorbitol) is required to be used for the formation of microemulsions (Flanagan & Singh 2006; McClements & Rao 2011; Yaghmur, Aserin, & Garti 2002b). According to McClements and Rao (2011), cosurfactants are described as weakly amphiphilic molecules (having a hydrocarbon chain and a hydroxyl group) that are surface active but that are not good at stabilizing emulsions themselves because of a small size of their polar head group. This implies that cosurfactants are always mixed and used together with primary surfactants (Chennamsetty, Bock, Scanu, Siperstein, & Gubbins 2005). Generally, cosurfactant has some functions of (1) reducing the interfacial tension; (2) increasing the fluidity of the interface; (3) increasing the entropy of the system; (4) adjusting the curvature of the interfacial film; and (5) optimizing the ratio of dispersed-to-continuous phase viscosity (Attwood 1994; Eccleston 1994; Garti, Yaghmur, Leser, Clement, & Watzke 2001; Gradzielski 1998; Shafiq-Un-Nabi et al. 2007). A wide variety of materials can be used as cosurfactants, including nonionic surfactants, alkanoic acids, alkanediols and alkyl amines (Lawrence & Rees 2000).

On the other hand, cosolvents are not necessarily amphiphilic, but they may change the physicochemical properties of emulsifier molecules and are always distributed between the aqueous phase, the oil phase and the interfacial layer (Aboofazeli, Lawrence, Wicks, & Lawrence 1994; Chennamsetty et al. 2005; McClements & Rao 2011). Usually, alcohols are considered as both cosurfactant and cosolvent and for many additives there is no clear-cut distinction between cosurfactant and cosolvent, and they will be distributed in the surfactant layer as well as in the surrounding solution (Chennamsetty et al. 2005). Ethanol is the only alcohol permissible for food application if the microemulsion were to be incorporated into an alcoholic cocktail in which the concentration can range from 10 to 20% (Flanagan & Singh 2006). After the formation of microemulsions, alcohol may be removed via evaporation or its level can be reduced via dilution with water. However, this can disrupt the structural and compositional properties of microemulsion systems causing a loss of microemulsions and/or phase changes (Garti et al. 2001).

## 2.4 Formation of emulsions

Various techniques are available to prepare emulsions. The formation of emulsions involves a single step or a series of consecutive steps, depending on the nature of starting materials and methods used and a type of emulsions desired to be prepared. In general, the methods used to form conventional, nano- or microemulsions can be divided into two categories: high-energy and low-energy methods, which can also be referred to as mechanical and chemical processes, respectively (Acosta 2009). High energy methods use mechanical devices, such as high pressure homogenizer, microfluidizer and ultrasonic homogenizer, to generate intense disruptive forces to break an oil phase up into a number of smaller oil droplets. However, there are some limitations in using high-energy methods to produce nanoemulsions and ultrafine microemulsions because of some factors, including high initial equipment and operating costs, high power requirements, potential for equipment breakdown, and difficulties in producing very fine droplets from certain kinds of food ingredients (e.g. highly viscous oils or slowly adsorbing emulsifiers) (McClements & Rao 2011). For example, nanoemulsions containing droplets smaller than 100 nm have been shown to be prepared by using a combined method of high pressure homogenization and solvent evaporation rather than only using high pressure homogenization (Chu, Ichikawa, Kanafusa, & Nakajima 2007; Horn & Rieger 2001; Lee & McClements 2010; Tan & Nakajima 2005). The formation of nanoemulsions by this approach is based on the size reduction of oil droplets taking place during evaporation of an inorganic solvent (e.g. ethyl acetate) added to the oil phase prior to emulsification. The process involves the removal of the solvent from oil droplets via diffusion and evaporation during and after the emulsion preparation.

Low energy methods, such as phase inversion methods, do not require high mechanical energy input and are based on the spontaneous formation of small oil droplets using chemical energy. As described earlier, the free energy of microemulsion system is much lower than that of the phase-separated components, thus microemulsions are thermodynamically stable (Lee & McClements 2010; Rao & McClements 2011a). Low energy methods are effective in producing very fine droplets, and have lower equipment and energy costs than high energy methods. However, low energy method also have some potential disadvantages and limitations, including requirements of certain types of oils and surfactants, certain chemical and environmental conditions, and minimal use and addition of other ingredients rather than required surfactants and oils (Acosta 2009; McClements 2005; Yang, Marshall-Breton, Leser, Sher, & McClements 2012).

The formation of microemulsion using small molecule surfactants can be explained by several principles and mechanisms as follows:

(1) Interfacial or mixed film theories

Prince (1967) described that negative interfacial tension is responsible for the formation of microemulsions. In this theory, the interfacial film is considered as a duplex film having different properties on the water side and the oil side (Paul & Moulik 1997). This means that in the small molecule surfactants, the surface tension differences between oil and water phases across the interfacial monolayer impose the bimolecular leaflets due to high film pressure to enable the micelle to swell and reach an appropriate degree of curvature on the dispersed droplets (Lawrence & Rees 2000; Schulman, Stoeckenius, & Prince 1959).

(2) Solubilisation theories

Shinoda and Kunieda (1973) pointed out that microemulsion is a swollen micellar solution which is stable. The solubilisation of non-polar molecules into surfactant micelles takes place when three components of surfactant, oil and water are mixed over a wide composition range.

(3)Thermodynamic treatments

According to Ruckenstein and Chi (1975), the free energy of microemulsion formation can be explained with reference to the following equation (1):

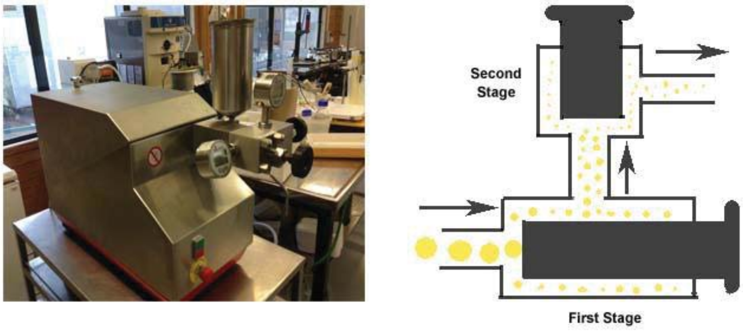
$$\mathbf{\Delta G_f = \gamma \Delta A - T\Delta S} \qquad \mathbf{(1)}$$

In this equation, $\Delta G_f$ is the free energy of microemulsion formation, $\gamma$ is the surface tension at the oil-water interface, $\Delta A$ is the change in interfacial area on microemulsification, T is temperature, and $\Delta S$ is the change in the system entropy. Due to an obvious reduction in droplet size in the process of microemulsion formation, the change of $\Delta A$ value is very large. The value of $\gamma$ is positive at all times and it is normally very low. Also, the value of $\Delta S$ rises in the process of microemulsion formation because of mixing of two immiscible phases. Therefore, a thermodynamically stable microemulsion is formed spontaneously due to a negative free energy achieved. However, it is worth to note that though microemulsion systems are thermodynamically stable, there may be kinetic barriers to the formation process, so some mechanical agitation or the input of heat will be required in some cases. In addition, the order of addition and mixing of components (surfactant, cosurfactant, cosolvent, oil and water) may also have influence on the formation and properties of microemulsion during preparation (Lawrence & Rees 2000).

## 2.4.1 High energy methods

### *2.4.1.1 High pressure homogenization*

High pressure homogenizer shown as an example in Figure 2.10 uses intense shear forces, including turbulence, cavitation, pressure gradient and recirculation, in order to break up a droplet into smaller size. Before the main homogenization process, all ingredients are mixed together to form a coarse pre-emulsion. The crude pre-emulsion is then introduced into the input chamber under high pressure. When the pressure exceeds a certain pre-set value, a valve at the end of the chamber opens to form an annular slit through which the emulsion is forced. The slit width is typically 15 – 300 μm, and the pressure drop across the slit is in the range 3 - 20 MPa. The passage through the slit and into the second chamber results in a reduction in droplet size, down to 0.1 μm, depending on the pressure applied (Robins 2000). The size of droplets produced by using this method usually depends on the homogenization pressure and /or the number of cycles (Cheong & Tan 2010). The size of droplets also depends on the characteristics of oil and emulsifier used together with their concentrations (McClements & Rao 2011). Due to the Laplace pressure, high pressure homogenizer is difficult to reduce the particle size further to below a certain size level (e.g. <100 nm) when they reach a minimum particle size (McClements 2005).



(A) (B)

**Figure 2.10** (A) A bench top scale of APV-2000 two-stage high pressure homogenizer and (B) schematic illustration of a two-stage high pressure homogenizer used to produce emulsions.
(B) adapted from McClements and Rao (2011).

### *2.4.1.2 Microfluidization*

Microfluidizer also uses a high pressure homogenization technique to create emulsions with tiny droplet size (Figure 2.11). It forces a mixture of oil and water to flow through a narrow channel into two streams and then direct into an interaction chamber. In the interaction chamber, high disruption force occurs when the two fast-moving streams of emulsion impinge upon each other (Kanafusa, Chu, & Nakajima 2007; McClements & Rao 2011). Microfluidizers have traditionally been used in the pharmaceutical industry or beverage industry (McClements & Rao 2011).

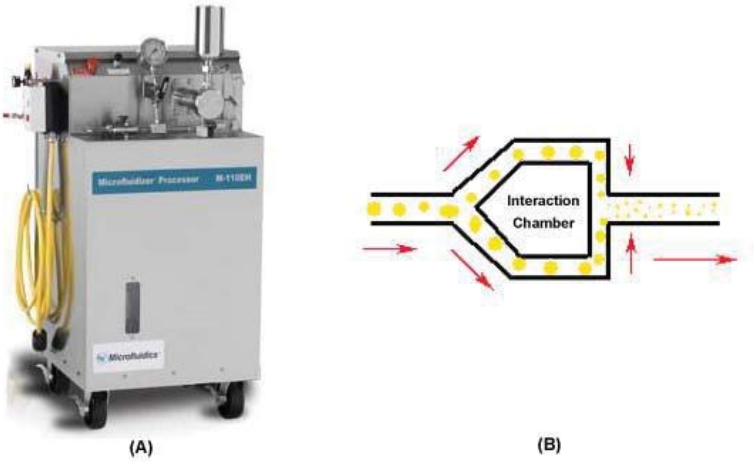


**Figure 2.11** (A) Microfluidics-M-110EH microfluidizer (Microfluidics 2013) and (B) schematic illustration of microfluidizer used to produce emulsions.
Adapted from McClements and Rao (2011).

### *2.4.1.3 Ultrasonication*

Ultrasonic homogenizers generate the intense disruptive forces with high-intensity ultrasonic waves through a sonicator probe rather than high pressure in order to break up the large droplets (Kentish et al. 2008; Leong, Wooster, Kentish, & Ashokkumar 2009; Lin & Chen 2008). The sonicator probe is placed within the liquids to form disruptive force by mechanical vibrations (Figure 2.12). The size of droplets formed also depends strongly on the type and amount of emulsifier used and the viscosity of liquids as well as the intensity of ultrasonic waves generated (Kentish et al. 2008; Leong et al. 2009; Maa & Hsu 1999). The limitations of this method are that the high local intensities may cause protein denaturation, polysaccharide depolymerization, or lipid oxidation (McClements & Rao 2011), and this method is limited in a laboratory batch process not for industrial production (Huang, Yu, & Ru 2010; Mason et al. 2006; Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma 2005).

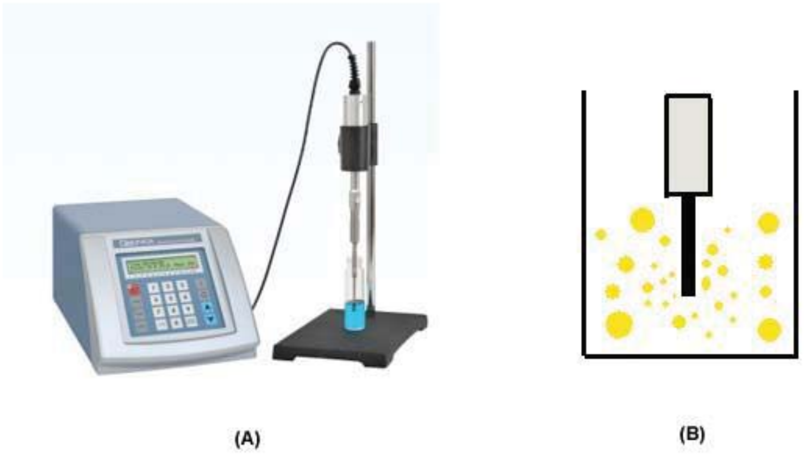


**Figure 2.12** (A) QSONICA-Q125 sonicator (Qsonica 2013) and (B) schematic illustration of ultrasonic probe homogenizer used to produce emulsions.
Adapted from McClements (2005).

#### *2.4.1.4 Homogenization and solvent displacement/evaporation method*

Normally, the size of emulsion droplets less than 100 nm in radius is difficult to achieve by using high-energy methods alone (Wooster et al. 2008). In recent years, nanoemulsions have been shown to be able to be produced by a combined method of high pressure homogenization and solvent displacement/evaporation (Chu et al. 2007; Chu, Ichikawa, Kanafusa, & Nakajima 2008; Lee, Choi, Li, Decker, & McClements 2011; Lee & McClements 2010; Ribeiro et al. 2008; Tan & Nakajima 2005). Briefly, as shown in Figure 2.13, an organic phase consisting of oil and solvent is homogenized with an aqueous phase consisting of water and emulsifier (e.g. proteins). The organic solvent is then removed from the emulsions by evaporation using a rotary evaporator, which causes the oil droplets to shrink in size, depending on the initial solvent concentration loaded into the oil phase. The organic solvents used to prepare nanoemulsions include ethyl acetate and hexane, which have a low boiling point and can thus easily be removed at low temperatures under reduced pressure. The advantage of the homogenization and solvent evaporation method is that various types of emulsifiers can be used to prepare nanoemulsions, including proteins (e.g. whey proteins and caseinate) and small molecule surfactants (Lee & McClements 2010).

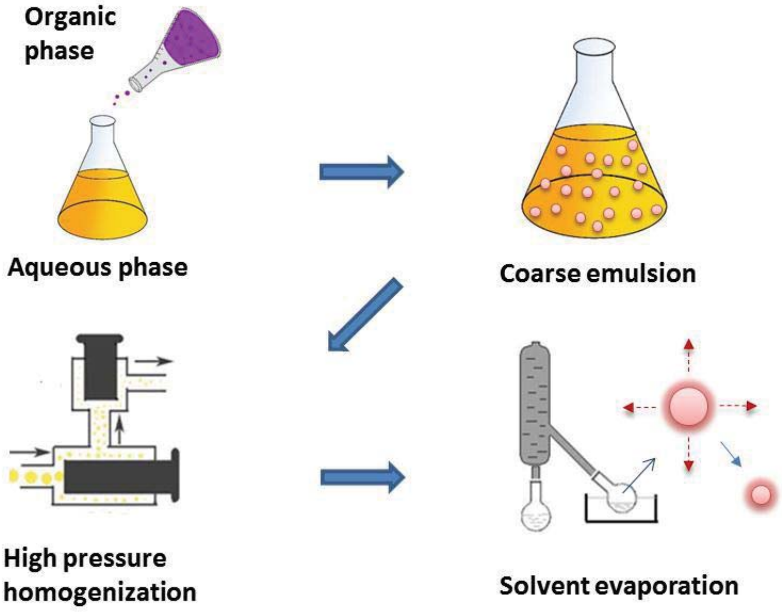


**Figure 2.13** Schematic illustration of the process of a combined method of homogenization and solvent evaporation to form nanoemulsions.

## 2.4.2 Low energy methods

The methods used in the preparation of nano- and microemulsions using phase inversion methods are referred to as low energy emulsification methods. Phase inversion methods can be divided into phase inversion temperature (PIT), phase inversion composition (PIC) and emulsion inversion point (EIP). In these methods, a system forms various phase systems and undergoes phase transition and conversion, including from a w/o emulsion to an o/w emulsion or vice versa, by changing the emulsion composition and/or environment conditions (e.g. temperature, pH and ionic strength) (Anton, Benoit, & Saulnier 2008; Chu et al. 2007; McClements & Rao 2011; Yin, Chu, Kobayashi, & Nakajima 2009).

### *2.4.2.1 Phase inversion temperature (PIT)*

The PIT method relies on changes in the physicochemical properties (e.g. molecular geometry or relative solubility) of non-ionic small molecule surfactants with changing temperature (Anton & Vandamme 2009; Lawrence & Rees 2000; McClements & Rao 2011). The temperature at which a system changes from a w/o emulsion to an o/w emulsion (or vice versa) according to changes in the curvature (molecular geometry) or solubility of surfactants is referred to as the ‘PIT’. The ability of surfactants to change their molecular geometry to form particular aggregates (e.g. surfactant monolayer via self-assembly) and various phase systems (e.g. lamellar phase, o/w emulsion and w/o emulsion) by changing the temperature of a system is associated with the critical packing parameter (CPP) of surfactants (Israelachvili, Mitchell, & Ninham 1976). The CPP is the ability of surfactants to self-assemble to form a surfactant monolayer in response to their molecular geometry, and it can be calculated using the follow equation (2):

$$CPP = \gamma / a\, l_c \qquad (2)$$

where $\gamma$ is the partial molar volume of the hydrophobic tail of surfactant, $a$ is the surface area of surfactant head group and $l_c$ is the hydrophobic chain length of surfactant tail group.

Sometimes, the packing parameter of surfactants is also described by the following equation (3):

$$p = a_t / a_h \qquad (3)$$

where $a_t$ and $a_h$ are the cross sectional area of the lipophilic tail group and hydrophilic head group of surfactant, respectively (Israelachvili 1992).

Figure 2.14 shows the effect of changing CPP caused by changing the temperature of a system on the phase inversion. This is due to changes in the curvature of non-ionic surfactant molecules. At temperature below the PIT, the CPP of surfactant is less than 1 and the surfactant becomes more soluble in water due to its relatively large head group. As a result, the surfactant has a large positive (convex) spontaneous curvature and favours the formation of an o/w emulsion. AT the PIT, the CPP equals to 1 where the o/w emulsion breaks down and the droplets become readily coalesced, and the system is changed into liquid crystalline or bi-continuous structures (e.g. lamellar phase) (McClements & Rao 2011). As the temperature increases above the PIT, the CPP is larger than 1 and the hydrophilic head group of the surfactant becomes smaller than the hydrophobic tail group. This makes the surfactant molecules more soluble in oil than in water and the spontaneous curvature becomes negative (concave), therefore, a w/o emulsion is formed (Ee, Duan, Liew, & Nguyen 2008; Lawrence & Rees 2000; McClements & Rao 2011)

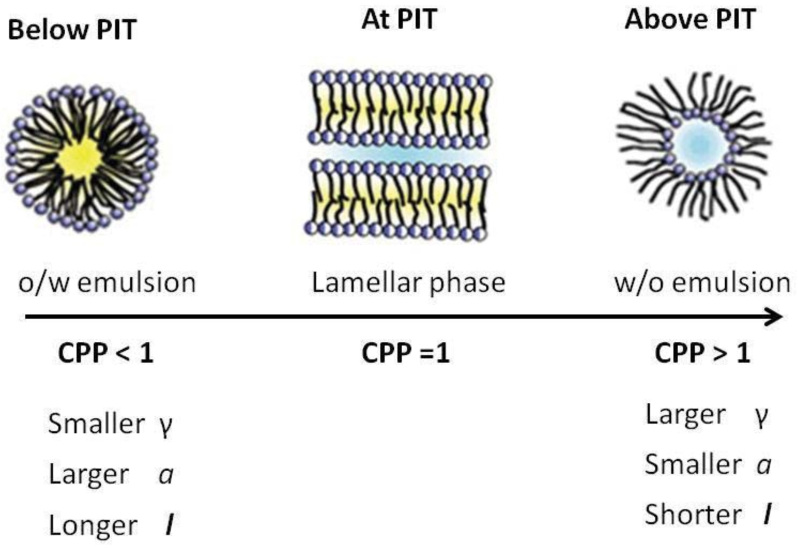


**Figure 2.14** Effect of changing CCP caused by temperature changes on the phase inversion of a system containing small molecule surfactant. $\gamma$ is the partial molar volume of the hydrophobic tail of surfactant, *a* is the head group area of surfactant and ***l*** is the hydrophobic chain length of surfactant tail group.
Adapted from Lawrence and Rees (2000).

.The typical advantage of the PIT method is that it is a low flow process, which does not require high energy input (Fryd & Mason 2012). However, there are some limitations including: (1) the system produced by the PIT is readily disrupted and changed as its temperature is altered; (2) only nonionic surfactants can be used though ionic surfactant can be mixed with nonionic surfactant; (3) it requires heat energy input and a limited stable temperature range (Maestro, Solè, González, Solans, & Gutiérrez 2008; Mei et al. 2011; Solè et al. 2006).

### *2.4.2.2 Phase inversion composition (PIC)*

Similar to the PIT method, the PIC method used to prepare nano- and microemulsion is also based on changes in the optimum curvature of surfactant molecules, but it is induced by changing the formulation of the system instead of by changing the temperature (Anton & Vandamme 2009; McClements & Rao 2011). For instance, by changing pH or adding salt to alter the electrical charge of surfactant head group, the surfactant curvature can be change. This implies that in the PIC, ionic surfactants can be used. Adding salt (i.e., increasing ionic strength) could adjust the CCP of ionic surfactant to be larger than 1 which favours the formation of a w/o emulsion because of the ability of salt ions to screen the electrical charge on the surfactant head group (McClements & Rao 2011). Similarly, with increasing the pH of a system greater than the $pK_a$ value of ionic surfactant, the carboxyl groups of ionic surfactants are more ionised. This makes the surfactants more water-soluble and the CPP becomes less than 1, thereby the system forms an o/w emulsion (Maestro et al. 2008; McClements & Rao 2011). This implies that at the pH below the pKa value for the ionic surfactant, the system forms a w/o emulsion. The w/o emulsion formed by increasing ionic strength can be also converted to the o/w emulsion when it is diluted in water (McClements & Rao, 2011).

#### *2.4.2.3 Emulsion inversion point (EIP)*

The EIP method is different from the PIT or PIC methods. As described above, in the PIT or PIC method, the curvature of surfactant is altered by changing the temperature, pH, or ionic strength, whereas in the EIP method, the only change involved is the volume fraction of ingredients (i.e. the ratio of oil and water) rather than the surfactant properties (Fernandez et al. 2004; McClements & Rao 2011). The critical step using this method is the transition between w/o and o/w emulsions via an intermediate bi-continuous phase by gradually adding the dispersed phase of water into an o/w emulsion initially formed or oil into the initial system of w/o emulsion) (Hessien, Singh, Kim, & Prouzet 2011). In this process, the surfactants form some monolayers at the interface of oil and water which lead to a bi-continuous microemulsion at the inversion point (Fernandez et al. 2004). Nanoemulsions can also be produced by slowly adding water to lamellar liquid crystalline phase, whereas rapid dilution can induce coarse emulsion with larger droplet size (Gutiérrez et al. 2008; Usón, Garcia, & Solans 2004; Wang, Li, Zhang, Dong, & Eastoe 2007).

The limitation of this method is that only the small molecule surfactants that can stabilize both w/o and o/w emulsions can be used as the emulsifier, and good mutual solubility of oil and water is needed (Fryd & Mason 2012; McClements & Rao 2011). Furthermore, Ostwald ripening is prone to occur due to the higher Laplace pressure inside small emulsion droplets (Fernandez et al. 2004; Fryd & Mason 2012). However, the advantages of EIP are that it is a low energy process and does not require changes in temperature (Fernandez et al. 2004; Fryd & Mason 2012; Pey et al. 2006; Sajjadi 2006).

### 2.4.3 Emulsion titration/dilution method

In recent years, it has been shown that some o/w nano- and microemulsions containing very fine oil droplets can also be formed by another approach which can be similar somehow to the EIP method. This method involves a two-step process in which a pre-made nonionic surfactant-stabilized (e.g. Tween 80) o/w conventional emulsion is diluted into an aqueous surfactant micellar solution (e.g. 1% Tween 80) as illustrated in Figure 2.15 (McClements 2012; Rao & McClements 2012a). This method can thus be called ‘emulsion titration or dilution method’, however, this method involves both high energy and low energy methods. Using the emulsion titration/dilution method, Ziani et al. (2012a, 2012b) have shown that colloidal delivery systems can be fabricated by using various types of surfactant (Tween 20, 60 and 80) and oil (lemon oil, vitamin E and vitamin D). The results showed no significant effect of non-ionic surfactant type on the formation and properties of the colloidal dispersions. However, using this method microemulsions could only be formulated by using lemon oil in 1% Tween solution due to its relatively small molecule dimensions rather than vitamin E and vitamin D. Rao & McClements (2012a) showed that high fold lemon oil had a larger extent of solubilisation and produced more stable system, which means the amount of lemon oil that could be incorporated into small molecule surfactant micelles increased with increasing oil fold (Rao & McClements 2012a).

It should be noted that the formation of nano- and microemulsions by the emulsion dilution method was shown to be highly dependent on the concentration of oil in the final mixture. This means that the level of oil concentration required to be diluted into the surfactant solution to enable to form nano- and microemulsions (e.g., oil droplet size smaller than 50 or 100 nm) is limited and determined by the amount of titrated conventional emulsion into a surfactant solution.

It was shown that the size of oil droplets smaller than 100 nm could be formed by this approach only when a oil concentration added was lower than 1.5 wt% oil in the final mixture (Rao & McClements 2012c). In other words, when the volume of emulsion added has a relatively low concentration of oil droplets, all of the oil molecules in the emulsion move out of the droplets and are incorporated into the surfactant micelles. It has been suggested that there are several possible mechanisms by which oil molecules move from emulsion droplets to surfactant micelles, thereby being transferred and entrapped into surfactant micelles. These are as follows: (1) oil molecules are directly solubilized in water, and then they are accommodated by micelles in the aqueous phase; (2) oil molecules are incorporated into micelles due to the collision of micelles with the surface of emulsion droplets; (3) there may be spontaneous "budding-off" of a number of oil and surfactant molecules from the surface of an emulsion droplet to form a micelle (Weiss et al. 1997).

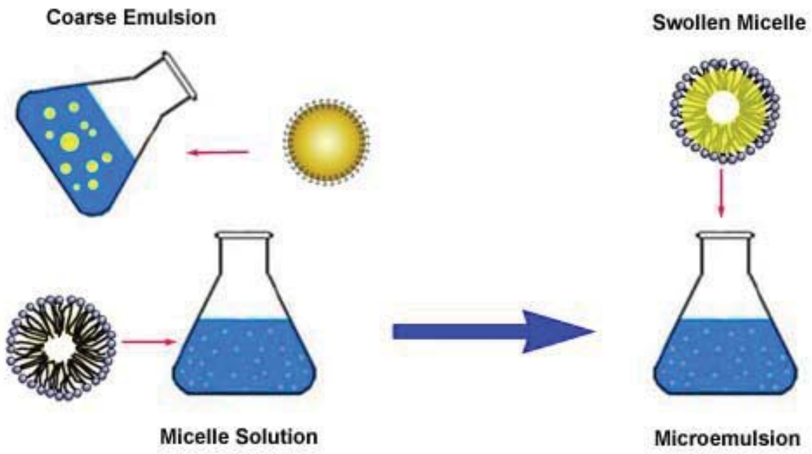


**Figure 2.15** A schematic representation of an experiment where emulsion droplets are titrated into a surfactant micelle solution to produce microemulsion.
Adapted from Rao and McClements (2012c).

McClements (2012) described that the structures of micelles containing solubilised oil molecules may be spheroid, cylinder-like, plane-like or sponge-like, but only thermodynamically stable isotropic liquids can be considered as microemulsion. For example, in an o/w microemulsion system, the lipid molecules can be incorporated into the hydrophobic interior of a surfactant micelle as a separate core or between the surfactant tails, as shown in Figure 2.16, whereas the hydrophilic head groups of the surfactant molecules are oriented toward the surrounding aqueous phase.

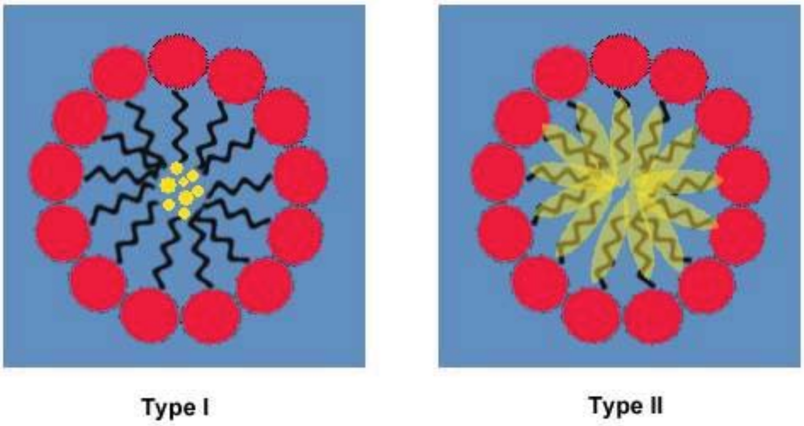


**Figure 2.16** Two types of an o/w microemulsion system.
Adapted from McClements (2012).

Emulsion titration/dilution method has not been extensively studied yet. In some articles, this method has also been called “oil exchange”, “swelling of o/w emulsion”, “oil solubilisation” or “molecular transport” (Binks et al. 1998; Evilevitch, Jönsson, Olsson, & Wennerström 2001; Evilevitch et al. 2000; McClements & Dungan 1993; Weiss et al. 2000; Weiss et al. 1997). Using this method, transparent nano- and microemulsions seem to be quite readily prepared. It may be possible to use the emulsion titration/dilution method to deliver some lipophilic components, such as nutraceuticals, colourants, flavouring or preservatives, for applications in food and beverages (McClements & Dungan 1993; Ziani et al. 2012a).

The disadvantage of emulsion titration/dilution method is that only certain types of emulsifiers can be used which are limited to mostly non-ionic small molecule surfactants (Rao & McClements 2012a, 2012c; Ziani et al. 2012a, 2012b). The types of oil that can be used seem to be also limited to certain types of low viscosity and non-triglyceride oils, and the level of oil concentration incorporated in a final nano- and microemulsions seems to be quite low as well.

## 2.5 Ternary phase diagram

The low energy method, such as phase inversion method, used to prepare microemulsions, which was described in the above, often involves the creation of ternary phase diagrams based on the physical states of mixtures of three components (i.e. oil, water and surfactant) referred to as ternary systems. If the system is comprised of four components, for instance, by the addition of lipophilic substance into oil, it is referred to as a pseudo-ternary system. A mixture of three components (i.e. oil, water and surfactant) can form one, two or more separate phases, depending on the relative proportion of three components.

According to Ruckenstein and Chi (1975), a microemulsion forms spontaneously when all the ingredients (oil, water and surfactant) are brought together by mixing them at appropriate concentrations and then it remains stable. However, it may not form spontaneously because of the presence of kinetic energy barriers or slow mass transport processes (McClements 2012). In practices, a mechanical agitation or heat is necessary to prepare a microemulsion. A nanoemulsion is very similar to a microemulsion, but their major distinction is their thermodynamic stability as shown in Figure 2.17.

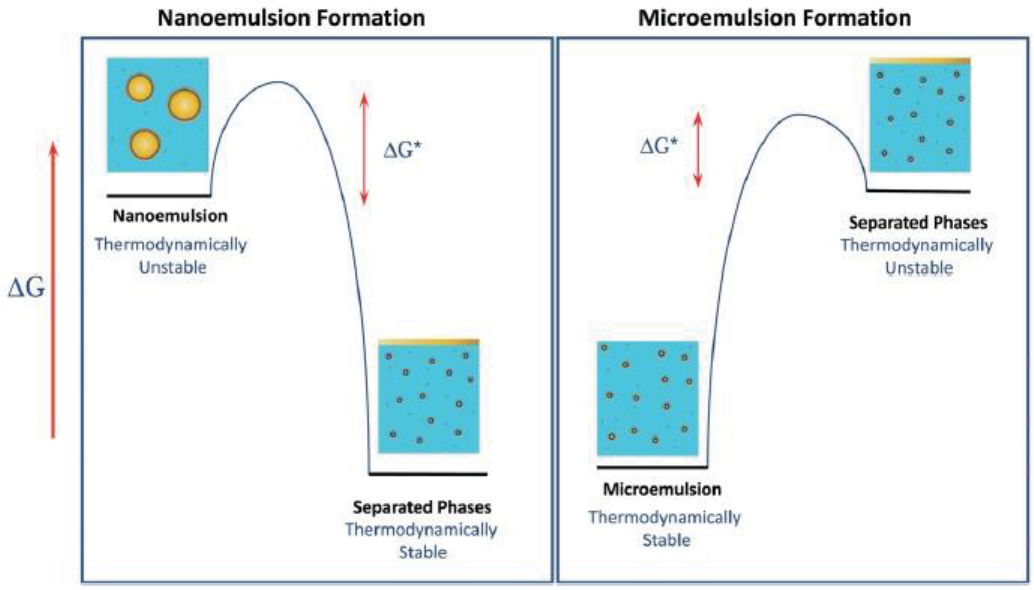


**Figure 2.17** Schematic diagram of the free energy of nanoemulsion and microemulsion systems compared to the phase separated state (McClements 2012).

Phase diagram is created to study the phase behaviour and transition of a mixture in response to its composition. In the case where a simple system consists of water, oil and surfactant, a ternary phase diagram is constructed where each corner of the diagram represents 100% of that particular component. A typical example of such representations is presented in Figure 2.18. When four or more components are involved, pseudo-ternary phase diagrams are used in which a corner represents a binary mixture of two components (Lawrence & Rees 2000).

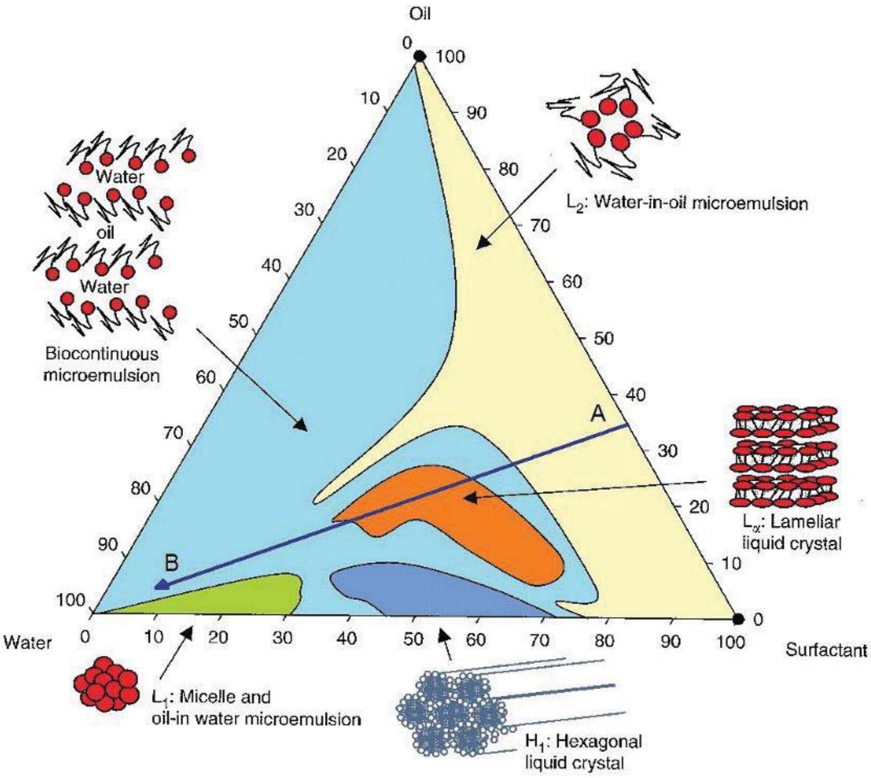


**Figure 2.18** Ternary phase diagram of an oil-surfactant-water system (Cannon 2011).

In a three-component system, as shown in Figure 2.18, transition from Point A to Point B can occur through a number of intermediate structural phases, including bicontinuous, lamellar, hexagonal and multiple phases, when a mixture of surfactant and oil at certain ratios is mixed gradually with water. Bicontinuous structure may exist in systems where the amount of water and oil are similar and both oil and water exist as a continuous phase. Multiple phases may exist where there is insufficient surfactant to form a single microemulsion phase, particularly for compositions close to the oil-water binary axis (Lawrence & Rees 2000). Each phase formed at different ratios of three components may have different consistencies over a region of ternary diagrams and show some different appearances with isotropic and nonisotropic characters (Paul & Moulik 1997). The transitions between the various phases mapped out in the phase diagram can be driven by changing the temperature or addition of a component (Lawrence & Rees 2000). Therefore, mapping a phase diagram needs a very careful experimentation due to its accuracy dependent significantly on the environmental conditions (e.g. temperature and additives).

Preparation of samples for analysis and construction of phase diagrams can be done by two approaches. One is titration of a mixture of two components with the third component, for example, using water to titrate the mixture of surfactant and oil. The other is preparation of a large number of samples with different compositions (i.e. different ratios of three components). If all mixtures do not reach equilibrium quickly, the latter method is recommended as the time taken for system to equilibrate can be greatly increased as the phase boundary is approached (Bhargava, Narurkar, & Lieb 1987; Lawrence & Rees 2000). In order to speed up the process, heat and sonication are often employed in the experimentation (Lawrence & Rees 2000).

The phase inversion method has been used to prepare nano- or microemulsions by a number of researchers in various fields (Fanun 2009; Fernandez et al. 2004; Garti et al. 2001). Finely dispersed oil droplets were achieved via emulsion phase inversion method using water, paraffin oil and mixture of non-ionic surfactants (Cremophor® A6 and A25) (Fernandez et al. 2004). They found that using the EIP method a critical surfactant concentration, an estimated value of about 10% for the surfactant-to-oil ratio, is necessary for emulsification to assure bicontinuous or lamellar structures. Fanun (2009) studied the phase behaviour of ternary systems consisting of water, non-ionic surfactant and peppermint oil in order to determine the single phase microemulsion regions using two types of surfactants: hydroxylated (sugar esters) and ethoxylated (ethoxylated mono-di-glyceride and polyoxyethylene sorbitan monooleate). It was shown that microemulsions formed using polyoxyethylene surfactants were sensitive to temperature while microemulsions formed with hydroxylated sugar esters were stable against temperature. The study also showed that the chain length, monoester content and head group type of surfactants had a significant influence on the total area of single phase microemulsion region (Fanun 2009). Garti et al. (2001) investigated food grade microemulsions with five-components, water, oil (R(+)-limonene and medium-chain triglycerides), surfactant (Tween 20, 60, 80), ethanol and polyols (propylene glycol and glycerol). The results showed that o/w microemulsions could not be formed from a mixture of three components, water, oil and single surfactant, unless polyols and short-chain alcohols were added.

## 2.6 Two main mechanisms of emulsion stability

### 2.6.1 Electrostatic repulsion

Electrostatic interactions (i.e. electrostatic attraction and repulsion) play an important role in determining the overall stability and physicochemical properties of emulsion droplets stabilized by ionic emulsifiers. When the surface of emulsion droplets is highly electrically charged with either high net positive or negative charges, droplet aggregation can be prevented by an electrostatic repulsive force. On the other hand, if the surface net charge of emulsion droplets is not high, they can be more readily attracted to each other by other mechanisms (e.g. hydrophobic interaction, etc). The electrical charge of emulsion droplets is discussed further later in the section of 2.8.3.

Figure 2.19 shows a schematic diagram illustrating the distribution of positive and negative ions around positively charged emulsion droplets. The thickness of a counter ion layer defines the strength of electrostatic interaction. This is determined by the distance that the counter ions have to move from the electrolyte solution before the charge of the counter ions completely neutralizes the charge on the droplet surface. McClements (2005) described that the strength of droplet interactions is affected by various factors and depends on electrical charges, (1) the magnitude and sign of electrical charge on droplets; (2) the ionic strength and dielectric constant of aqueous solution containing electrolytes which surround the droplets; (3) the electrical characteristics of droplet surfaces, namely, the number of emulsifier molecules adsorbed per unit surface area and the number of ionized groups per emulsifier molecule; and (4) the size of emulsion droplets.

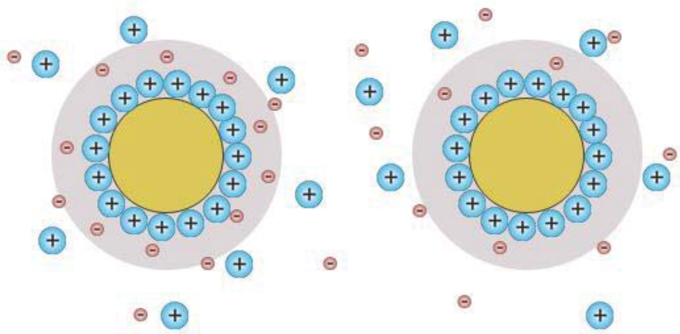


**Figure 2.19** Schematic diagram illustrating the distribution of ions around charged emulsion droplets.
Adapted from McClements (2005).

### 2.6.2 Steric hindrance

The oil droplets in emulsions are coated by an interfacial membrane formed by emulsifier molecules, such as small molecule surfactants, protein and polysaccharide emulsifiers (Figure 2.20). When two droplets approach each other very closely, the interfacial layers start to intermingle or overlap with each other and become compressed. As a result, steric hindrance, which is a repulsive force, can occur between the oil droplets, thus preventing them from aggregation. Sterically-stabilized emulsions are less sensitive to changes in pH and ionic strength than electrostatically-stabilized emulsions (Hunter 1986). The effect of droplet stability driven by steric hindrance can be more pronounced in emulsions stabilized by protein or polysaccharide emulsifiers than small molecule surfactants due to their large molecular dimension and flexible conformational structure than small molecule surfactants (McClements 2005). McClements (2005) pointed out that the strength of steric hindrance depends on (1) the thickness of the adsorbed layer; (2) the size of the emulsion droplets; (3) the precise molecular characteristics of the interfacial membrane, such as packing, flexibility and rheology.

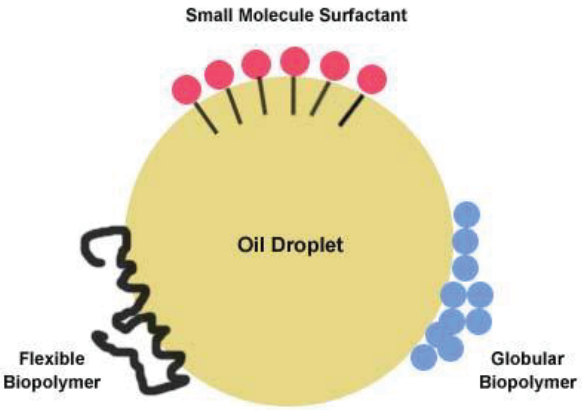


**Figure 2.20** Adsorption of emulsifiers (small molecule surfactants, flexible biopolymers and globular biopolymers) at the surface of oil droplet.

## 2.7 Emulsion stability

Thermodynamics describes whether a given process will occur or not, while kinetics indicates the rate at which it will proceed if it does occur (Atkins 1994). Microemulsions defined as an emulsion containing droplets smaller than 50 nm are thermodynamically stable. On the other hand, nanoemulsions and conventional emulsions are a thermodynamically unstable system and undergo physicochemical changes over time during storage by various types of destabilization mechanisms, including droplet aggregation, flocculation, coalescence, creaming and Ostwald ripening. An example of thermodynamic instability of emulsions is a tendency for oil with a lower density to move to the top of emulsion because the oil phase is lower in density than the water phase (McClements 2005).

Nanoemulsions (< 100 nm) are kinetically more stable than conventional emulsions due to their smaller droplet size and relatively smaller difference in densities between the oil and water phases. However, nanoemulsions are not small sufficiently enough to be thermodynamically stable.

In this literature review, five main mechanisms related to the instability of emulsions as shown schematically in Figure 2.21 are discussed, including gravitational separation, flocculation, coalescence, Ostwald ripening and phase inversion.

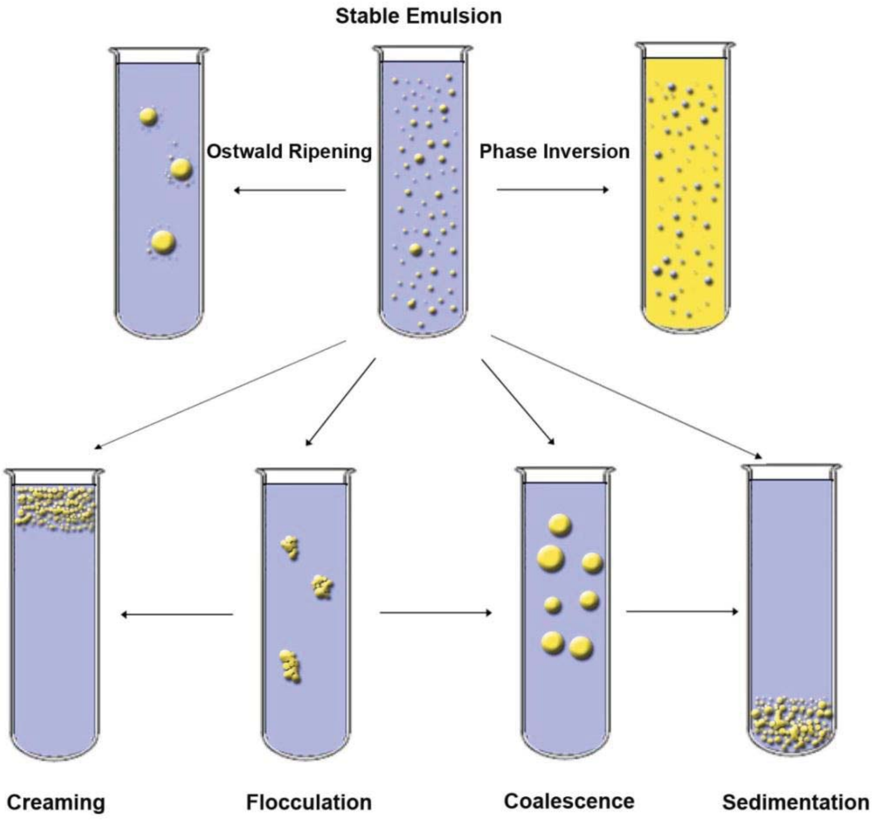


**Figure 2.21** Schematic illustrations of different types of emulsion destabilization. Adapted from McClements and Rao (2011).

### 2.7.1 Gravitational separation

Gravitational separation can be divided into creaming and sedimentation. Creaming is the upward movement of droplets due to their density being lower than the surrounding liquid (Figure 2.21). On the other side, sedimentation is the downward movement of droplets resulting from the droplets being higher in their density than the surrounding liquid (McClements 2005, 2010). Because the densities of most edible oils are lower than that of water, droplets in o/w emulsions tend to form a cream layer, whereas droplets in w/o emulsions tend to sediment to the bottom of emulsions. Conventional emulsions are particularly prone to gravitational separation and droplet aggregation due to their relatively large droplet size (Robins 2000). The rate of gravitation separation is also influenced by many other factors, including types of oil and emulsifier, their ratio, concentrations of oil and water, pH, ionic strength, temperature and viscosity.

The stability of an emulsion to gravitational separation can be estimated using the following equation (4) known as Stokes' law:

$$\boldsymbol{v_{Stokes} = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1}} \qquad \textbf{(4)}$$

where $r$ is the radius of particle, $g$ is the gravitational constant, $\rho_1$and $\rho_2$ refer to the density of continuous and dispersed phases, respectively, $\eta$ is the viscosity of the continuous phase, and $\upsilon$ is the separation velocity and its sign determines whether the droplet moves upward or downward (McClements 2005).

There are a number of ways of retarding gravitational separation by minimizing density difference, reducing droplet size and/or modifying continuous phase rheology (viscosity) (McClements 2005). Minimizing the density difference between the oil and aqueous phases is based on reducing the driving force for gravitational separation. For most natural triacylglycerol-based edible oils, they have all similar densities (e.g. 910 kg $m^{-3}$) being lower than the density of water. Therefore a number of lipid soluble weighting agents, such as ester gum and sucrose acetate isobutyrate, can be added into the oil phase for density matching (McClements 2005; Tan 2004). Modifying continuous phase rheology can be achieved by adding some thickening agents to increase the viscosity of the liquid surrounding droplets, thereby decreasing the velocity of droplet movements (McClements 2005).

### 2.7.2 Flocculation

Flocculation is a process where two or more droplets come together to form aggregations in emulsions, but the droplets retain their individual integrity (Figure 2.21) (McClements 2005). Flocculation occurs when the kinetic energy released during droplet collision brings the droplets over the repulsive force barrier into a region where attractive forces operate, causing the droplets to attach to each other (Bergenstahl & Claesson 1990). Flocculation increases the effective droplet size and accelerates the rates of gravitational separation, which reduces the stability of emulsions (Luyten, Jonkman, Kloek, & van Vliet 1993).

At low or intermediate droplet concentrations, flocculation tends to increase the creaming velocity due to a larger effective size, whereas in concentrated emulsions, flocculation retards creaming due to its three-dimensional network structure, which could prevent the individual droplets from moving (McClements 2005). It also causes an increase in the emulsion viscosity and may even lead to form a gel (Demetriades, Coupland, & McClements 1997).

Evans and Wennerstrom (1994) indicated that the flocculation rate depends on the frequency of droplet collisions and the fraction of collisions that lead to aggregation, according to the equation (5) as follows**:**

$$\boldsymbol{\frac{dn_T}{dt} = -\frac{1}{2} FE} \qquad \textbf{(5)}$$

where $dn_T/dt$ is the flocculation rate, $n_T$ is the total number of particles per unit volume, $t$ is the time, $F$ is the collision frequency, and $E$ is the collision efficiency. Collision frequency may be influenced by the movement of droplets due to Brownian motion, gravitational separation or applied mechanical forces (McClements 2005). Usually, the flocculation rate can be controlled by decreasing the density difference between the droplet and surrounding liquid or increasing the viscosity of the continuous phase (McClements 2005).

### 2.7.3 Coalescence

Coalescence is a process where two or more liquid droplets merge together to form a single larger droplet (Figure 2.21) (McClements 2005). The term “droplet flocculation” described in the above can lead to coalescence. Coalescence can only occur when the droplets are close to each other and the interfacial membranes are disrupted. It also causes the increase of separation velocity of droplets due to an increase in their sizes.

In o/w emulsions, coalescence eventually leads to creaming and/or the formation of a layer of free oil on the top, which is also called oiling off (McClements 2005). In many food emulsions, the presence of impurities, for example, gas bubbles, solid particles and crystals, can cause droplet coalescence (van Aken 2004). Moreover, physical forces, such as freezing, drying and centrifuging, can cause the stretch and tear of the interfacial membrane, which result in an exposure of emulsifier-depleted surface area of droplets (Fribcrg, Larsson, & Sjoblom 2004). Emulsion droplets become deformed as they approach each other, which is an important factor of droplet coalescence in emulsions containing relatively large droplets (Ivanov, Danov, & Kralchevsky 1999; Petsev 1998; Walstra 2003).

The method of controlling coalescence is highly dependent on the type of emulsifier used to stabilize the emulsion system due to the physicochemical properties of the interfacial membranes, and the environmental conditions because of their influence on the colloidal and hydrodynamic interactions (McClements 2005). The stability of droplets against coalescence can be changed by emulsifier type, pH, ionic strength or temperature. For example, protein or polysaccharide emulsifiers have been found to be highly resistant to coalescence. Strong repulsive forces between droplets provided by a combination of electrostatic and steric interaction form membranes to resist the rupture. Small molecule surfactant keeps droplets apart by a large steric overlap and hydration repulsion (Euston, Finnigan, & Hirst 2001; McClements 2005; van der Ven, Gruppen, de Bont, & Voragen 2001).

### 2.7.4 Ostwald ripening

Ostwald ripening is a process whereby large droplets grow at the expense of smaller ones because of the higher Laplace pressure inside small emulsion droplets (Figure 2.21) (Binks et al. 1998; Kabalnov & Shchukin 1992; McClements 2005; Weiss et al. 2000). It occurs because the solubility of substance within a spherical droplet in the continuous phase increases as the droplet size decreases. Therefore, the substance moves from the smaller droplets to the larger droplets due to the concentration gradient, leading to the shrinkage of smaller droplets and the growth of larger droplets (McClements 2005). It should be noted that when o/w emulsions contain more water soluble lipids, such as flavour oils, or when the water phase contains alcohol, such as cream liqueurs, Ostwald ripening can be significant in emulsions. This is because the velocity of Ostwald ripening mainly depends on the solubility of dispersed oil in the aqueous continuous phase (Binks et al. 1998; McClements 2005).

### 2.7.5 Phase inversion

Phase inversion is a process whereby a system changes from an o/w emulsion to a w/o emulsion or vice versa (McClements 2005), as shown in Figure 2.21. It is a complex process which involves flocculation and coalescence. Phase inversion in food emulsions can be divided into three categories: i) surfactant induced phase inversion, ii) catastrophic phase inversion, and iii) fat crystallization-induced phase inversion (Brooks, Richmon, & Zerfa 1998; McClements 2005).

In emulsions stabilized by small molecule surfactants, surfactant-induced phase inversion occurs when the molecular geometry of surfactant molecules is altered through by changing the temperature, ionic strength, or effective hydrophile-lipophile balance (HLB) number in solution or environmental conditions (Binks 1998; Evans & Wennerstrom 1994; McClements 2005). This kind of phase inversion is usually reversible, and undergoes a transition from an o/w emulsion, to a bicontinuous system, and then to a w/o emulsion, or vice versa (Binks 1998).

Catastrophic phase inversion is a kind of irreversible process, and there is suddenly a dramatic change in the system when the disperse phase volume fraction is gradually increased above a critical level (Binks 1998; Brooks et al. 1998). Fat crystallization-induced phase inversion occurs in an o/w emulsion containing partial crystalline droplets when the liquid droplets are cooled to a temperature (McClements 2005; Walstra 2003). The reason for this phase inversion is that the partial coalescence of the droplets leads to the formation of a continuous fat crystal network, which encapsulates water droplets within it (Dickinson & Stainsby 1982).

Although phase inversion is an essential step for butter and margarine manufacture, it is undesirable in other foods because it has an adverse effect on their appearance, texture, stability, and taste (McClements 2005). According to McClements (2005), phase inversion can be induced through increasing or decreasing the temperature of emulsions. Cooling an o/w emulsion to a temperature at which the oil partly crystallizes and shearing causes fat crystallization-induced phase inversion. On the other hand, heating an o/w emulsion stabilized by a surfactant may cause surfactant-induced phase inversion above the PIT as described earlier.

### 2.7.6 Factors affecting the stability of emulsion

The rate of emulsion destabilization is affected by a number of factors, including composition (e.g. concentration and type of emulsifier, oil, salts, acid, etc) and environmental (e.g. pH, ionic strength, temperature) and processing (e.g. shearing, pressure, temperature, etc) conditions.

#### *2.7.6.1 pH*

The pH of the continuous phase of emulsions influences significantly the stability of emulsions, particularly those stabilized by emulsifiers (anionic, cationic and Zwitterionic) that have ionizable groups, due to its effect on the ionization and surface charges of emulsion droplets. For examples, milk proteins (caseins and whey proteins) except lactoferrin have their isoelectric points (pI) in the range of pH between 4 and 6. At pI, the magnitude of positively charged groups becomes equal to the magnitude of negatively charged groups in proteins, and eventually the electrical net charge (zeta-potential) value of such protein-coated droplet surface at pI becomes zero (McClements, 2005). This mean when the pH of the continuous phase closes to pI, the emulsion is susceptible to extensive flocculation because the electrostatic repulsion between the droplets are not strong enough to overcome the predominant attractive interactions, such as van der Waals, hydrophobic and depletion forces (McClements, 2004; McClements, 2005).

At pH below pI, most proteins have the positive zeta-potential as amino groups become positively charged because of them being more protonated ($NH_3^+$) while carboxylic groups become neutral (COOH). On the other hand, at pH above pI, the magnitude of positive charge on the droplets decreases as the pH increases. This is because carboxyl groups become deprotonated, thus more negatively charged ($COO^-$) and amino groups become neutral ($NH_2$). Therefore, the emulsions stabilized by ionic emulsifiers, such as proteins, maintain better stability and resistance to flocculation or coalescence when the pH values are away from the pI of proteins, because the droplets surfaces are highly charged (Hunt & Dalgleish, 1994; Demetriades & McClements, 1998). Lee et al. (2011) investigated the effects of pH on the stability of conventional emulsions and nanoemulsions stabilized by whey protein isolates. They showed that both types of emulsions destabilized due to droplet aggregations when the pH values were around the isoelectric point of the adsorbed proteins, whereas at higher and lower pH values emulsions remained stable.

#### *2.7.6.2 Ionic strength*

Ionic strength has a significant impact on the stability of emulsions in various ways, primarily depending upon the valency, charge, concentration and nature of ions available in the continuous phase (McClements, 2005). As the ionic strength increases, the electrostatic repulsion between droplets is progressively screened, until eventually it is no longer strong enough to prevent flocculation (McClements, 1998). A number of studies have shown that the presence of monovalent ions (e.g. $Na^+$ and $K^+$) or multivalent ions (e.g. $Ca^{++}$) influence the stability of emulsions, especially when they are stabilized by ionic emulsifiers (e.g. proteins). It has also been reported that multivalent ions have more significant impact on the instability of emulsions than monovalent ions. Multivalent ions cause flocculation by both bridging flocculation and electrostatic screening effects, whereas monovalent ions cause flocculation mainly by the screening of electrostatic interactions (Keowmaneechai & McClements, 2002).

## 2.8 Characterization of emulsion properties

### 2.8.1 Light scattering

Light is scattered by particles in dispersions (e.g. emulsions). The degree of light scattering is dependent on the size of particles in dispersions. In theory, when the emulsion particle size is larger than the wavelength of light, the light is scattered by particles in all directions through reflection and refraction, the emulsion appears white in colour and is opaque in its optical properties. On the other hand, when the size of particles is smaller than the wavelength of light, the incident light scatters weakly.

When the size of emulsion droplets is small in diameter less than ¼ λ where λ is the wavelength of light, the light can pass through the dispersion. The emulsion appears translucent or opalescent due to the reduced light scattering effect (Prince 1977). Accordingly, the optical properties and visual appearance of emulsions, in terms of transparency, translucency and opacity, may be used to identify if emulsions are nanoemulsion, microemulsion or conventional emulsion by measuring the turbidity of emulsions (McClements & Dungan 1995; Prince 1977).

### 2.8.2 Particle size and size distribution

The average particle size and particle size distribution of emulsions are measured to evaluate the formation, properties and stability of emulsions using various techniques, including light scattering and microscopy techniques (e.g. optical microscopy, confocal laser scanning microscopy and electron microscopy). The most direct method is a light microscopy when the size of emulsion droplets to exam is larger than > 3 μm (Flint 1994) and electron microscopy can be applied to smaller droplets (Robins 2000).

However, microscopy technique may not be as accurate as light scattering techniques and can be a qualitative measurement. Particles in an emulsion are not uniform in their size and are made up of a range of different sizes and the shape and pattern of particle size distribution also differ between emulsions (Gregory 2005; Paul Van der, Koen, & Hans 2004). For example, the particle size distributions of nanoemulsions usually have single or multiple peaks which are narrow or broad, but microemulsions tend to have a single narrow peak (McClements 2012).

Light scattering techniques used to measure the size of particles can be divided into static light scattering (SLS) and dynamic light scattering (DLS) techniques. These two techniques are the most widely used in measuring the particle size of emulsions. The SLS technique involves a measurement of the angular dependence of the scattered light intensity when a laser beam passes through a dilute emulsion (McClements & Dungan 1995). In contrast, the DLS measures the size of particles based on the frequency shift of light scattered by particles in solution during their random movement due to Brownian motion which depends on the velocity of particles moving in solution. This means the size of a particle is related to its velocity via the diffusion coefficient and calculated by measuring the frequency shift (McClements & Dungan 1995). The range of particle size measured by SLS technique is between 0.1 and 1000 µm while DLS measures a much smaller range of particles between 3 and 5000 nm (McClements & Dungan 1995).

The particle size of emulsions containing particles smaller than 100 nm in diameter, such as nano- and microemulsions, should be analysed by DLS technique. The particle size results analysed by DLS are expressed in various ways, such as Z-average, volume mean, number mean and intensity mean. The Z-average, also known as the cumulants mean, is the primary and most stable parameter in DLS, and this mean is defined as the "harmonic intensity averaged particle diameter" by ISO 22412 (Malvern 2011). According to Horiba (2013), the Z-average is calculated by the following equation (6):

$$\boldsymbol{D_z} = \frac{\sum \boldsymbol{S_i}}{\sum\left(\boldsymbol{S_i}/\boldsymbol{D_i}\right)} \qquad \textbf{(6)}$$

where $D_z$ is the Z-average, $S_i$ is the scattered intensity from particle $i$ and $D_i$ is the diameter of particle $i$. Because this mean is calculated from the intensity weighted distribution, the Z-average is the intensity weighted harmonic mean. Due to Rayleigh scattering, $S_i \sim D_i^6$, the Z-average can be approximated as:

$$\boldsymbol{D_z} \approx \frac{\sum \boldsymbol{D_i}^6}{\sum \boldsymbol{D_i}^5} \qquad \textbf{(7)}$$

The calculation of the Z-average is mathematically stable, and insensitive to noise. However, it should be noted that the Z-average is a hydrodynamic parameter and is only applicable to particles in dispersion or molecules in solution (Malvern 2011).

### 2.8.3 Zeta potential

The droplets in emulsions often have an electrical charge, which plays an important role in their functional performance and stability (McClements 2010). The electrical charge of emulsion droplets (particles) is expressed as "zeta potential" which is the electrical potential at the shear plane defined as the distance away from the droplet surface below which the counter-ions remain strongly attached to the droplets when they move in an electrical field (Figure 2.22). Hence, the value of zeta potential is useful for understanding and predicting the stability of particles in terms of interactions between particles in a system.

Practically, zeta potential measured at the shear plane is a better representation of the electrical characteristics of an emulsion droplet than the surface potential because it inherently accounts for the adsorption of any charged counter-ions (Hunter 1986). The electrical characteristics of emulsion droplets can be controlled, depending on the selection of particular emulsifier types (McClements 2005). In order for the droplets of emulsions to remain stable against droplet aggregation by electrostatic repulsion, the values of zeta potential are normally required to be a minimum of ± 30 mV (Müller, Jacobs, & Kayser 2001).

The zeta potential values can be determined using Zetasizer, which measures the distribution of the electrophoretic mobility of particles with sizes ranging from 3 nm to 10 µm using the laser doppler velocity technique. The analyzer calculates the zeta-potential from the measured velocity using the Smoluchowski equation (Cheong & Tan 2010; Chu et al. 2007, 2008; Yin et al. 2009).

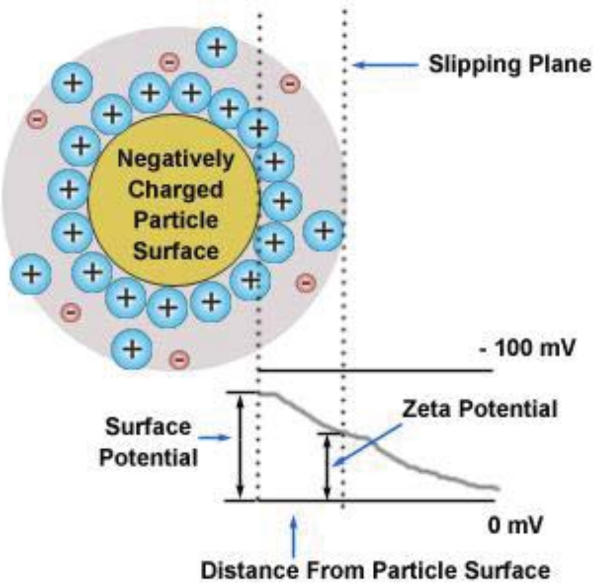


**Figure 2.22** A schematic diagram of the electrical charge (zeta potential) of droplets in emulsion measured in mV at the slipping plane.
Adapted from Malvern (2013).

### 2.8.4 Birefringence

Birefringence is defined as the optical property of a transparent, molecularly ordered material having the double refraction (Murphy, Spring, Fellers, & Davidson 2013). When very small aggregates are not isotropic, thus having different physical properties, i.e., one dimension is longer than the other, as in rod-like or disc-like aggregates, dispersions of them cause a ray of incident light to become doubly refracted (i.e. double refraction) when they are stirred or allowed to stream (Prince 1977). Thus, if a drop or two of an anisotropic dispersion is placed between two microscope slides and squeezed to produce flow, upon examination between crossed nicols, the illuminated field will light up into beautifully coloured patterns. This is due to the scattering and repolarization of the polarized light (Prince 1977). This phenomenon is used to explain what happens when a system inverts through a viscoelastic gel stage (liquid crystal & lamellar phase) from a w/o to an o/w type (or vice versa) during the microemulsion preparation by using low energy methods (e.g. phase inversion/transition). Thus, as a general rule, during the development of a clear, transparent microemulsion by phase inversion methods, the viscoelastic gel stage can be formed and will appear transparent or opalescent (Prince 1977) which are easy to differentiate visually from the form of microemulsions because both systems are clear and has no phase separation.

The presence of birefringence from some systems during the preparation of microemulsions thus needs to be monitored to identify if they are liquid crystals or microemulsions. This means that the appearance of birefringence when examined using a polarised light microscope indicates that the dispersed phase of a ternary (oil-water-surfactant) system is no longer in a spherical form and that a liquid crystalline phase is present (Prince 1977). Polarised light microscopy is a simple technique to use, therefore, it becomes essential to confirm findings based on visual appearance between various anisotropic liquid crystalline systems and isotropic microemulsion systems. However, it is worth noting that the cubic mesophase is the only type of liquid crystalline systems, which is isotropic, and would not display birefringence (Alany, Wen, & Gad 2010).

### 2.8.5 Electrical conductivity

Electrical conductivity measurements can provide valuable information on the continuity of the aqueous phase, and they have been used to study the structure and the phase behaviour of microemulsion systems (Alany et al. 2010; Baptista & Tran 1997;

Kahlweit et al. 1987). The ionic conductance in an o/w microemulsion is like a normal aqueous medium, whereas it is very low in a w/o microemulsion, and significantly high in a bicontinuous condition (Moulik & Paul 1998). In several w/o microemulsion systems, a small change in water concentration leads to a significant change in the electrical conductivity, which has been attributed to percolation (Alany et al. 2010; Alexandridis, Holzwarth, & Hatton 1995; Baptista & Tran 1997; Lagues & Sauterey 1980). Percolation is defined as the phenomenon that a conducting microheterogeneous dispersion in a very weakly conducting or non-conducting medium may show a rapid rise in electrical conductance above a threshold concentration (Alexandridis et al. 1995; Kirkpatrick 1973).

There are some debates on the mechanisms of the percolation in the literature. Grest, Webman, Safran, and Bug (1986) suggested that ion hopping between micelles may be responsible for this phenomenon. Fast material and water exchange in dispersed systems when micelles coalesce has also been suggested as the cause for percolation (Baptista & Tran 1997; Goffredi, Liveri, & Vassallo 1993; Jada, Lang, & Zana 1990). Some authors recognised that the formation of bicontinuous structures in the solution may explain the percolation phenomenon due to the formation of random and momentary continuous water channels (Alany et al. 2010; Baptista & Tran 1997; Prince 1977; Scriven 1976). Because of the non-conducting nature of the continuous phase of w/o emulsion system, the electrical conductivity remains low, but as the fraction of water increases and exceeds the percolation threshold, the conductive droplets begin to contact each other and form clusters. Consequently, an efficient transfer of charge carriers between the dispersed droplets leads to an explosion of conductance from an almost zero value to much higher levels (Alany et al. 2010).

### 2.8.6 Viscosity

Measurements of viscosity play a significant important role among the fundamental physicochemical studies on colloidal dispersions. It has been reported that emulsions may be stabilized by a multilayer arrangement, which can modify van der Waals interactions between particles, increase steric repulsions and interfacial viscosity, retard destabilization processes, and provide long-term stability to the emulsions (Vilasau et al. 2011). At the same oil content, the viscosity of microemulsions is much larger than that of conventional emulsions due to the larger interfacial layer surrounding very small oil droplets (Mason et al. 2006; Tadros et al. 2004). The viscosity of microemulsion systems is also sensitive to structural changes (Alany et al. 2010). During the preparation of microemulsions, when the spherical aggregates begin to transform to cylindrical or lamellar ones, the new forms tend to obstruct the flow of aggregates past one another in the dispersion medium, thus increasing the viscosity (Prince 1977).

Some measurements confirm that viscosity increases in the vicinity of the percolation transition state of phase inversion systems due to droplet clustering, droplet shape changes, and droplet ionization (Berg, Moldover, & Huang 1987). Holmberg, Hansson, Piculell, and Linse (1999) reported that when the surfactant concentration in ternary systems for the formation of microemulsions increased gradually, the viscosity increased to a maximum, and then decreased. Falco, Walker, and Shah (1974) investigated the effect of phase volume ratio and phase inversion on the viscosity of microemulsions and liquid crystals. They found that the phase inversion region showed a remarkable increase in viscosity, and the dispersion had extremely high viscosity when the water to oil ratio was between 2.0 and 3.5. Moreover, they proposed that the change of phase inversion was from water spheres, to water cylinders, then to water lamellae and continuous water. According to Moulik and Paul (1998), the observation of spherical aggregates consisting anisotropic particles was in good agreement with the theoretical predictions for the viscosity of systems composed of spherical particles. Microemulsion systems are usually low in their viscosity and exhibit Newtonian flow behaviour (Alany et al. 2010) compared to bicontinuous phase which has non-Newtonian flow behaviour and plasticity (Berg et al. 1987; Moulik & Paul 1998).

### 2.8.7 Turbidity

Turbidity is the optical properties of dispersions and its measurement in nano- and microemulsions is used to study the degree of transparency in relation to the particle size of dispersions. The measurements of turbidity of emulsions are rapid and simple to carry out and require relatively small quantities of sample, and it can be measured using ultraviolet-visible (UV-vis) spectrophotometers, which are widely available in many research laboratories (Chantrapornchai, Clydesdale, & McClements 1998; Lee et al. 2010; McClements & Dungan 1995). The turbidity of emulsion systems depends on the size and concentration of oil droplets (McClements & Dungan 1995). Chantrapornchai et al. (1998) indicated that when the droplet diameter is around 1 μm, a maximum in emulsion turbidity can be observed at a wavelength of 650 nm. They also found that emulsions with the same droplet concentration but different droplets sizes have dramatically different turbidity spectra.

## 2.9 Conclusions

Nano- and microemulsions whose particles are smaller than 100 nm have a number of valuable properties, such as high optical clarity, long-term stability and increased bioavailability. Therefore, they have a high potential to encapsulate, protect and deliver lipophilic bioactive compounds extracted from plant or animal origin.

These kinds of nano- and micro delivery systems could be formed spontaneously when all the ingredients are brought together by using low energy emulsification method due to a negative free energy (Lawrence & Rees 2000). Phase inversion method is widely used to produce microemulsions. However, due to their limitations to the certain types of oils and surfactants, transparent emulsion systems are not widely used for food applications. Very little work has focused on researching the formation, phase behaviour and properties of them using food grade materials. Hence, it is necessary to investigate some optimal conditions for food-grade microemulsion systems by using phase inversion method based on ternary phase diagram.

Use of high pressure homogenization is the most commonly used method in making emulsions. However, it is difficult to generate very fine droplets due to the Laplace pressure (McClements 2005). Some other homogenization methods, such as microfluidization and ultrasonication, may produce ultrafine emulsions. However, due to the low throughput capacity, these methods are usually used in a laboratory batch, thus not suitable for use in the industrial production. In recent years, nanoemulsions containing tiny droplets smaller than 80 nm in diameter have been shown to be able to be produced by a method of high pressure homogenisation combined with solvent evaporation. However, there are some concerns about toxicity and environmental issues associated with organic solvent being used in food application although solvent is removed via evaporation (Lee & McClements 2010). Also, anther technique of making nano- or microemulsions has been introduced recently by some research groups as alternative methods without using organic solvent (Rao & McClements 2012a; Ziani et al. 2012a, 2012b). This technique produces transparent emulsions without using organic solvent by titrating/diluting conventional emulsions, which were prepared by high pressure homogenisation, into surfactant micelle solution. This method seems to be more suitable for application in food and beverage products, but it has not been well studied yet.

Both phase inversion and emulsion titration methods require small molecule surfactants as emulsifiers to stabilize the transparent emulsion system (McClements & Rao 2011). In this study, food grade non-ionic surfactants, Tween 20, 40, 60 and, 80, were used to study the influence of surfactant types on the formation of transparent emulsions. Types of lipids used in transparent emulsion system formulations can vary but are in general limited to small molecule non-triglyceride lipid compounds prepared by using phase inversion and emulsion titration methods. In this study, different types of oils were selected and used to investigate their effects, including sunflower oil, lemon oil, isopropyl myristate, tributyrin and Imwitor 308. These oils have different chemical composition and physical properties. Lemon oil, isopropyl myristate and tributyrin are low in viscosity at 20°C. Tributyrin has density higher than water. Imwitor 308 is solid at room temperature. Beta-carotene is one of the major carotenoids commonly found in some diets, but its chemical properties are characterised to be poor water solubility, chemical instability and low bioavailability as a functional ingredient (Johnson 2002; Liang, Shoemaker, Yang, Zhong, & Huang 2013). It was incorporated in oil phase as a core lipophilic bioactive model in preparing some nano- and microemulsions, especially by using phase inversion method but it was not analysed for its oxidative stability and degradation.

In summary, the goal of the study was to investigate the formation, stability and properties of transparent emulsion systems by using two different methods, such as phase inversion method and emulsion titration method. Several different types of non-ionic surfactants (Tween series) and oils were used and studied as factors to determine their influence on the formation and stability of nano and microemulsions. Samples of emulsions prepared were visually monitored and also determined or their particle size, zeta potential, turbidity, etc., using instrument analyses. Also, some nano- and microemulsions prepared were characterised for their stability that could be affected by some environmental conditions (pH, salt and thermal treatment).

# Chapter 3
# Formation and Characterisation of Transparent Microemulsions by Phase Inversion Method

## 3.1 Introduction

Microemulsions are defined as a thermodynamically stable system because of their very fine droplet sizes smaller than 50 nm in diameter. Microemulsions offer some potential benefits as delivery systems for bioactive compounds, such as long-term stability, high optical clarity and enhanced solubility and bioavailability (Gutiérrez et al. 2008). Microemulsions are prepared by low energy emulsification methods which use the potential chemical energy of a ternary system consisting of water, surfactant and oil or a pseudo-ternary system if a lipophilic substance is also incorporated into oil phase (Fernandez et al. 2004; Usón et al. 2004). Therefore, the low energy methods are able to form microemulsions spontaneously by gentle mixing of ternary systems and do not require use of high mechanical shear forces (e.g. high pressure) which are commonly used to prepare conventional emulsions.

The low energy methods can be divided into phase inversion temperature (PIT), phase inversion composition (PIC) and emulsion inversion point (EIP) methods. These methods involve phase inversion from w/o microemulsion to o/w microemulsion or vice versa through phase transition into liquid crystal or bicontinuous phase during the microemulsion preparation (Fernandez et al. 2004). In fact, a number of different systems are formed during phase inversion and transition, such as microemulsion, coarse emulsion, micelles, liquid crystals, lamellar phase and gels (Zargar-Shoshtari, Wen, and Alany (2010). These different phase systems may coexist, depending on three component ratios (oil, surfactant and water). Ternary phase diagrams based on three components with different ratios are created to study the phase behaviours of each system through visual observation and measurements of droplet size, zeta potential, electrical conductivity and/or viscosity (Alany et al. 2010; Zargar-Shoshtari et al. 2010). The PIT or PIC methods rely on changes in small molecule surfactant properties, such as their curvature at the interface between oil and water, by changing temperature, pH or ionic strength of a ternary system. In the EIP method the main change involved is the volume fraction of three components (oil, surfactant and water) (McClements & Rao 2011).

Low energy methods have some limitations because a certain type of oils (e.g. small molecule lipids with low viscosity) can only be used to form microemulsions with small molecule surfactants (e.g. mostly nonionic) at high concentration. Most research studies on microemulsions have focused on their use in the pharmaceutical field as drug delivery systems. In many cosmetic applications, microemulsions are also used in skin-care products, because microemulsion formulation can result in a faster uptake into the skin. However, the application of microemulsions in foods is relatively very limited, mainly due to some limiting factors associated with phase inversion methods as already described above. Only a few researchers have attempted to develop food grade microemulsions using limonene, medium-chain triglycerides, short-chain alcohols, polyols and several surfactants (Garti et al. 2001; Spernath, Yaghmur, Aserin, Hoffman, & Garti 2002; Yaghmur, Aserin, & Garti 2002a).

In recent years, there has been growing interest in the development of beverages and soft drinks containing nutraceutical compounds extracted from plants or animals (Ziani et al. 2012b). Many nutraceutical compounds (e.g. carotenoids) conferring health benefits are lipophilic and have poor solubility (Given Jr 2009). As a result, they cannot be readily incorporated into aqueous products owing to their poor solubility in both water and oil as well as their chemical instability (Liang et al. 2013). A suitable delivery system, which can increase their solubility, stability and bioavailability, needs to be developed for their incorporation in aqueous solution (e.g. water and beverages) without affecting clarity and transparency.

The objective of this study was to investigate the phase behaviours of ternary systems of lemon oil, surfactant (Tween 20 or Tween 80) and water. Ternary phase diagrams were constructed to find optimal composition ratios that would result in the formation of microemulsions. The preparation of microemulsions was carried out by diluting the mixtures of oil containing β-carotene and surfactant with water at different ratios.

## 3.2 Materials and Methods

### 3.2.1 Materials

Two different types of non-ionic small molecule surfactants, such as polysorbate 20 (Tween 20) and polysorbate 80 (Tween 80), were purchased from Pharmachem Ltd. (Auckland, New Zealand) and used as emulsifiers. Lemon oil (cold-pressed, FG) and β-carotene (Type 1, ≥ 93% UV) were purchased from Sigma-Aldrich Co. (St. Louis, USA). All other chemicals and reagents used were of analytical grade and were purchased from Fisher Scientific (Pittsburgh, PA), Sigma-Aldrich (St. Louis, USA) or other chemical suppliers. Milli-Q quality water (Milli-Q apparatus, Millipore Corporation, France) was used in preparing all aqueous solutions. In this study, the concentrations of chemicals in solutions and emulsions used were on the basis of weight per weight (% w/w) unless stated otherwise.

### 3.2.2 Preparation and construction of phase diagrams

Ternary phase diagrams were constructed to define the region of microemulsion that determines the appropriate concentration ranges of three components (oil, water and surfactant) resulting in the formation of microemulsion. The preparation of samples (ternary systems) was carried out at room temperature (20°C) through the water dilution method by adding water gradually from 0 to 90% at 10% increments to the mixture of lemon oil and surfactant (Tween 20 or Tween 80) and then mixing gently using vortex. The ratio of the oil and surfactant mixture was varied as 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:50 and 90:10 (w/w), which are denoted as Lines 1-9 in the phase diagram as shown in Figure 3.1. In this study, 0.05 wt% β-carotene was added, as a lipophilic bioactive model core, into lemon oil, which was lower than its maximum solubility (0.16 wt%) in lemon oil that was pre-determined through a preliminary study.

After equilibrium by storing the prepared ternary systems overnight at 20°C, the samples were first analysed by visual observation. All samples, which exhibited a single phase (i.e. monophasic) with no phase separation and optical transparency, were further analysed to identify and determine their properties: types of microemulsion (o/w or w/o), presence of bicontinuous lamellar, particle size and electrical charge.

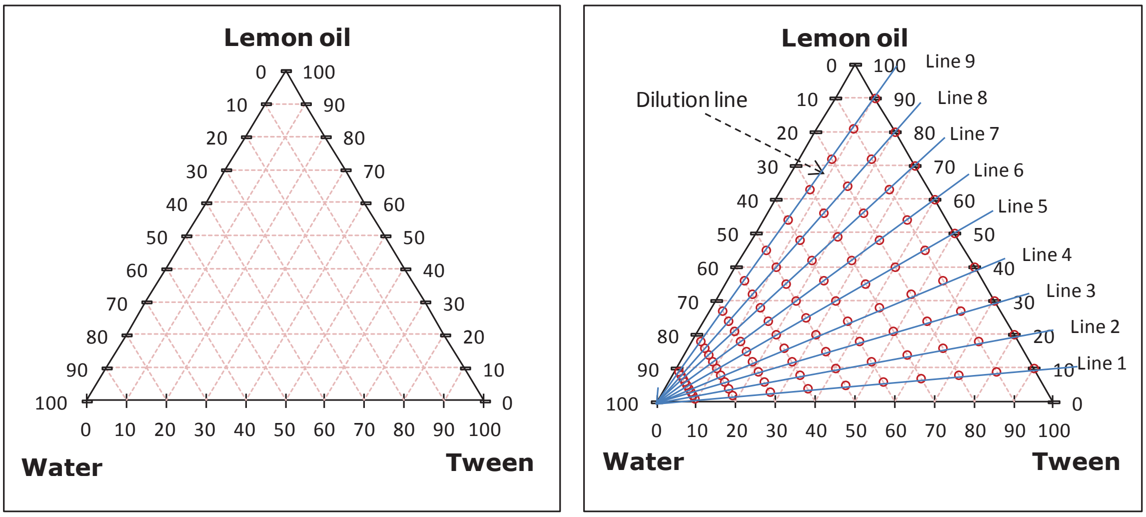


**Figure 3.1** Ternary phase diagram composed of lemon oil, surfactant (Tween 20 or 80) and water.

## 3.2.3 Characterisation of samples

### *3.2.3.1 Type of microemulsions*

The type of microemulsion (o/w or w/o) was identified by a dye solubility test using an oil-soluble dye (Sudan Red) and a water-soluble dye (Methylene Blue). Two drops of Sudan Red or Methylene Blue were dropped into 2 ml microemulsion samples. If an oil-soluble red dye spreads faster than a water-soluble blue dye, it is w/o microemulsion. On the contrary, if a water soluble dye diffuses faster, it is o/w microemulsion (Xu et al. 2010).

### *3.2.3.2 Droplet size and size distribution*

The mean droplet size and size distribution of emulsions were determined by a dynamic light scattering technique using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK), which had a measuring range of 0.6 nm – 6 µm (Yin et al. 2009), at a fixed detector angle of 173. Refractive indices of lemon oil (1.476) and water (1.33) were used to determine the particle sizes of emulsions. The size measurements of all samples were carried out in triplicate. The mean particle size of emulsions is reported as volume mean diameter.

### *3.2.3.3 Polarized light microscopy*

Plane polarized light microscopy was used to identify the type of mesophase (e.g. liquid crystal) formed in some of the prepared samples by the presence of birefringence. A drop of sample was placed between a cover slip and a glass slide and then observed at 25°C under cross-polarized light (Leica DMR, GmbH, Germany). Isotropic materials such as microemulsion, in contrast to anisotropic liquid crystals, do not interfere with the polarized light and the field of view remains dark because the analyzer absorbs light passing through the polarizer (Mrunali, Rashmin, Jolly, Kashyap, & Ajay 2010).

### *3.2.3.4 Electrical conductivity measurements*

Electrical conductivity measurements were conducted at 25°C on samples which were a single phase system using Waterproof Conductivity ECTester (Eutech Instruments, USA). The mixtures of surfactant and oil at different ratios were gradually titrated with distilled water by adding 0.2 ml water at a time under magnetically stirring until a two-phase system formed. The samples were equilibrated for at least 1 min before a reading was taken. All measurements were carried out at least in duplicate.

### *3.2.3.5 Viscosity measurements*

The dynamic viscosities of microemulsions, which were formed along dilution line 1 of the ternary phase diagram from the lemon oil-Tween 80-water ternary system, were measured by using AR-550 Rheometer (TA Instruments, New Castle, Delaware, USA) equipped with a cone (40 mm diameter with an angle of 2°) and plate geometry. The temperature was maintained at 25 ± 0.5 °C throughout the experiment. Steady shear tests were performed over a shear rate range of 1-100 $s^{-1}$. The viscosity values were recorded at 10 $s^{-1}$ shear rate. Samples were analysed in duplicate.

## 3.3 Results and Discussion

### 3.3.1 Phase diagrams

A total of 110 samples formulated with different concentrations of lemon oil, surfactant and water were prepared by mixing them in glass vials. In this study, two types of nonionic small molecule surfactants (Tween 20 and Tween 80) were used to investigate their influence on the phase behaviour of a three component system (surfactant/oil/water) and identify the optimum area of ternary phase diagrams corresponding to the formation of microemulsions. After one day of sample preparation, the samples were visually inspected for optical properties and phase separation. Samples, which exhibited transparency and monophasic with no phase separation, creaming or gelation, were further analysed for their properties (i.e. particle size, electrical conductivity and viscosity) to define the phase behaviour and construct the ternary phase diagrams.

Figure 3.2 shows the phase diagrams constructed from the two different systems with different types of surfactants (Tween 20 and Tween 80). The results showed that the area of regions representing o/w and w/o microemulsions between the two phase diagrams had some differences. This means the compositional ratios and ranges that allowed the formation of microemulsions differed between the two systems made from Tween 20 and Tween 80. Also, the results showed that the regions in the phase diagrams represented by liquid crystals, creaming, coarse emulsion, nanoemulsion and bi- or multiphase systems were different between the two systems (Tween 20 and Tween 80).

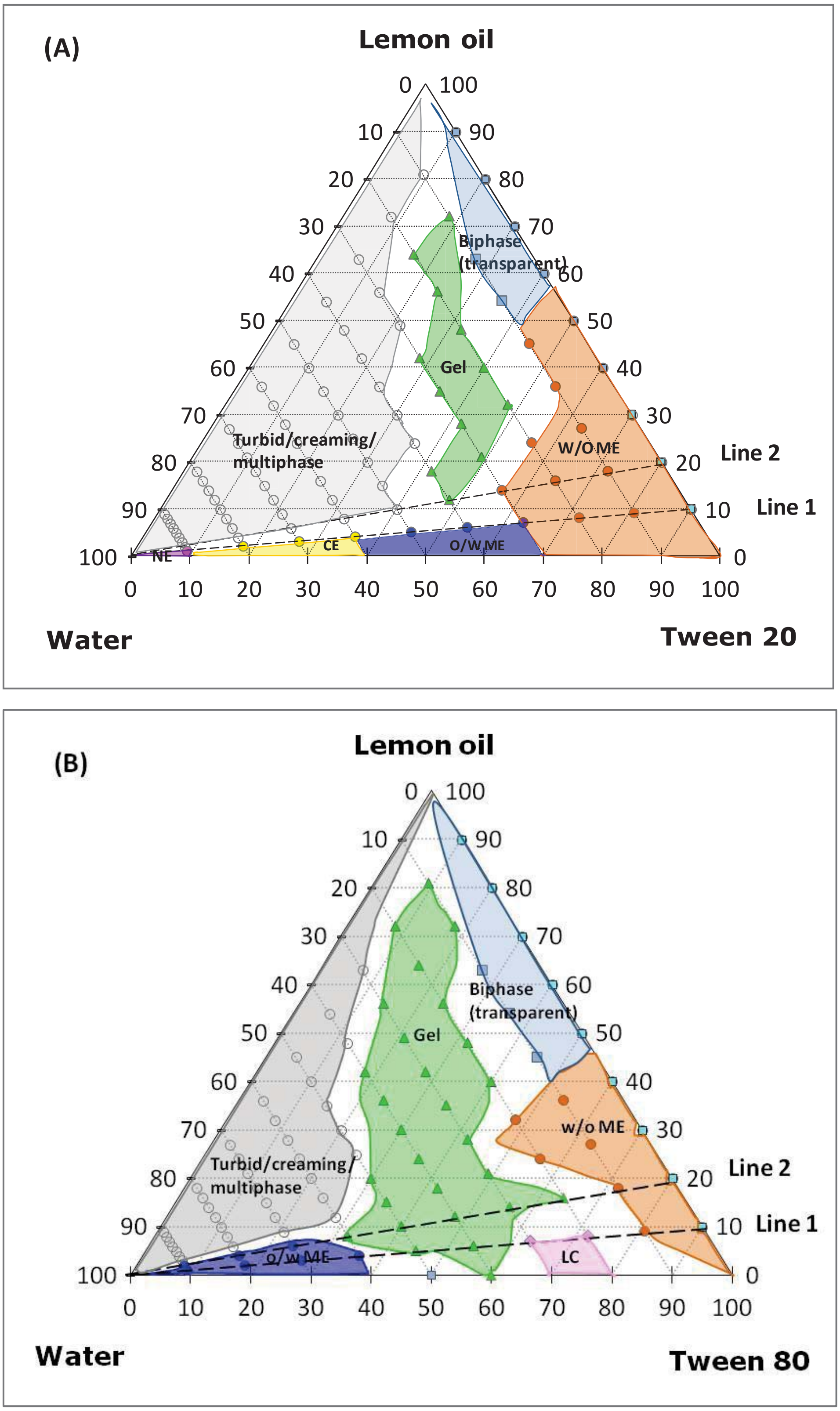


**Figure 3.2** Phase diagrams created for ternary systems formulated with different levels of lemon oil, water and Tween 20 (A) or Tween 80 (B). Abbreviations: ME represents microemulsion, CE conventional emulsion, NE nanoemulsion and LC liquid crystals.

As described earlier, the ternary phase diagrams were constructed based on the results of ternary systems prepared by a two-step process in which lemon oil and surfactant (Tween 20 or Tween 80) were mixed first at different ratios and then diluted with water. The results shown in Figure 3.2 indicated that the formation of o/w microemulsion only occurred at a small range of composition, compared to w/o microemulsion, which was on or near to the dilution Line 1 and Line 2 in the phase diagrams. For the Tween 20 system, o/w microemulsion was formed when the concentration of three components was made up of oil <10%, surfactant 0-70%, and water 40-80%. For the Tween 80 system, o/w microemulsion was formed at oil <10%, surfactant <40%, and water >60%. In contrast, w/o microemulsion was formed at a relatively larger range of composition in both systems as indicated by a much larger region in the phase diagrams around the dilution Lines 1-5 where the concentration of oil, surfactant and water was <50%, >50% and <30% , respectively.

Overall, the results imply that when the mixture of lemon oil and Tween 20 with a low ratio of lemon oil (<50%) was diluted with water, the system started to form a w/o microemulsion spontaneously but as the dilution progressed more than 30%, it changed to an o/w microemulsion. In case of Tween 80, the o/w microemulsion started to form when the oil and surfactant mixture was highly diluted (>60%) through the transition of the w/o microemulsion to liquid crystals resulting from a bicontinuous structure or gelation. The latter was observed to form in some samples from the Tween 20 system when it was not sufficiently diluted. The presence of liquid crystals was detected through the observation of birefringence under a polarised light microscope but it was not observed from the samples prepared for the Tween 20 system, probably due to the transition occurring sharply at a narrow range of composition. The observation of birefringence using the polarised light microscope is further discussed later.

Figure 3.3 illustrates the mechanisms typically involved in the formation of o/w and w/o microemulsions during the emulsification process by low energy methods. Initially, when a small amount of water is added into the mixture of oil and non-ionic surfactant, w/o microemulsion is formed. As the dilution progresses, the volume fraction of water increases and therefore water droplets become merged. Further dilution with water causes the formation of bicontinuous structures, also known as lamellar phase, followed by its conversion into an o/w microemulsion when the emulsion inversion point is reached (Fernandez et al. 2004; Forgiarini, Esquena, González, & Solans 2001). According to Morales, Gutiérrez, García-Celma, and Solans (2003), the formation of microemulsion depends on the solubilisation of oil used. Microemulsion is easier to form in a high surfactant concentration due to a complete solubilisation of oil near the phase inversion point. However, an incomplete oil solubilisation leads to larger droplets, which results in a coarse emulsion and even multiphase systems at low or medium surfactant concentration.

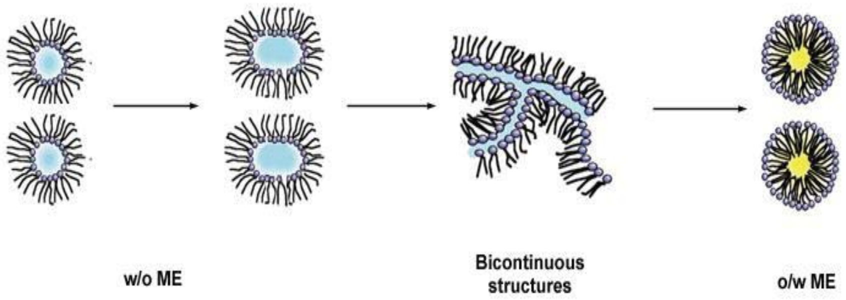


**Figure 3.3** Schematic representation of phase inversion from w/o to o/w microemulsion through bicontinuous structure formation (e.g. liquid crystalline lamellar phase). Adapted from Fernandez et al. (2004).

Another noticeable difference between the two systems was that using Tween 20 as surfactant resulted in the formation of translucent nanoemulsion and opaque conventional emulsion, defined in this study according to their particle size range. These emulsions containing larger droplets than transparent microemulsions were also stable with no occurrence of creaming or phase separation unlike did some turbid coarse emulsions.

It should also be noted that in the central part of the ternary phase diagrams for both systems, there was a large region corresponding to a single phase gel-like structure with mostly opaque appearance and high viscosity. The formation of a gel occurred much less extensively in the ternary system containing Tween 20 compared to that of Tween 80. This is probably attributed to the higher HLB value and lower viscosity of Tween 20. Tween 20 has a monolaurate group ($C_{12:0}$) in its lipophilic tail while Tween 80 has a monooleate group ($C_{18:1}$) with a double bond. The arrangement of polyoxyethylene groups in Tween 80 is relatively complex with stereochemistry as compared to that in Tween 20. Hence, the viscosity of Tween 80 is higher than that of Tween 20 (Bajaj, Rao, Khole, & Munjapara 2013; Garti 2000, 2002; Sirshendu & Sourav 2012; Symon & William 2001; Ziani et al. 2012a).

## 3.3.2 Analysis of ternary systems

### *3.3.2.1 Visual observation*

Figures 3.4 and 3.5 are the visual appearance of ten samples derived from each of dilution lines 1, 2 and 3 in the ternary phase diagrams for the two systems, lemon oil/Tween 20/water and lemon oil/Tween 80/water, respectively. The samples along the dilution lines 1, 2 and 3 were constant for their ratio of oil to surfactant as 1:9, 2:8 and 3:7, respectively, prior to the water dilution, and had a gradual increase in dilution with water by 10% from 0 to 90% where w/o and o/w microemulsions were predominantly formed. The chemical composition and properties of samples from dilution lines 1, 2 and 3, including a few samples from dilution lines 4 and 5, are shown in Table 3.1. The visual images and composition of samples for the other dilution lines of ternary phase diagrams are shown in Appendices 1-62 along with their properties based on visual observation.

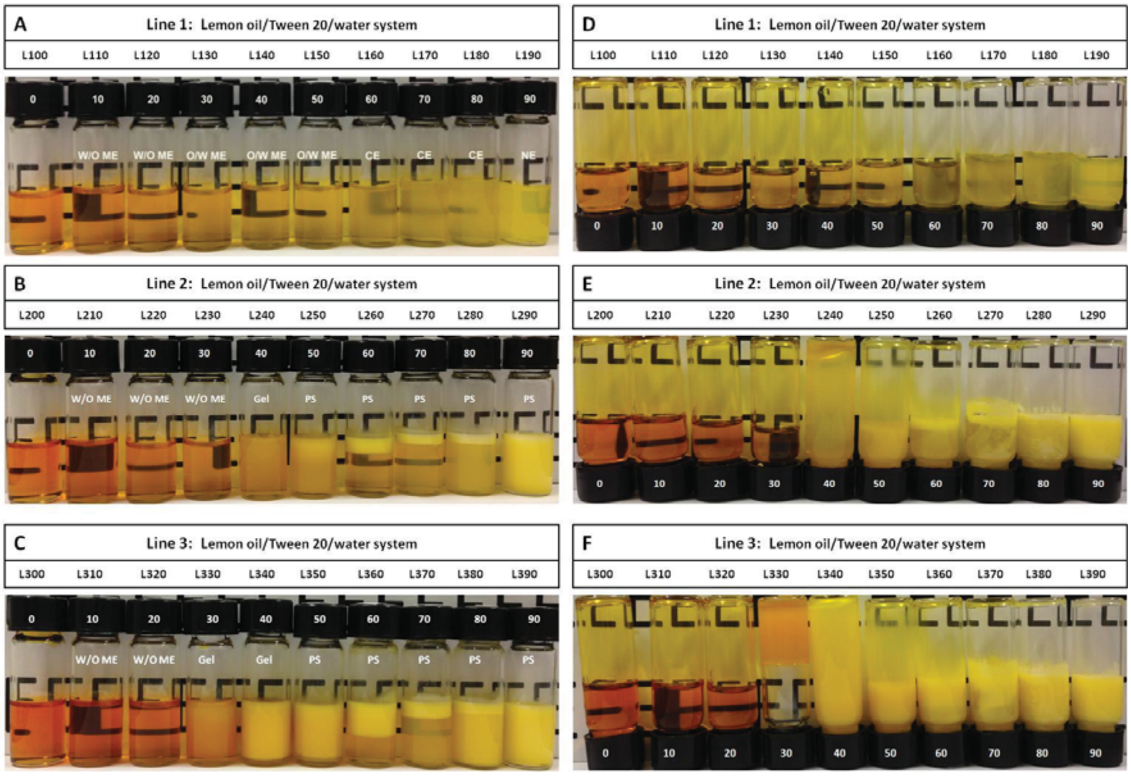


**Figure 3.4** Visual appearance of samples derived from dilution lines 1, 2 and 3 (1:9, 2:8 and 3:7 ratio of oil to surfactant) of the phase diagram for lemon oil/Tween 20/water ternary systems. Numbers from 0 to 90 labelled on vial caps indicate water weight fraction (% w/w); ME represents microemulsion; CE coarse emulsion; NE nanoemulsion and PS phase separation including creaming. The pictures shown in D, E and F indicate some differences in the rheological properties (e.g. viscosity and gelation) between samples. A pattern used in the photo background was to show and compare the degree of clarity and opacity between samples.

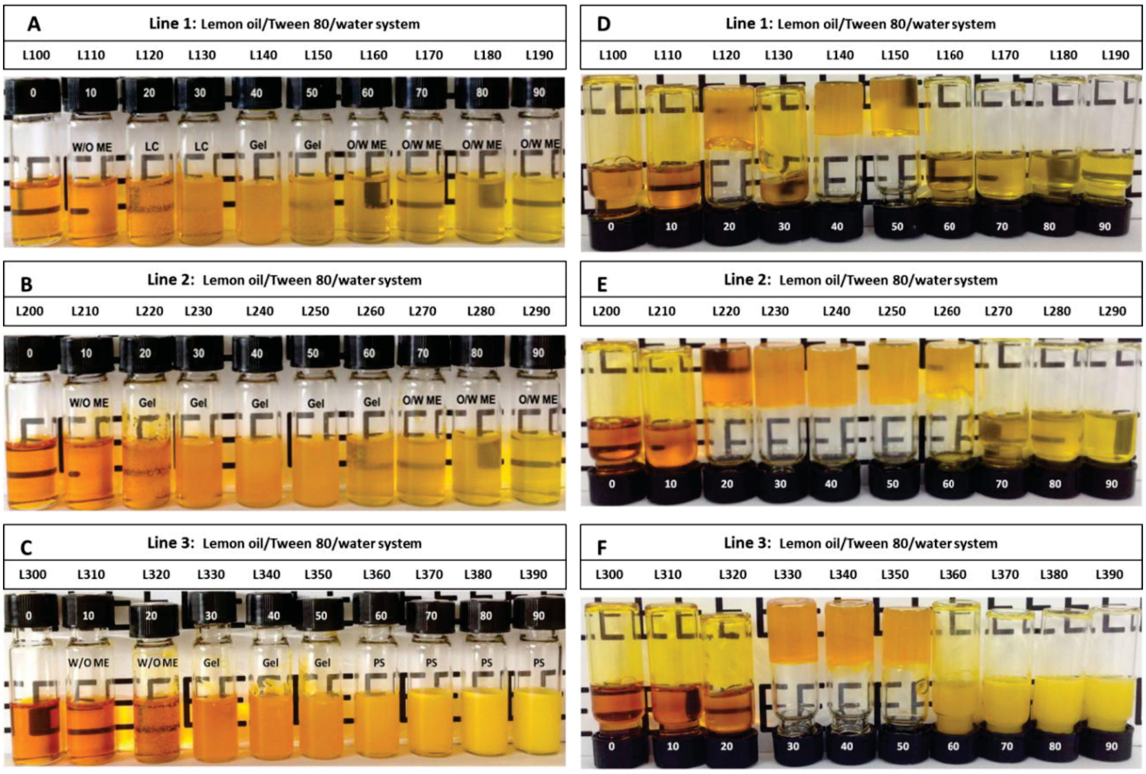


**Figure 3.5** Visual appearance of samples from dilution lines 1, 2 and 3 (1:9, 2:8 and 3:7 ratio of oil to surfactant) of the phase diagram for lemon oil/Tween 80/water ternary systems. Numbers from 0 to 90 labelled on vial caps indicate water weight fraction (% w/w). Abbreviations: ME represents microemulsion, CE coarse emulsion, LC liquid crystals and PS phase separation including creaming. The pictures shown in D, E and F indicate some differences in the rheological properties (e.g., viscosity and gelation) between samples.

**Table 3.1** Composition and types of phase behaviour of ternary systems composed of lemon oil, surfactant (Tween 20 or Tween 80) and water from dilution lines 1, 2 and 3 of ternary phase diagrams. A few samples from dilution lines 4 and 5 that formed w/o microemulsions are also included.

| | Sample | Weight fraction (% w/w) | | | Types of phase system | |
|---|---|---|---|---|---|---|
| | code | Lemon oil | Surfactant | Water | Tween 20 | Tween 80 |
| Line 1 (oil:surfactant=1:9) | L100 | 10 | 90 | 0 | CSP | CSP |
| | L110 | 9 | 81 | 10 | w/o ME | w/o ME |
| | L120 | 8 | 72 | 20 | w/o ME | LC |
| | L130 | 7 | 63 | 30 | o/w ME | LC |
| | L140 | 6 | 54 | 40 | o/w ME | Gel |
| | L150 | 5 | 45 | 50 | o/w ME | Gel |
| | L160 | 4 | 36 | 60 | o/w CE | o/w ME |
| | L170 | 3 | 27 | 70 | o/w CE | o/w ME |
| | L180 | 2 | 18 | 80 | o/w CE | o/w ME |
| | L190 | 1 | 9 | 90 | o/w NE | o/w ME |
| Line 2 (oil:surfactant=2:8) | L200 | 20 | 80 | 0 | CSP | CSP |
| | L210 | 18 | 72 | 10 | w/o ME | w/o ME |
| | L220 | 16 | 64 | 20 | w/o ME | Gel |
| | L230 | 14 | 56 | 30 | w/o ME | Gel |
| | L240 | 12 | 48 | 40 | Gel | Gel |
| | L250 | 10 | 40 | 50 | PS | Gel |
| | L260 | 8 | 32 | 60 | PS | Gel |
| | L270 | 6 | 24 | 70 | PS | o/w ME |
| | L280 | 4 | 16 | 80 | PS | o/w ME |
| | L290 | 2 | 8 | 90 | PS | o/w ME |
| Line 3 (oil:surfactant=3:7) | L300 | 30 | 70 | 0 | CSP | CSP |
| | L310 | 27 | 63 | 10 | w/o ME | w/o ME |
| | L320 | 24 | 56 | 20 | w/o ME | w/o ME |
| | L330 | 21 | 49 | 30 | Gel | Gel |
| | L340 | 18 | 42 | 40 | Gel | Gel |
| | L350 | 15 | 35 | 50 | PS | Gel |
| | L360 | 12 | 28 | 60 | PS | PS |
| | L370 | 9 | 21 | 70 | PS | PS |
| | L380 | 6 | 14 | 80 | PS | PS |
| | L390 | 3 | 7 | 90 | PS | PS |
| L4 | L410 | 36 | 54 | 10 | w/o ME | w/o ME |
| | L420 | 32 | 48 | 20 | - | w/o ME |
| L5 | L510 | 45 | 45 | 10 | w/o ME | - |

Abbreviations: w/o (w/o microemulsion), o/w (o/w microemulsion), CE (coarse emulsion), NE (nanoemulsion) and PS (phase separation including creaming) and CSP (clear single phase)

For the lemon oil/Tween 20/water system, Figure 3.4 shows that microemulsions with optical transparency were produced from the sample data points located on dilution lines 1, 2 and 3 of the ternary phase diagram. At low weight fraction of water (10 and 20%) on Line 1 (Figure 3.4A), the type of microemulsion formed was a w/o microemulsion. When the w/o microemulsion was diluted with water to 30%, it was converted into an o/w microemulsion (L130) as indicated in Table 3.1. As the sample was more diluted to 60% water, the o/w microemulsion was changed to a coarse o/w emulsion (L160, L170 and L180) or an o/w nanoemulsion (L190), depending on the extent of water dilution. In terms of transparency and opacity, these emulsion samples were not completely opaque and had some translucency in their appearance.

For the samples from dilution line 2 in the phase diagram which had a higher ratio of oil to surfactant (2:8), w/o microemulsion was also formed with its dilution with water up to 30% water (Figure 3.4B). However, the phase inversion of microemulsion from w/o to o/w did not occur, regardless of the extent of water dilution applied to the samples from the dilution line 2 (Figure 3.4B). Instead, at the level of 40% water dilution, the sample was found to become a viscous gel-like structure (sample code L240) (Figure 3.4E). As dilution progressed further, the sample was changed into a turbid dispersion that had some phase separation and/or creaming. These samples were collectively labelled as phase separation (PS) in Figures 3.3 and 3.4 and Table 3.1 but some of them were actually coarse emulsions, thus resulting in creaming. Among them, the sample diluted to 90% water (L290) had significantly less creaming compared to the others. For the samples along the dilution line 3 (i.e. 3:7 ratio of oil to surfactant), a similar pattern of phase behaviour was observed, except that the formation of a more solid gel-like structure (L330) occurred at a lower water dilution (<30% water).

For the ternary systems with Tween 80 from dilution lines 1, 2 and 3 (Figure 3.5), the results showed a similar pattern of phase behaviour that at low water fraction, the systems formed a w/o microemulsion. However, when the dilution increased to 20-30% at a 1:9 ratio of oil to surfactant (i.e. dilution line 1) (Figure 3.2B), the system had a change from w/o microemulsion to another system defined as liquid crystals, which was viscous and gel-like structures (L120, L130) as shown in Figure 3.5D. Due to the anisotropic nature of a liquid crystalline phase, this can easily be distinguished by the presence of birefringence under the polarized microscopy (Basheer, Noordin, & Ghareeb 2013). The microscopic appearance of birefringence resulting from the presence of liquid crystals in these samples is shown in Figure 3.6. With the increasing water fraction, the liquid crystals was more likely transited to a gel phase (L140, L150) and then inverted to a transparent o/w microemulsion (L160, L170, L180, L190) when the water fraction added was higher than 60%.

For the samples of dilution line 2 (Figure 3.2), a similar pattern of changing phase behaviour was observed. However, the formation of liquid crystal phase was not found in samples on the dilution line 2 and more samples were observed to form gels at 20-60% of water (Figure 3.5E). The samples from dilution line 3 also exhibited a similar pattern of phase inversion, i.e. transition of a w/o microemulsion into a gel at 30-50% of water. However, at the more dilution up to 60% water or higher, the system did not undergo a phase inversion into an o/w microemulsion. Instead, it was indeed converted into opaque dispersions with creaming to some extent, which can be corresponding to coarse emulsions but denoted as samples classified as phase separation in Figure 3.5C and Table 3.1.

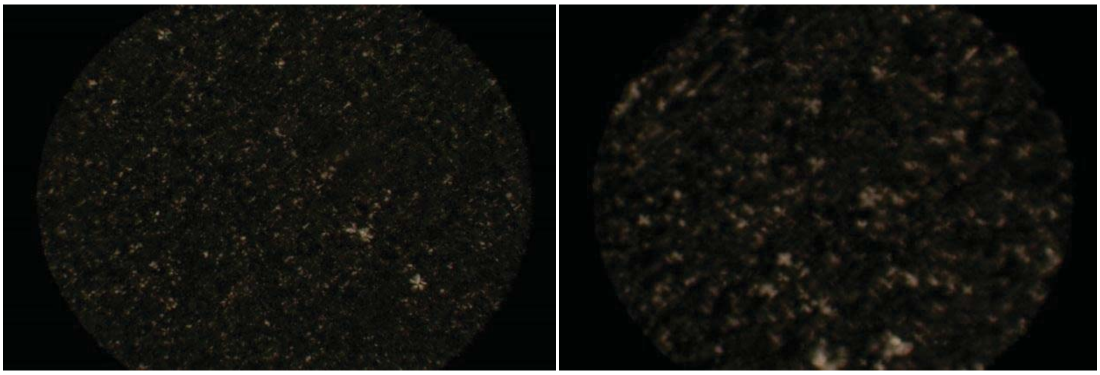


**Figure 3.6** The microscopic appearance of liquid crystals, represented by birefringence, found in some samples (L120 and L130 of dilution line 1) of a ternary system of lemon oil, Tween 80 and water. The image was taken at 20 × magnification under a polarized light microscopy.

### *3.3.2.2 Particle size measurements*

The mean particle size and particle size distribution of w/o and o/w microemulsion samples selected from the two ternary systems containing Tween 20 or Tween 80 were analysed using dynamic light scattering technique, and the results are shown in Table 3.2. The droplet size of all microemulsions was in a range of 2.56-18.88 nm in diameter for Tween 20 system and 2.64-15.55 nm for Tween 80 system, depending on type (o/w or w/o) and composition of emulsion. From the results shown in Table 3.2, it can be notable that the proportion of water for Tween 20 system enabling the formation of microemulsion was not very high, being below 50% even for the o/w microemulsion. In contrast, the water weight fraction was much higher for o/w microemulsions from Tween 80 system. The polydispersity index (PdI) of all microemulsions from the Tween 20 and Tween 80 systems was in a range of 0.3-1.0 with no particular pattern in their differences in the PdI between samples that could be attributed to a certain variable. A PdI of <0.1 is typically regarded as monodisperse and a PdI of >0.5 is referred to as a very broad distribution (Anantachoke et al. 2006). This indicates that overall the distribution of particles in microemulsions obtained in this study was very broad. Figure 3.7 shows the particle size distributions of microemulsions, which were monomodal (i.e. single peak) in all cases.

**Table 3.2** Average particle size in diameter of selected microemulsions from ternary systems of lemon oil, surfactant (Tween 20 or Tween 80) and water.

| | Sample code | Type of emulsion | Oil (%) | Surfactant (%) | Water (%) | Particle size[a] (nm) | PdI[b] |
|---|---|---|---|---|---|---|---|
| Tween 20 | L110 | w/o | 9 | 81 | 10 | 11.81 ± 0.64 | 0.93 ± 0.06 |
| | L120 | w/o | 8 | 72 | 20 | 4.70 ± 2.98 | 0.69 ± 0.14 |
| | L320 | w/o | 24 | 56 | 20 | 8.18 ± 1.87 | 0.44 ± 0.06 |
| | L130 | w/o | 7 | 63 | 30 | 3.82 ± 1.43 | 0.39 ± 0.04 |
| | L230 | w/o | 14 | 56 | 30 | 18.88 ± 11.41 | 0.70 ± 0.03 |
| | L140 | o/w | 6 | 54 | 40 | 3.28 ± 0.24 | 0.86 ± 0.05 |
| | L150 | o/w | 5 | 45 | 50 | 2.56 ± 0.20 | 0.47 ± 0.08 |
| Tween 80 | L160 | o/w | 4 | 36 | 60 | 2.64 ± 0.18 | 0.66 ± 0.04 |
| | L170 | o/w | 3 | 27 | 70 | 6.74 ± 3.24 | 1.00 ± 0.00 |
| | L270 | o/w | 6 | 24 | 70 | 15.55 ± 3.92 | 0.92 ± 0.03 |
| | L180 | o/w | 2 | 18 | 80 | 11.64 ± 1.73 | 0.58 ± 0.00 |
| | L280 | o/w | 4 | 16 | 80 | 13.78 ± 1.17 | 0.97 ± 0.04 |
| | L190 | o/w | 1 | 9 | 90 | 10.14 ± 0.43 | 0.93 ± 0.12 |
| | L290 | o/w | 2 | 8 | 90 | 11.62 ± 0.03 | 0.30 ± 0.02 |

[a] Each value represents the mean ± SD (n ≥ 3).

[b] PdI: polydispersity index

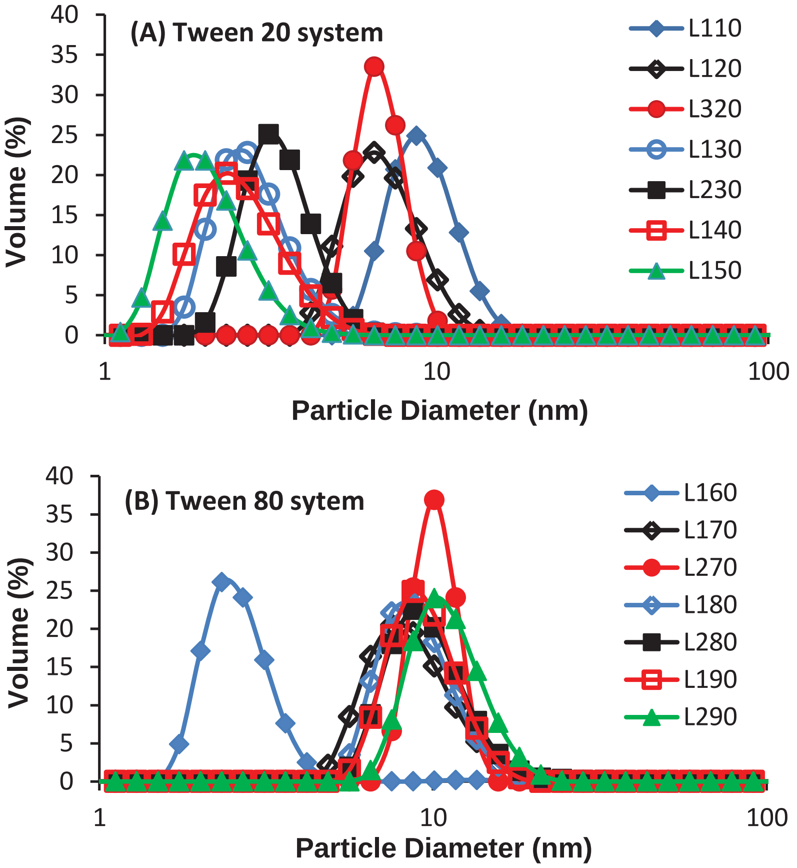


**Figure 3.7** Particle size distributions of microemulsions prepared from ternary systems of lemon oil, surfactant (Tween 20 or Tween 80) and water.

### 3.3.3 Electrical conductivity measurements

Electrical conductivity is a structure-sensitive property which is affected by the composition of a system along the dilution line (Mehta and Bela, 2000). A measurement of electrical conductivity can thus be used to understand the phase behaviour of ternary systems according to change in their composition along the dilution lines and to identify the boundary between phase transition or phase inversion of systems (e.g. w/o to o/w microemulsion or vice versa) (Fasolin, Santana, & Cunha 2012).

Figure 3.8 shows the results of electrical conductivity measured from samples for the lemon oil-Tween 20-water system along the dilution line 1 (Figure 3.2A) and the lemon oil-Tween 80-water system along the dilution lines 1 and 2 (Figure 3.2B), where microemulsions were found to be mostly formed. The graphs were divided into three regions according to the extent of changes in the conductivity associated with the results shown in Table 3.1. In case of Tween 80 system (Figure 3.8B), the first region was where the electrical conductivity was practically zero or very small when the water content was below 10%. This region was followed by a gradual and then steep increase at the second region in which the water weight fraction varied from 20% to 55%. With further dilution with water, the conductivity reached a plateau with a very minor insignificant change. In the first region, w/o microemulsions were formed whereas o/w microemulsion were formed in the third region. This behaviour was also depicted in the phase diagram as shown in Figure 3.2. However, the reason for the relatively slow increase in conductivity observed in the second region for the samples from the dilution line 2 (Tween 80, 2:8) when compared to the samples from the dilution line 1 (Tween 80, 1:9) may be due to the formation of firmer gels.

In case of the Tween 20 ternary system (Figure 3.8A), the conductivity of samples was also low in the first region when the content of water was lower than 20%, suggesting the presence of w/o microemulsion. This region was followed by a gradual continuous increase at the second region denoted as the phase system of o/w microemulsions, which appeared to be proportional to the content of water added, rather than by a dramatic sharp increase. With further dilution (>50% water), the increase in conductivity was also observed to be marginal similar to the pattern observed from the Tween 80 system. This was despite of the fact that its system was changed from the o/w microemulsion to coarse emulsion, which might have been due to the presence of significant larger oil droplets. It should also be mentioned that although it is not clearly understood, the overall conductivity values measured between the two systems differed and were much higher in the Tween 20 system than the Tween 80 system. This could also be confirmed by the conductivity measured from some samples between the two systems by using the Zeta Nanosizer (data not shown).

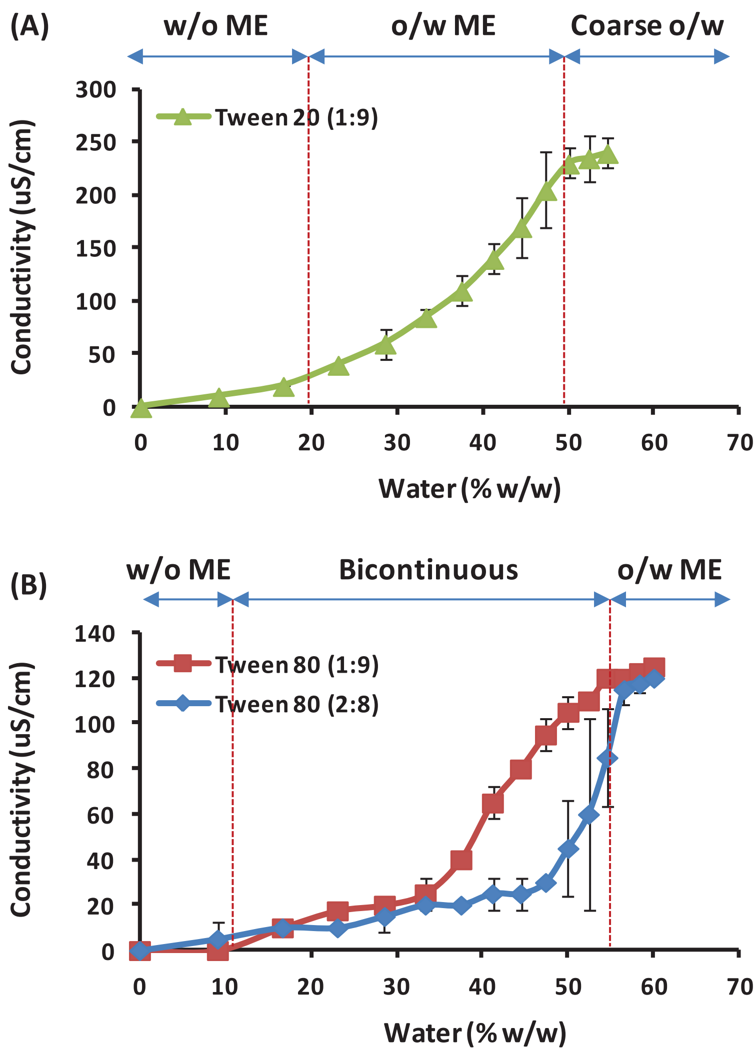


**Figure 3.8** Electrical conductivity versus water weight fraction along (A) the dilution line 1 of lemon oil-Tween 20-water system and (B) the dilution lines 1 and 2 of lemon oil-Tween 80-water system.

The results obtained can be explained to some extent by the concept of percolation, which has been considered for the transfer of charges (Basheer et al. 2013; Fasolin et al. 2012; Fisher, Wachtel, Aserin, & Garti 2013; Garti, Amar, Yaghmur, Spernath, & Aserin 2003; Moulik & Paul 1998; Wang, Fang, Zhang, & Zhang 2008; Zargar-Shoshtari et al. 2010). At low water phase fraction where discrete water droplets are immobilized and dispersed within a continuous oil phase, the system would have low electrical conductivity as the microemulsion system is based on nonionic surfactants. In the Tween 80 system of dilution line 1, 10% water can be considered as a critical point, which is called a percolation threshold (Zargar-Shoshtari et al. 2010). At the percolation threshold, w/o microemulsions are converted into a bicontinuous microstructure where water is more mobile and the conductivity increases. At high water content, the continuous phase is changed to water phase, and water channels are formed across the system, thus the conductivity reached a plateau (Wang et al. 2008).

### 3.3.4 Viscosity measurements

The measurement of rheological properties of ternary systems is one of the important parameters that can be used to understand changes in the phase behaviour of ternary systems which can be largely influenced by their composition and conditions (e.g. type and shape of aggregates) (Garti, Spernath, Aserin, & Lutz 2005; Zargar-Shoshtari et al. 2010). In this study, the lemon oil-Tween 80-water ternary system was used to determine the viscosity of samples along dilution line 1 at 25°C using a rheometer equipped with a cone and plate geometry. The results are shown in Figure 3.9A. All microemulsions (L110, L160, L170, L180 and L190) and the mixture of oil and surfactant with no added water (L100) consisting of reverse micelles demonstrated Newtonian flow behaviour at shear rates from 1 to 100 $s^{-1}$. However, it should be mentioned that two o/w microemulsion samples (L180 and L190) containing more than 80% water showed non Newtonian behaviour at low shear rates from 1 to 10 $s^{-1}$. On the other hand, the other samples, except for L150, that were characterised as liquid crystals and gel-like structures (L120, L130 and L140) exhibited non-Newtonian shear-thinning behaviour as their viscosities decreased with increasing shear rate from 1 to 100 $s^{-1}$. The sample L150 that was measured to be non-Newtonian (Figure 3.9A) suggests that this sample could exist in an o/w microemulsion state although it formed a gel-like structure as shown in Figure 3.5 A and D.

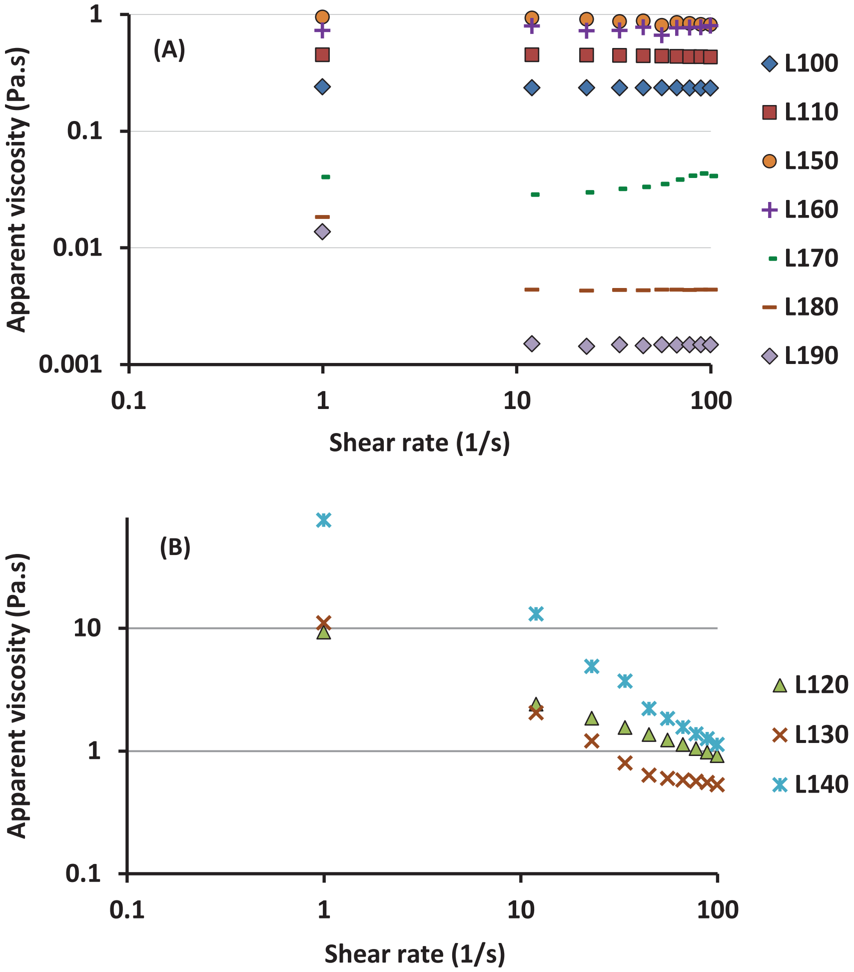


**Figure 3.9** Viscosity of samples along the dilution line 1 for the lemon oil-Tween 80-water system measured at 25°C. Samples: (A) Newtonian fluid and (B) non-Newtonian fluid.

A representative dynamic viscosity versus water weight fraction of samples at dilution line 1 of the lemon oil-Tween 80-water that was measured at a shear rate of 12 $s^{-1}$ is shown in Figure 3.10. At water weight fraction below 10%, the dynamic viscosity of samples was low. This was followed by a moderate increase in the viscosity as the water content increased up to 20 wt%. Moulik and Paul (1998) described that an increase in viscosity with dilution with water can be due to an increase in the diameter of water filled conduits in the bicontinuous structure as well as network formation in the system. For the water content between 20% and 30%, the viscosity of samples did not show a significant change possibly due to the formation of liquid crystals as indicated in Table 3.1. Then, there was a dramatic increase in the viscosity when the level of water added was increased to 40% because of the gel-like structure that was formed. Upon further dilution the viscosity decreased markedly, and the transition seemed to be completed at about 50-60% water content, which means the systems were changed from bicontinuous structures to o/w microemulsions. This phenomenon may be due to a decrease in the hydrophobic interaction of the surfactant tails  (Fanun 2007). Above 60 wt% of water, the viscosity remained in a low value and was practically close to zero, which indicates that no anisometric aggregates were existed and the systems were composed of individual spherical droplets (Quemada & Langevin 1985). If the nanometre-sized droplets of oil or water are non-interacting, the resulting microemulsion tends to be low in viscosity, while the interaction of cylindrical or worm-like micelles in microemulsions gives rise to high viscosity systems (Lawrence & Rees 2000).

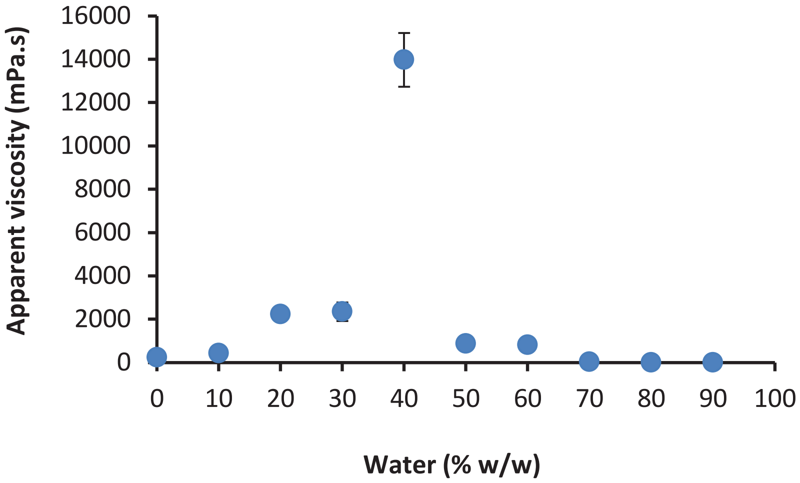


**Figure 3.10** Variation in dynamic viscosity as function of water content along the dilution line 1 for the lemon oil-Tween 80-water system at 25°C. The values of viscosity were taken at a shear rate of 12 $s^{-1}$.

## 3.4 Conclusions

The results showed that non-ionic surfactant-stabilised transparent microemulsions as a potential nutrient delivery system can be prepared by using a three component system, referred to as a ternary system, consisting of an optimum concentration of lemon oil, surfactant (Tween 20 or Tween 80) and water, all of which are food-grade materials. In this study, β-carotene was incorporated into the lemon oil phase as a lipophilic core model although the storage stability of β-carotene against oxidative degradation and its impact on the phase behaviour of ternary systems were not measured. This is because the main objective of the study was to develop and compare ternary phase diagrams using two types of surfactant (Tween 20 and Tween 80), focusing on identifying the regions of ternary phase diagrams that allow the formation of o/w or o/w microemulsions. This was conducted by visual observation of samples and measurements of birefringence using a polarized light microscope, particle size, electrical conductivity, and viscosity. From the ternary phase diagrams constructed, w/o microemulsions were identified to form mainly at the corner of surfactant-rich area along dilution lines 1, 2, 3 and 4. The formation of o/w microemulsions was found to be relatively limited to a smaller region of the phase diagrams along dilution lines 1 and 2 for both systems. It was also found that the viscosity of most microemulsions remained stable without much change with increasing shear rates, suggesting microemulsions had Newtonian flow behaviour. Between the two systems (Tween 20 and Tween 80), there were also some differences in other types of phases, such as bi- and multiphase, gel, coarse emulsions, etc. A much larger area corresponding to gel-like structures was found in Tween 80 system compared to Tween 20 system. Also, a small area representing samples of liquid crystals was determined to be present in Tween 80 system.

# Chapter 4
# Formation and Characterisation of Transparent Nano- and Microemulsions by Emulsion Dilution Method

## 4.1 Introduction

Mechanical devices, such high shear mixer and high pressure homogenizer, used in making o/w emulsions usually do not allow the formation of oil droplet size smaller than 100 nm in diameter. Using this high energy method alone, the formation of very fine droplets (< 100 nm) is not readily achieved. As a consequence, conventional emulsions are opaque in appearance. This is due to the scattering of light caused by emulsion droplets that are larger than the visible wavelength of light.

In Chapter 3, the formation of transparent microemulsions containing very fine oil droplets was investigated by using a method called phase inversion method. This method is also referred to as a low energy emulsification method as it does not require a high mechanical shear force. The method involves mixing of three components (water, small molecule surfactant and lemon oil) at various ratios and then characterizing their properties. A ternary phase diagram is then created to identify the composition of ternary system representing o/w or w/o microemulsion, coarse emulsion, liquid crystal, multiphase systems or gel.

In recent years, some studies have shown that nano- and microemulsions can also be produced by approaching a combined method of high and low energy emulsification methods (Bernardez & Ghoshal 2008; Binks et al. 1998; Evilevitch et al. 2001; Evilevitch et al. 2000; McClements & Dungan 1993; Rao & McClements 2012a, 2012c; Weiss et al. 2000; Weiss et al. 1997; Ziani et al. 2012a, 2012b). The method involves dilution of a conventional o/w emulsion, which was stabilised by small molecule surfactants and prepared by high pressure homogenisation, into aqueous surfactant solutions. This method can also thus be mentioned as a dilution (or titration) method, which is similar to the phase inversion method used in Chapter 3. The principle of this method is based on the solubilisation (e.g. transport) of oil molecules from emulsion droplets into surfactant micelles (Bernardez & Ghoshal 2008; Ziani et al. 2012a, 2012b).

In a study reported by Ziani et al. (2012b), it was shown that the formation and stability of microemulsions containing lemon oil and Tween 80 were affected by surfactant-to-oil ratio and it was more favourable at higher surfactant-to-oil ratios. Bernardez and Ghoshal (2008) showed that the chain length of an alkyl portion of non-ionic surfactants (e.g. Brij 30, Brij 35, Tween 80, Triton X-100 and Tergitol NP-10) and their micelle volume had influence on the kinetics of a solubilisation process of oil molecules. Rao and McClements (2012a) examined the influence of lemon oil fold (1, 3, 5 and 10×) on the formation of microemulsions and nanoemulsions by titration of lemon oil droplets into 1 % Tween 80 solution. It was shown that high fold lemon oil (10×) resulted in a high rate of solubilisation and more stable system. In another study by the same authors that investigated the influence of surfactant concentration, type and mixing ratio of sucrose monopalmitate (SMP) and Tween 80 on lemon oil solubilisation (Rao & McClements 2012c), the solubilisation capacity was higher in SMP than in Tween 80 due to its molecular structure, and the capacity increased with surfactant concentration.

Although some studies have shown the preparation of nano- and microemulsions by diluting a pre-formed conventional emulsions into surfactant micellar solutions, there is not much scientific information available. Therefore, the objectives of the study were to investigated for the formation and stability of microemulsion and nanoemulsion with regards to: (1) the influence of non-ionic surfactant type (Tween 20, 40, 60 and 80) and concentration; (2) the effect of oil type (lemon oil, isopropyl myristate, tributyrin, Imwitor 308 and sunflower oil) and concentration; (3) the effects of pH, ionic strength and heat treatment.

## 4.2 Materials and Methods

### 4.2.1 Materials

Four different types of non-ionic small molecule surfactants, such as polysorbate 20 (Tween 20), polysorbate 40 (Tween 40), polysorbate 60 (Tween 60) and polysorbate 80 (Tween 80), were purchased from Pharmachem Ltd. (Auckland, New Zealand) and used as emulsifiers in making o/w emulsions. Different types of oils were used as the oil phase of emulsions, including lemon oil, isopropyl myristate, tributyrin, Imwitor 308 and sunflower oil. Isopropyl myristate (98%), lemon oil (cold-pressed, food grade) and tributyrin (97%, food grade) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Imwitor 308 was supplied by Sasol (Germany). Sunflower oil (Pams, New Zealand) was purchased from a local supermarket. Sodium azide purchased from Merck KGaA (Germany) was used as an antimicrobial agent. All other chemicals used were of analytical grade and were purchased from Fisher Scientific (Pittsburgh, PA), Sigma-Aldrich (St. Louis, USA) or other chemical suppliers. Milli-Q quality water (Milli-Q apparatus, Millipore Corporation, France) was used in preparing all aqueous solutions. In this study, concentrations of chemicals in solutions and emulsions used were on the basis of weight per weight (% w/w) unless stated otherwise.

## 4.2.2 Preparation of stock and secondary emulsions

### *4.2.2.1 Stock emulsion*

An o/w emulsion, denoted as stock emulsion, containing 10% oil (i.e. 1:9 ratio of oil to water) was prepared by homogenizing isopropyl myristate (IPM) into aqueous emulsifier solution (1% Tween 80). Homogenisation of emulsion was carried out in a two-step process. In the first step, IPM was mixed with 1% Tween 80 solution using an Ultra-turrax (Model: Tempest 500 W, VirTis Company Inc, New York) at 11,000 rpm for 1 minute to form a coarse o/w emulsion. In the second step, the coarse emulsion was homogenized with a two-stage high pressure homogeniser (APV-2000, Invensys® APV®, Denmark) operated at 500 bar and 50 bar for the first and second stages, respectively, by passing four times to form a fine o/w emulsion. Then, the fine emulsion (stock emulsion) was added with 0.02% sodium azide to prevent the growth of microorganisms.

### *4.2.2.2 Secondary emulsion*

An aqueous surfactant solution containing 1% Tween 80 was prepared by dispersing Tween 80 in Milli-Q water. Then, aliquots of the stock emulsion (10% IPM and 1% Tween 80) prepared were added into the aqueous surfactant solution (1% Tween 80 and pH 5.23) and mixed thoroughly using a vortex mixer. The dilution of oil droplet concentration of stock emulsion formed a series of mixtures (denoted as secondary emulsions) containing different concentrations of oil (IPM). The secondary emulsions were analysed for their particle size and turbidity after preparation and also after 1 day of storage at 20$^{o}$C.

## 4.2.3 Analyses of stock and secondary emulsions

### *4.2.3.1 Particle size and size distribution of emulsions*

The mean particle diameter and particle size distribution of emulsions were measured by a dynamic light scattering technique using a particle size analyser (Nano-ZS; Malvern Instruments Ltd., Malvern, Worcestershire, UK) as described in Section 3.2.3.2. For the particle size analysis of emulsions containing IPM, sunflower oil, lemon oil, tributyrin and Imwitor 308, the refractive index values used were 1.434, 1.465, 1.476, 1.435 and 1.450, respectively. The mean particle size of emulsions reported in this study is Z-Average size (or Z-Average mean) which is also known as the cumulants mean defined as harmonic intensity averaged particle diameter (Malvern 2011).

### *4.2.3.2 Analysis of emulsion turbidity*

The optical properties of emulsions, in terms of turbidity, were analysed by measuring absorbance at 600 nm against blank (distilled water) using a UV-visible spectrophotometer (UV-1700, Shimadzu Corp., Japan). Due to adsorption and scattering, the intensity of light would decrease exponentially when light spreads through a medium. Therefore, the turbidity of emulsion systems depends on the size and concentration of scatters (e.g., oil droplets) (McClements & Dungan 1995).

## 4.2.4 Effects of some variables on stock and secondary emulsions

### *4.2.4.1 Effect of surfactant type*

Four different non-ionic surfactants (Tween 20, 40, 60 and 80) were used to prepare stock emulsions (10% IPM and 1% surfactant) as described above. Subsequently, these four stock emulsions were diluted at various oil concentrations with each of 1% surfactant solutions (Tween 20, 40, 60 and 80) in order to produce secondary emulsions as also described in the above. All samples were stored for 1 day at 20$^{o}$C prior to analysis.

#### *4.2.4.2 Effect of surfactant micelle solution*

The effect of concentration of surfactant solution used to dilute stock emulsions was studied. The stock emulsion (10% IPM and 1% Tween 80) prepared as described in Section 4.2.2.1 was used for dilution into each of three different concentrations (0.5, 1 and 2%) of Tween 80 surfactant solutions. The stock emulsion was also diluted with water as a control sample. All samples were stored at 20$^o$C for 1 day prior to analysis of particle size and turbidity.

#### *4.2.4.3 Effect of oil type*

In addition to IPM, four other types of oils, such as sunflower oil, tributyrin, lemon oil and Imwitor 308, were used as oil phase in preparing stock emulsions (10% oil and 1% Tween 80). Each of these stock emulsions was diluted in 1% Tween 80 solution to make a series of secondary emulsions containing different oil concentrations. All samples were stored at 20$^o$C for 1 day prior to their particle size and turbidity measurements.

#### *4.2.4.4 Effects of environmental factors*

Effects of pH, ionic strength and thermal treatment on the particle size of secondary emulsions were analysed. For this study, secondary emulsions containing three different oil concentrations of 0.05, 0.15 and 0.5% IPM were prepared by diluting the stock emulsion containing 10% IPM and 1% Tween 80 with 1% Tween 80 surfactant solution. The initial pH of this stock emulsion was pH 5.16.

##### *(1) Effect of pH*

Secondary emulsions (0.05, 0.15 and 0.5% IPM; 1% Tween 80) were adjusted to pH 1, 3, 5, 7, 9 and 11 by using HCl or NaOH solutions (100 mM and/or 1 M) appropriately in order to minimize variability in the oil and surfactant concentrations between samples after pH adjustment. The pH of samples was monitored at 20$^o$C using a pH meter (Sartorious Basic pH Meter PB-20, Sartorius AG, Germany). The secondary emulsions were placed into glass test tubes, capped, and then stored at 20$^o$C for 1 day prior to the analysis of particle size.

##### *(2) Effect of salt concentration*

The effect of ionic strength (100-500 mM NaCl) on the stability of secondary emulsions was analysed. Secondary emulsions were mixed with an equal volume of salt solutions containing 200, 400, 600, 800 and 1000 mM NaCl, thereby, the concentration of oil and surfactant was maintained constant between all samples though it was reduced to half. After mixing, the samples were placed in glass test tubes, capped, and stored at 20°C for 1 day, and then analysed for their particle size.

***(3) Effect of heat treatment***

Thermal treatment of secondary emulsions was carried out by heating a 10 ml of secondary emulsion samples (0.05, 0.15, and 0.5% IPM: 1% Tween 80) and stock emulsion (10% IPM and 1% Tween 80) in glass test tubes at 50˚C or 80°C for 15 minutes using a water bath (Grant GLS400, Grant Instruments, UK). After heating, the emulsions were cooled to ambient temperature and stored at 20˚C for 7 days. The particle size of emulsions was measured after 1, 3 and 7 days.

## 4.3 Results and Discussions

### 4.3.1 Dilution of stock emulsion in 1% Tween surfactant solution

Initially, an o/w stock emulsion containing 10% IPM and 1% Tween 80 was prepared by using a high pressure homogenizer. Then, the stock emulsion was diluted by gradually adding more stock emulsion per addition each time into 1% Tween 80 surfactant micelle solutions. The particle size, size distribution, turbidity and appearance of the mixed stock emulsion-surfactant solutions (secondary emulsions) were analysed after storage for 1 day at 20°C.

The initial mean droplet diameter of the stock emulsion was 202.46 nm in diameter but the size was increased to 239.79 nm after 1 day storage (Table 4.1). In the absence of oil, the size of surfactant micelles in 1% Tween 80 solution was around 10 nm. McClements (2012) stated that the initial size and shape of micelles are determined by the molecular geometry and packing of surfactant molecules which are in turn affected by the effective head group or tail group dimension. In other studies, the size of surfactant micelles in 1% Tween 80 solution was reported to be around 12 nm in diameter (Ziani et al. 2012a), which was in agreement with the results of this study.

For the secondary emulsions, the particle size was increased gradually as increasing IPM concentration (Table 4.1). This can also be seen in Figure 4.1 that shows the particle size distributions of the stock emulsion containing 10% IPM, some secondary emulsions containing 0.05-1% IPM and the surfactant micelle solution (1% Tween 80). The particle size distribution was monomodal and narrow for the secondary emulsions containing 0.15% IPM or less while it was bimodal and broader for the secondary emulsions containing 0.5% oil or higher.

**Table 4.1** Mean particle diameter (Z-Average) and polydispersity index (PdI) of surfactant solution (1% Tween 80), stock emulsion (10% IPM and 1% Tween 80) and secondary emulsions (different concentrations of IPM and 1% Tween 80) measured after storage for 1 day at 20$^{o}$C.

| Samples | Oil concentration (%) | Z-Average size (d.nm) | PdI |
|---|---|---|---|
| Surfactant solution | 0 | 9.72 ± 0.17 | 0.06 ± 0.02 |
| Stock emulsion (day 0) | 10.00 | 202.46 ± 8.80 | 0.23 ± 0.01 |
| Stock emulsion (day 1) | 10.00 | 239.79 ± 1.79 | 0.20 ± 0.03 |
| Secondary emulsions | 0.05 | 13.73 ± 3.59 | 0.17 ± 0.12 |
| | 0.06 | 15.59 ± 3.08 | 0.24 ± 0.08 |
| | 0.07 | 13.73 ± 2.28 | 0.23 ± 0.08 |
| | 0.08 | 16.20 ± 4.33 | 0.32 ± 0.17 |
| | 0.09 | 17.21 ± 3.59 | 0.40 ± 0.08 |
| | 0.10 | 17.24 ± 3.43 | 0.41 ± 0.13 |
| | 0.11 | 18.16 ± 2.45 | 0.41 ± 0.05 |
| | 0.12 | 25.43 ± 8.27 | 0.56 ± 0.18 |
| | 0.13 | 30.56 ± 8.33 | 0.61 ± 0.16 |
| | 0.14 | 49.63 ± 18.49 | 0.65 ± 0.06 |
| | 0.15 | 70.94 ± 24.25 | 0.46 ± 0.10 |
| | 0.16 | 93.57 ± 28.78 | 0.41 ± 0.09 |
| | 0.17 | 102.46 ± 22.62 | 0.55 ± 0.37 |
| | 0.18 | 111.87 ± 18.77 | 0.42 ± 0.16 |
| | 0.19 | 123.39 ± 18.10 | 0.34 ± 0.15 |
| | 0.20 | 140.69 ± 6.12 | 0.34 ± 0.15 |
| | 0.25 | 152.34 ± 4.44 | 0.17 ± 0.01 |
| | 0.50 | 172.80 ± 3.29 | 0.13 ± 0.02 |
| | 1.00 | 190.13 ± 15.70 | 0.15 ± 0.04 |

| | | | |
|---|---|---|---|
| | 1.50 | 185.30 ± 1.69 | 0.12 ± 0.03 |
| | 2.00 | 204.94 ± 16.06 | 0.16 ± 0.05 |

Each value represents the mean ± SD (n > 5).

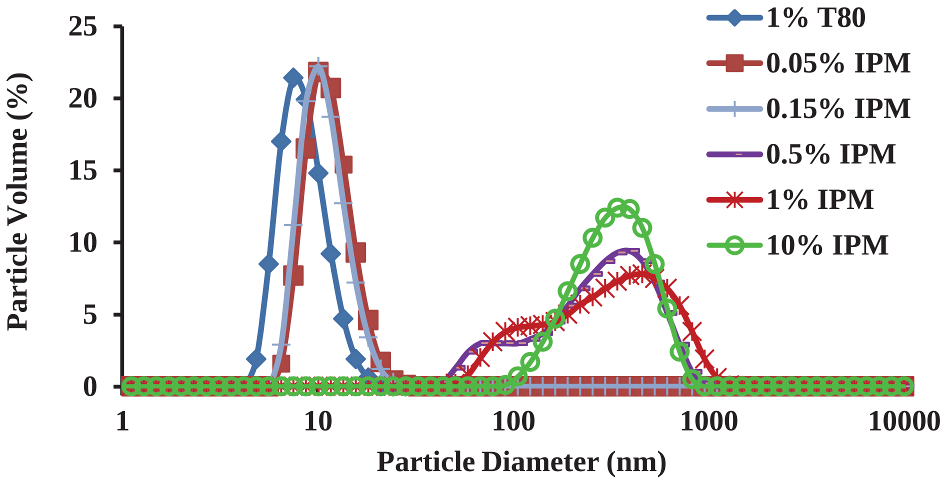


**Figure 4.1** Particle size distributions of mixed emulsion-surfactant solutions (1% w/w Tween 80) with different IPM oil concentrations after overnight storage at ambient temperature.

Secondary emulsions prepared had some differences in their optical properties (turbidity), depending on their oil concentration. The appearance of secondary emulsions changed from transparent gradually to translucent and then to opaque with the increasing of IPM concentration as shown in Figure 4.2. At oil concentration less than 0.16% IPM, all secondary emulsions were observed to be transparent while they became slightly translucent when the oil concentration increased up to 0.2% IPM. In contrast, at oil concentration higher than 0.5%, the secondary emulsion turned to be opaque.

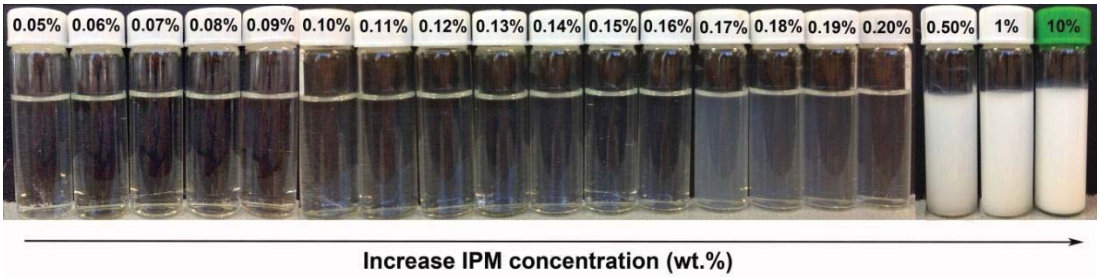


**Figure 4.2** Appearance of secondary emulsions (1% w/w Tween 80) with increasing IPM concentration and stock emulsion (1% w/w Tween 80 and 10% IPM). Pictures were taken after one day storage at 20°C. Numbers refers to IPM concentration.

Figure 4.3 shows changes in the particle size of secondary emulsions as a function of oil concentration, which can be divided into three regions according to the extent of particle size change and visual appearance of secondary emulsions, as follows:

1) 0 to 0.12% IPM. The secondary emulsions had small mean particle diameters (10-25 nm), transparent appearance (absorbance of turbidity ≈ 0 at 600 nm), and unimodal and narrow particle size distribution (Figure 4.1). This implies that when the stock emulsion was added into the surfactant solution, the molecules of IPM oil moved from the oil droplets into the micelles, which might lead to the formation of a swollen micelle or microemulsion. The droplet size of secondary emulsions in this region was marginally larger than the size of micelles ($d \approx 10$ nm). In fact, there was only a modest increase because the oil may be incorporated in between the tails of surfactant molecules, rather than within the core of the surfactant micelle (Rao & McClements 2012a).

2) 0.12 to 0.25% IPM. In this region, the mean particle diameter of secondary emulsions was increased dramatically from 25 nm to 152 nm. The appearance of the secondary emulsions changed from transparent to translucent, and the turbidity was increased slightly due to the presence of larger particles that scatter the light more strongly (Rao & McClements 2011a; Ziani et al. 2012b). The reason for these changes in particle size and optical properties may be because the saturation limit of Tween 80 micelles was exceeded, and some of the IPM molecules could not be incorporated into micelles and had to stay in the emulsion droplets.

3) > 0.25% IPM. The change of the mean particle diameter of secondary emulsions was relatively minor, which was from 172 to 240 nm. As more IPM droplets were added, the particle size distribution became broader and changed to biomodal and the appearance of the secondary emulsions was opaque. This suggests that all the surfactant micelles were completely saturated, and any additional oil droplet could not change the system. There might be a population of small particles, but it could not be observed since the overall signal in the

dynamic light scattering instrument was mainly influenced by the light scattered from larger particles (Rao & McClements 2012a).

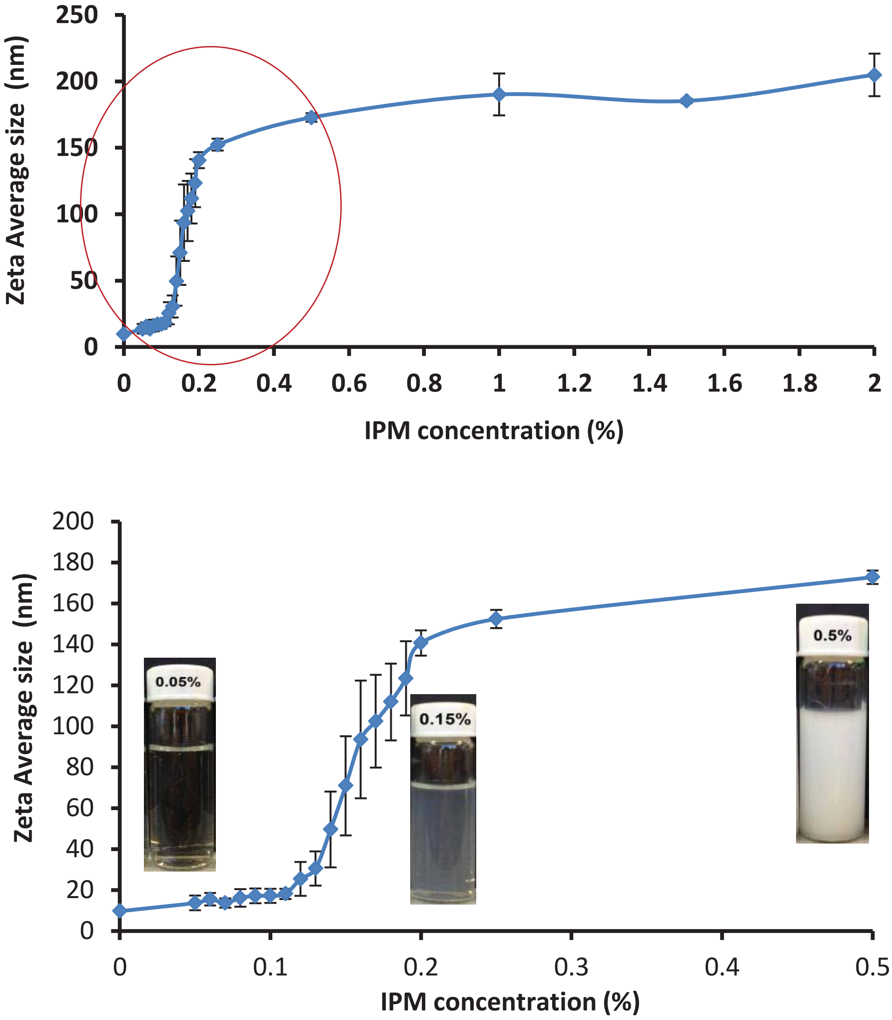
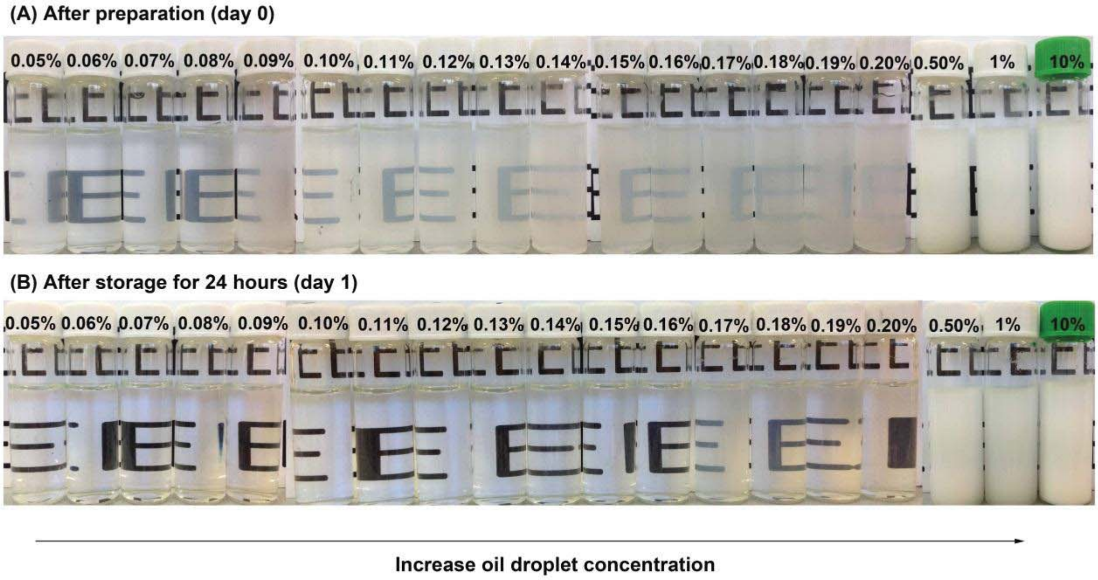


**Figure 4.3** Influence of oil (IPM) concentration on the mean particle diameters of mixed stock emulsion-surfactant solutions (i.e. secondary emulsions with 1% Tween 80) after overnight storage at ambient temperature.

### 4.3.2 Particle size of secondary emulsions with time after preparation

During the storage of secondary emulsions for 1 day prior to the analysis of their particle size, it was observed that the appearance of some secondary emulsions had been considerably changed over time after the emulsion preparation, and became transparent. The pictures of secondary emulsions taken before and after 1 day of storage are shown in Figure 4.4. A gradual decrease in turbidity over time might have been due to a decrease in their particle size with time. The possible reason is due to gradual solubilisation of oil droplets into surfactant micelles in certain samples rather than their spontaneous dissolution and incorporation into micelles. Therefore, another experiment was carried out to monitor the changing of droplet sizes over time during 24 hours after mixing stock emulsion and surfactant solution (1% Tween 80). For this experiment, secondary emulsions used were those containing three different oil concentrations of 0.05%, 0.15% and 0.5% IPM. These samples were chosen from each of three regions defined in Figure 4.3 according to the influence of oil (IPM) concentration on the particle size of secondary emulsions.


(A) After preparation (day 0)

(B) After storage for 24 hours (day 1)

**Figure 4.4** Visual appearance of secondary emulsions (1% w/w Tween 80) with increasing IPM concentration and stock emulsion (1% w/w Tween 80 and 10% IPM). Pictures were taken before (A) and after 1 day (B) storage at 20$^{o}$C. Numbers refers to IPM weight fraction.

The particle sizes of samples measured with time during 24 hours are shown in Figure 4.5A. At 10 min after the mixing of two liquids (stock emulsion and surfactant solution), the mean particle size of secondary emulsions containing, 0.05%, 0.15% and 0.5% IPM was 103 ± 6.77, 164 ± 4.76 and 189 ± 4.10 nm, respectively. Subsequently after 10 min, the particle size of secondary emulsion containing 0.5% IPM remained relatively constant over 24 hours. For the sample containing 0.15% IPM, there was a modest decrease in the particle size from 164 ± 4.76 nm to 132 ± 18.86 nm. On the hand other, it was worth noting that there was a steep reduction in the mean particle size for the secondary emulsion containing 0.05% IPM within the initial first 2 hour storage time, which was from 103 ± 6.77 nm to 11.2 ± 0.21 nm. This indicated that the solubilisation process of this system was relatively rapid after which the particle size remained constant. A further experiment was performed with this system by monitoring changes in its particle size every one or two minutes during 2 hours after the mixing of stock emulsion and surfactant solution, which is shown in Figure 4.5B. A similar result was observed with a rapid decline in the size of particles, particularly within 20 minutes after mixing the two liquids of stock emulsion and surfactant solution. These results imply several points as follows: (1) stock emulsion oil droplets were able to solubilise rapidly into 1% Tween 80 surfactant solution, (2) the oil droplet concentration of 0.05% IPM did not exceed the oil solubility limit of surfactant micelles present in the system, that is, the saturation of surfactant micelles, and (3) the water solubility of IPM was high at the level of 0.05% IPM.

The changes in the turbidity of secondary emulsions over time were confirmed by measuring the values of absorbance at 600 nm using a spectrophotometer (Figure 4.5C). The relatively low initial absorbance values measured from secondary emulsions containing 0.05% and 0.15% and a gradual decrease in absorbance from these two samples were in agreement of the visual observation of the samples changing from translucent to transparent with time. The other sample containing a relatively higher concentration of 0.5% IPM also exhibited a decrease in its absorbancy but due to its high absorbance value, the secondary emulsion remained opaque.

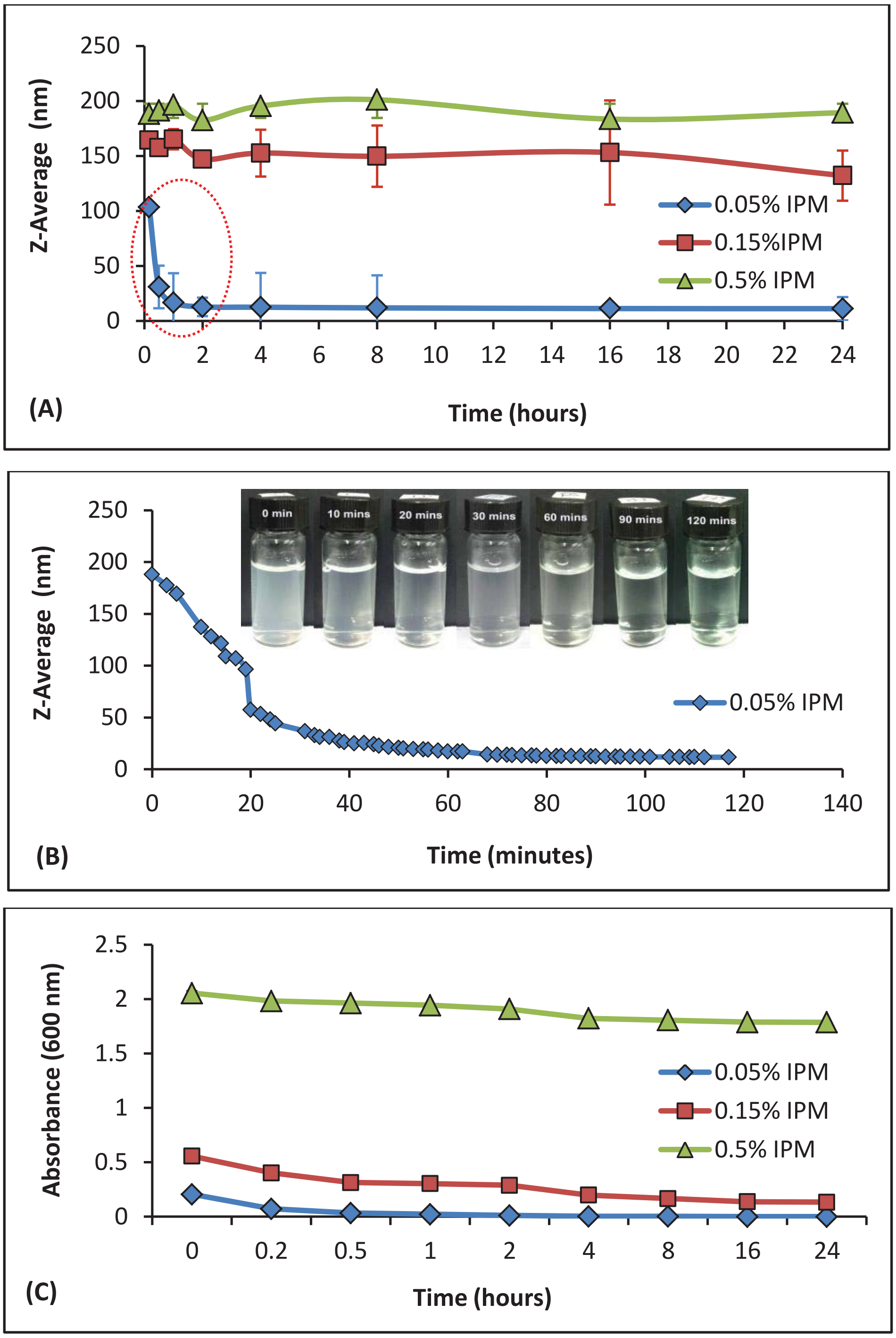


**Figure 4.5** Changes in the mean particle diameters (A and B) and turbidity of secondary emulsions (C) over time during 24 hrs of storage at 20°C.

According to the literature, the droplets of emulsion shrink in size with time as the solubilisation process of droplets proceeds, since oil molecules move from the emulsion droplets into the surfactant micelles. Several studies showed that these physicochemical phenomena can be attributed to thermodynamic effects (Rao & McClements 2012a; Weiss et al. 2000; Weiss et al. 1997; Ziani et al. 2012a, 2012b). The tendency for surfactant monolayers to attain their optimum curvature is the major thermodynamic driving force in the solubilisation process (Israelachvili 1992). When surfactant micelles and emulsion droplets are mixed together, oil molecules from the emulsion droplets are incorporated into the surfactant micelles until the optimum curvature of the surfactant monolayer is attained. Once the swollen micelle reaches its optimum size, additional oil molecules cannot make the size of micelle increased further, due to the additional energy required to bend the monolayer away from its optimum curvature and to increase the contact area between oil and water (Weiss et al. 1997). Due to the positive free energy associated with the oil-water interface, it is thermodynamically unstable when the oil molecules are in the emulsion droplets. On the other hand, the micelle solution is thermodynamically stable since the free energy decrease in the removal of non-polar molecules from water is larger than the increase of that due to a reduction in the entropy of mixing of the surfactant molecules (McClements & Dungan 1995; Rao & McClements 2012a; Ziani et al. 2012b). Therefore, the IPM molecules used in this study can be thought to be able to move from the stock emulsion droplets into the Tween 80 micelles until the micelles reached their optimum curvature and became saturated. The saturation level of surfactant micelles in 1% Tween 80 solution could thus be around 0.25% for IPM molecules, based on the results shown in Figures 4.3 and 4.4. According to Weiss et al. (1997), there is a balance between some factors which favour solubilisation, such as entropy of mixing and achievement of the optimum monolayer curvature, and oppose solubilisation, such as protrusion of the hydrocarbon molecule part into a hydrophilic region or the loss of configurational entropy associated with the restricted volume available to larger hydrocarbons. These factors determine the amount of hydrocarbon that can be solubilised in a micelle.

In summary, transparent microemulsions were able to be produced via solubilisation (dilution) of a pre-formed o/w emulsion containing IPM oil into Tween 80 surfactant solution. The concentration of IPM oil droplets that could result in the formation of microemulsions with transparency was up to around 0.25%.

### 4.3.3 Effect of surfactant type on droplet solubilisation

The effect of surfactant type on the solubilisation of emulsion oil droplets into surfactant solutions (Tween 20, 40, 60 and 80) was studied. These non-ionic small molecule surfactants are commonly used as hydrophilic surfactants in making o/w emulsions. These Tween surfactants are all produced via esterification of polyoxyethylene sorbitan with a fatty acid (Garti 2002), thus, they have a similar molecular structure. The main difference is the type of fatty acids esterified, which differ in chain length and unsaturation. Tween 20, 40, 60 and 80 have a hydrocarbon fatty acid tail esterified with lauric acid ($C_{12:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), and oleic acid ($C_{18:1}$), respectively (Garti 2000, 2002; Sirshendu & Sourav 2012; Symon & William 2001; Ziani et al. 2012a). The HLB values are similar between them, being 16.7, 15.6, 14.9 and 15.4 for Tween 20, 40, 60 and 80, respectively (Ćirin et al. 2012; Griffin 1946).

The mean particle diameters of individual surfactant solutions (1% Tween) and stock emulsions containing 10% IPM prepared with four types of Tween at 1% concentration are shown Table 4.2. The size of micelles in surfactant solutions was in the range of 8-17 nm which was larger in Tween 40 and Tween 60 than in Tween 20 and Tween 80. The size of oil droplets in stock emulsions varied from 230 to 255 nm in diameter which was larger in stock emulsions prepared with Tween 20 and Tween 40 than with Tween 60 and Tween 80. The size of surfactant micelles were in agreement with some studies reported by other researchers (Rao & McClements 2012a; Ziani et al. 2012a, 2012b).

The mean particle size of secondary emulsions prepared by dilution of stock emulsions in surfactant solutions is shown in Figure 4.6. It can be noted that surfactant type had some impact on the solubilisation behaviour of IPM oil droplet. The chain length of the fatty acid hydrocarbon tail of surfactants had a major impact on the formation of microemulsions and properties of the systems, in other words, the size and shape of micelles determined by the molecular geometry of surfactant molecules (Weiss et al. 1997).

**Table 4.2** Mean particle size of stock emulsions, expressed as Z-average in diameter (d.nm), that were prepared with 10% (w/w) IPM and 1% (w/w) Tween surfactants by using a two-stage high pressure homogenizer at 500/50 bar (first/ second stage pressure).

| | Oil phase/ Surfactant | Z-Average (d.nm) | PdI[a] |
|---|---|---|---|
| Surfactant solution | Tween 20 | 8.02 ± 0.24 | 0.12 ± 0.07 |
| | Tween 40 | 12.15 ± 0.24 | 0.45 ± 0.01 |
| | Tween 60 | 16.10 ± 0.30 | 0.48 ± 0.01 |
| | Tween 80 | 9.72 ± 0.17 | 0.06 ± 0.02 |
| Stock emulsion | IPM/Tween 20 | 255.17 ± 2.97 | 0.17 ± 0.02 |
| | IPM/Tween 40 | 254.47 ± 1.93 | 0.19 ± 0.01 |
| | IPM/Tween 60 | 229.63 ± 1.16 | 0.20 ± 0.02 |
| | IPM/Tween 80 | 239.79 ± 1.79 | 0.20 ± 0.03 |

Each value represents the mean ± SD ($n \geq 3$).

[a] PdI, polydispersity index

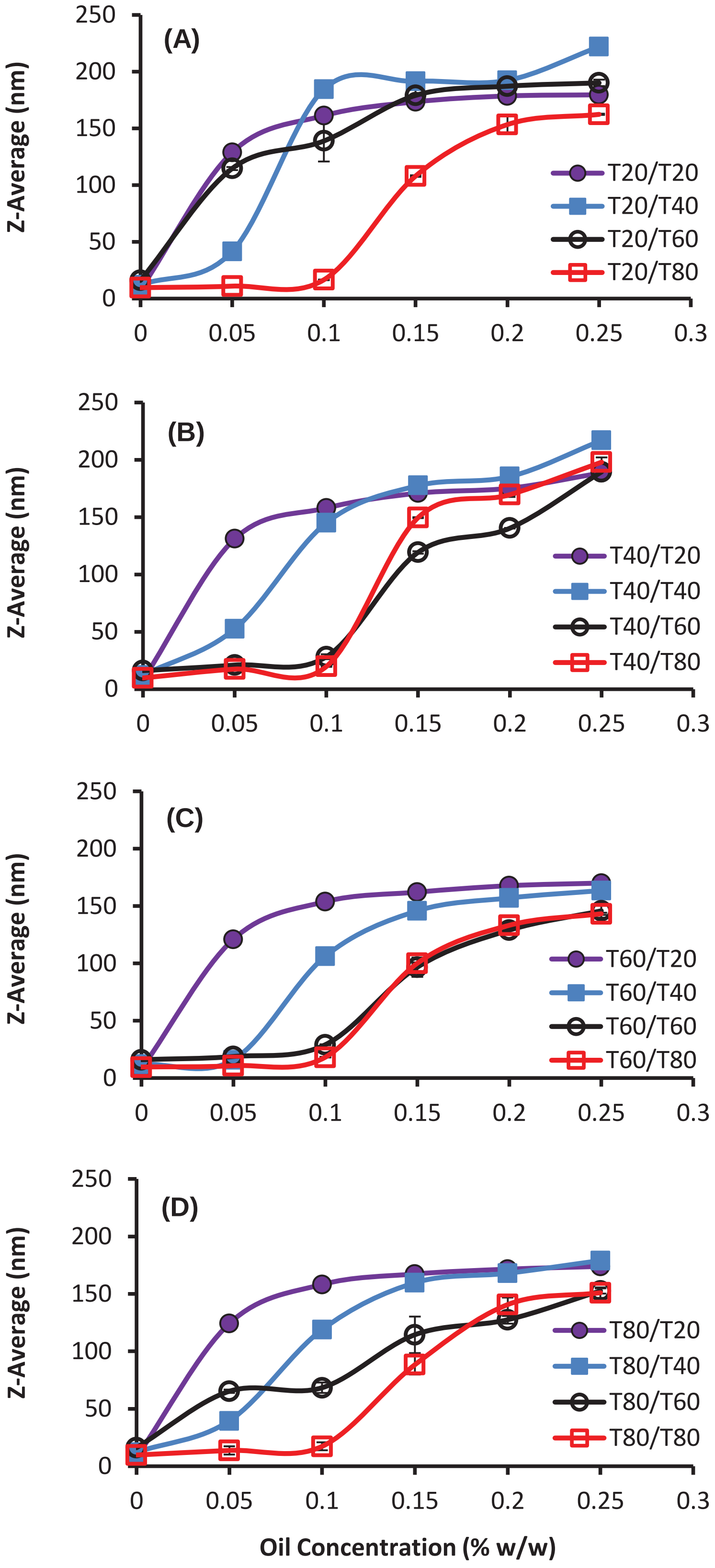


**Figure 4.6** Influence of surfactant type and oil concentration on the mean particle diameter of secondary emulsions prepared by titrating an emulsion (10% w/w IPM, 1% w/w surfactant) into a 1% w/w surfactant solution (Tween 20, 40, 60, and 80).

Figure 4.6 shows that Tween 60 or Tween 80 micelles were able to incorporate significantly more oil droplets within them than Tween 20 or Tween 40 micelles, regardless of the type of surfactants used in making stock emulsions. Between Tween 20 and Tween 40, at a lower IPM concentration, less oil molecules were solubilised within Tween 20 micelles than Tween 40 micelles. However, as more oil was added, the amount of oil droplets solubilised into micelles between them was almost same and remained constant.

When the stock emulsions stabilised by Tween 20 was titrated into four different surfactant solutions (Figure 4.6A), Tween 80 micelles could incorporate the largest amount of oil droplets within it before they were saturated, and they had the smallest particle size diameters. On the other hand, only a little oil concentration made Tween 20 micelles saturated, and reached a large droplet size rapidly compared to the other surfactant micelles. This observation can be seen clearly in Figure 4.7, which compares the patterns of particle size changes between secondary emulsion systems. The same surfactant molecules were used in both making stock emulsions and solubilisation of the stock emulsions in surfactant solutions.

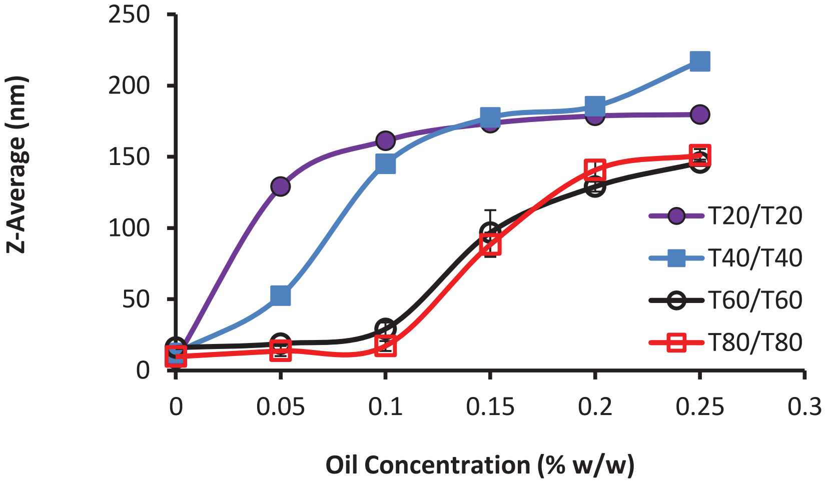


**Figure 4.7** Influence of surfactant type and oil concentration on the mean particle diameter of secondary emulsions prepared by titrating an stock emulsion (10% w/w IPM, 1% w/w Tween 20, 40, 60, or 80) into the same surfactant solution (1% w/w surfactant).

The particle size distributions shown in Figure 4.8 indicated that almost all secondary emulsions except for a few cases were monomodal. As described above, the results showed that among the fixed amount of oil droplet concentrations, there were more microemulsions formed in Tween 60 or Tween 80 surfactant solutions than Tween 20 or Tween 40 solutions. In case of Tween 60 and Tween 80, the formation of nano- and microemulsions with particle size smaller than 100 nm in diameter was obtained at oil concentrations lower than 0.15% IPM while the formation of nano- and microemulsions with particle size smaller than 100 nm occurred at the oil concentration below 0.05% IPM for Tween 40. On the hand other, a mean particle size smaller than 100 nm was not observed even at the low level of 0.05% IPM concentration from a system with Tween 20.

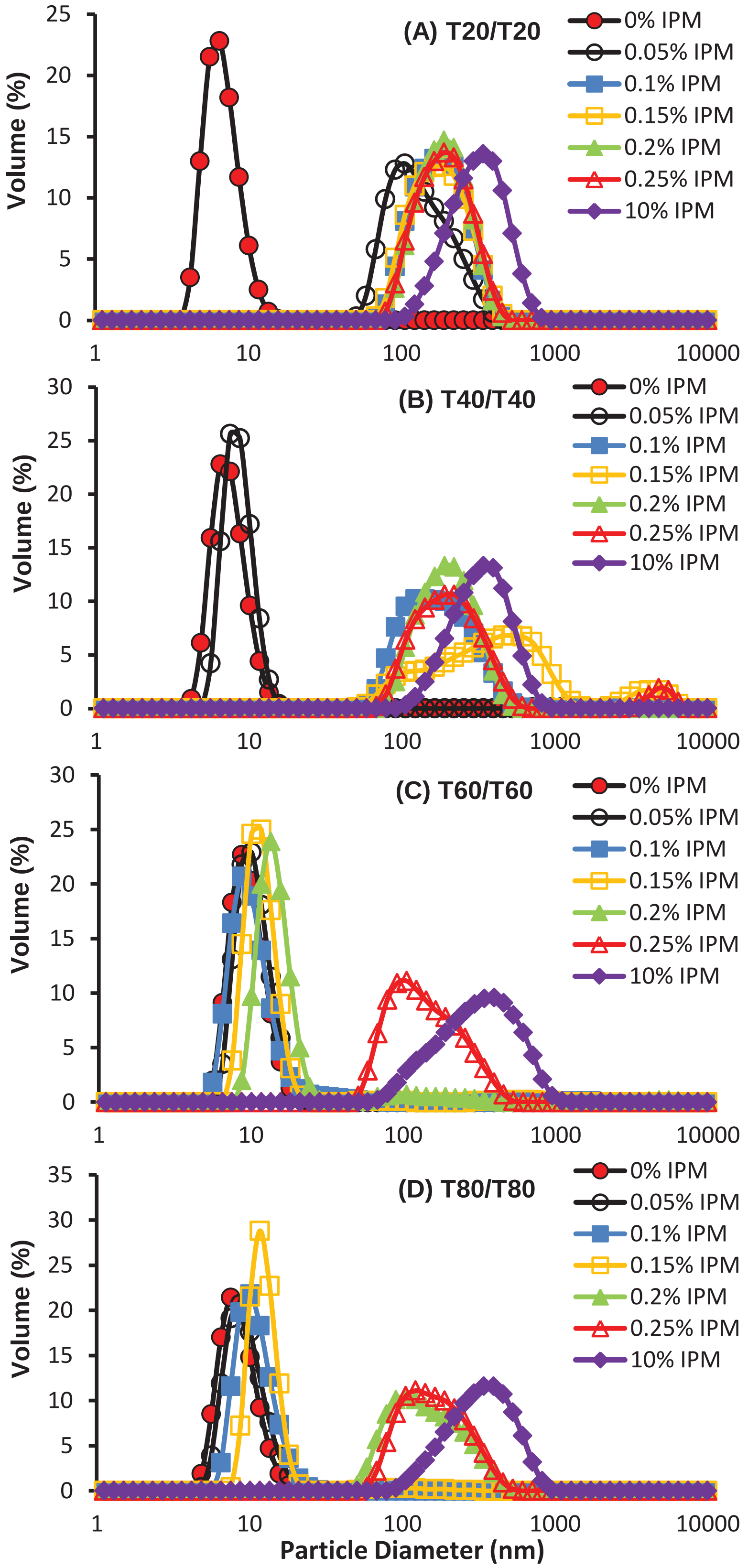


**Figure 4.8** Influence of surfactant type and oil concentration on the particle size distributions of secondary emulsions prepared by titrating stock emulsions produced by 10% w/w IPM and 1% w/w surfactant into 1% w/w same surfactant solutions. (A) Tween 20, (B) Tween 40, (C) Tween 60 and (D) Tween 80.

The visual observation of secondary emulsions shown in Figure 4.9 was also in agreement with the results of particle size analysis discussed above (Figure 4.8). At relatively low oil content, the secondary emulsions were transparent while they became turbid gradually with increasing oil levels. Among these four surfactants, Tween 60 and Tween 80 had the higher capacity of oil droplet solubilisation as described above, which can be attributed to the fact that they have a longer hydrocarbon tail. Due to the oil molecules incorporated between the non-polar tails, Tween 60 or Tween 80 was able to solubilise more IPM within them than did Tween 40 or Tween 20 (Rao & McClements 2012a; Ziani et al. 2012a, 2012b). Overall, the ability of a surfactant micelle to incorporate non-polar molecules depends on the size and shape of the hydrocarbon tails of surfactant molecules because non-polar molecules are packed between the tails of surfactant molecules rather than the core in the micelle interior.

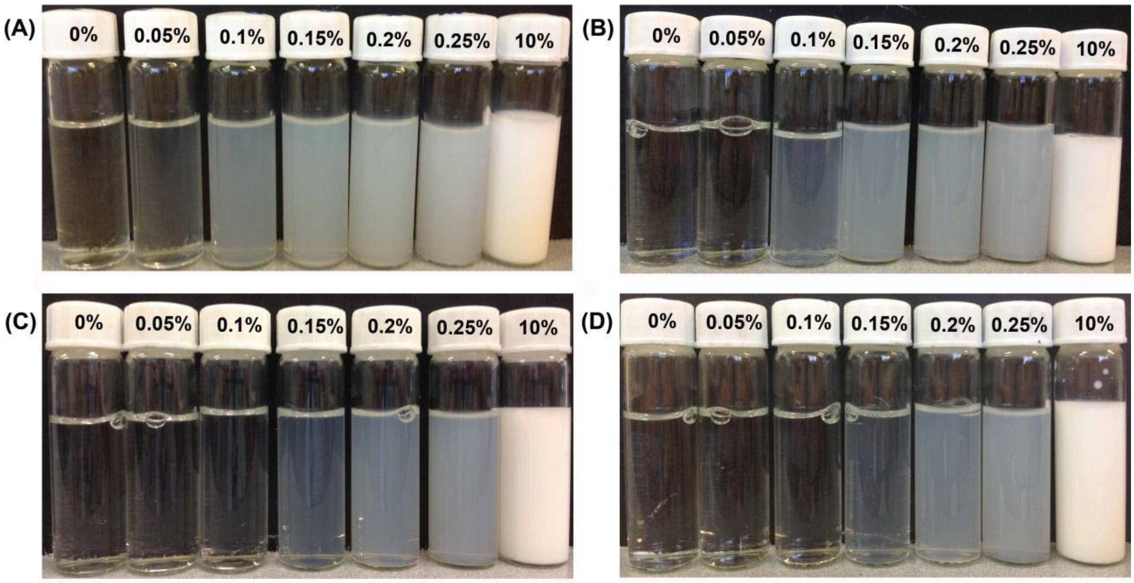


**Figure 4.9** Visual appearance of secondary emulsions prepared by titrating the stock emulsion produced by 10% w/w IPM and 1% w/w surfactant into 1% w/w surfactant solutions. (A) Tween 20, (B) Tween 40, (C) Tween 60, and (D) Tween 80. Numbers refer to IPM oil weight fraction (wt%). These pictures were taken after 1 day storage at 20$^{o}$C.

### 4.3.4 Effect of concentration of surfactant micelle solutions on droplet solubilisation

Solubilisation of emulsion oil droplets in surfactant micelles involves the transport of oil molecules from the emulsion droplets to the micelles. As a consequence, the properties of micelles play a very important role in the whole process. In the previous section, the results suggested that the longer hydrocarbon fatty acid tail of surfactant (i.e. Tween 60 and Tween 80) could incorporate more oil molecules than the shorter hydrocarbon tail of surfactant (i.e. Tween 20 and Tween 40). It was also expected that the concentration of surfactant solution would affect the solubilisation of oil droplets into micelles, meaning that the higher micelle concentration could also incorporate more oil molecules. For this reason, the influence of surfactant concentrations on the droplet solubilisation was investigated. A series of secondary emulsion samples with different IPM oil concentrations (0-0.5% w/w) were produced by mixing stock emulsions (10% IPM and 1% Tween 80) with Tween 80 surfactant solutions at different concentrations (0, 0.5, 1 and 2%) as shown in Figure 4.10. The turbidity and mean particle size of samples were measured after overnight storage at 20$^{o}$C and the results are shown in Figure 4.11 and Figure 4.12, respectively.

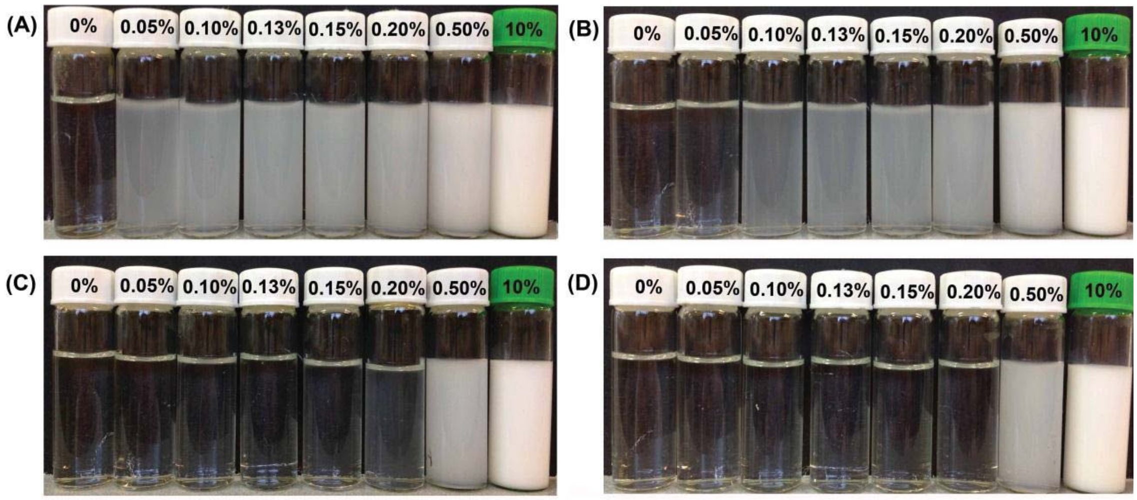


**Figure 4.10** Physical appearance of secondary emulsions prepared by titrating an emulsion (10% w/w IPM, 1% w/w Tween 80) into four different concentrations of surfactant solutions: (A) 0% w/w Tween 80 (water); (B) 0.5% w/w Tween 80; (C) 1% w/w Tween 80; (D) 2% w/w Tween 80. Numbers refer to IPM weight fraction (wt%). The pictures were taken after overnight storage at 20$^{o}$C.

There was an appreciably different behaviour observed when the stock emulsion droplets were titrated into a series of aqueous solutions containing different concentrations of Tween 80 micelles by increasing the amount of stock emulsion added into aqueous surfactant solution. In the absence of surfactant micelles, that is water, all the samples were turbid (Figure 4.10A), which suggested the oil molecules were not solubilised by the aqueous solution (i.e. water). On the other hand, in the presence of Tween 80 micelles, the appearance of samples was transparent at low levels of oil concentration and then changed from transparent to opaque as the oil droplet concentration was increased, indicating some of IPM oil molecules moved from the droplets into the micelles. The extent of this movement and solubilisation of oil droplets into the micelles increased with increasing concentration of surfactant micelles.

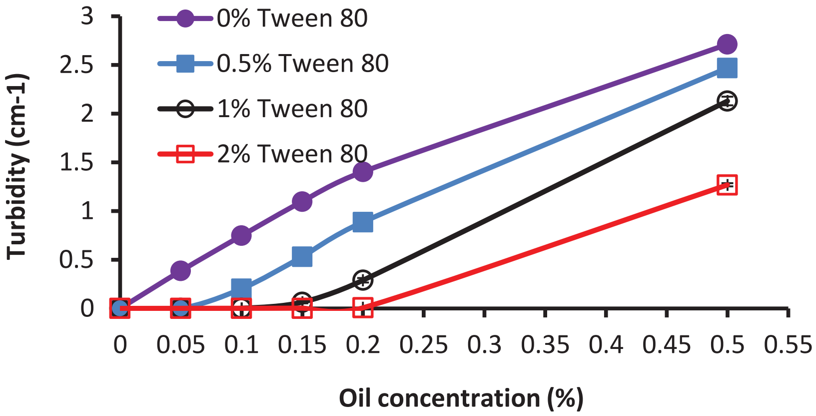


**Figure 4.11** Influence of surfactant micelle and oil concentrations on the turbidity of secondary emulsions prepared by titrating an emulsion (10% w/w IPM, 1% w/w Tween 80) into four different concentrations of surfactant solutions (0%, 0.5%, 1%, and 2% w/w Tween 80). The data was obtained by measuring absorbance at 600 nm after overnight storage at 20$^{o}$C.

The visual observations in terms of transparency were confirmed by turbidity measurements as shown in Figure 4.11. In the absence of surfactant micelles (0% Tween 80) when water was used for the titration of stock emulsion, the turbidity increased linearly with increasing oil droplet concentration. On the other hand, in the presence of Tween 80 micelles, the turbidity remained very low up to a certain oil concentration (e.g. up to 0.2% for 2% Tween 80 as shown in Figure 4.11), and then increased gradually when the droplet concentration was increased further. However, the maximum level of oil concentration at which the secondary emulsions still remained transparent increased with increasing concentration of Tween 80 micelles. This confirmed that the concentrations of Tween 80 micelle solution also played an important role in the formation of microemulsion.

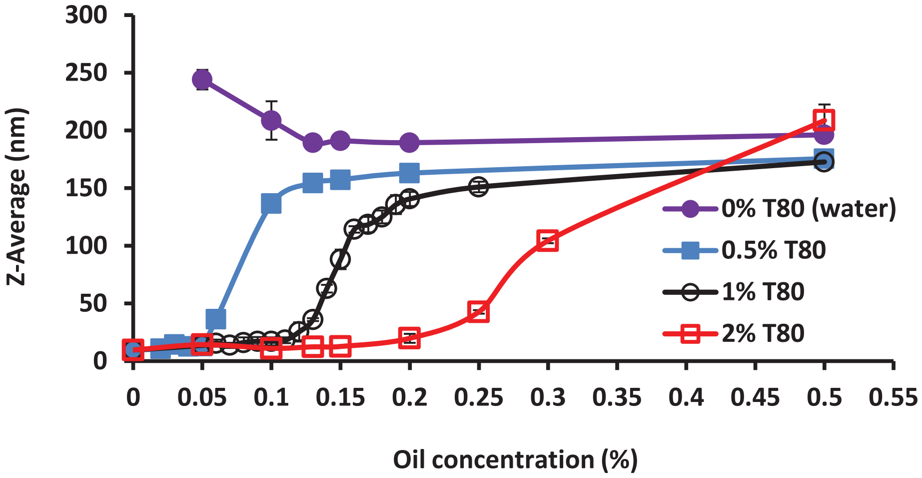


**Figure 4.12** Influence of surfactant micelle solution and oil concentration on the mean particle diameter of secondary emulsions prepared by titrating an emulsion (10% IPM and 1% Tween 80) into four different concentrations of surfactant solutions (0%, 0.5%, 1%, and 2% Tween 80). The samples were measured after overnight storage at ambient temperature.

The mean particle size of secondary emulsions is shown in Figure 4.12. The samples of secondary emulsions prepared by titration of the stock emulsion into water had a large mean particle size in a range of 200-240 nm, regardless of the final oil concentration. The size was similar to the original particle size of the stock emulsion. The results showed that the formation of secondary emulsions containing small particles (<100 nm) which can be considered 'nano- and microemulsion' could not be formed by titration (e.g. dilution) in water, regardless of the final oil concentration after dilution.

In contrast, some of the secondary emulsions prepared by titration into the Tween 80 solution containing micelles with approximately 10 nm in diameter (Table 4.2) had a very small mean particle size (<100 nm). This indicated that due to the presence of micelles, the oil molecules moved from the emulsion droplets to the Tween 80 micelles. The formation of smaller than 100 nm particles in the secondary emulsions was achieved at different levels of oil concentrations, depending on the surfactant concentration of Tween 80 solutions used to titrate (dilute) the stock emulsion. For example, in the presence of 0.5, 1.0 and 2.0% Tween 80, the particle size of smaller than 100 nm was produced at the final oil droplet concentrations of up to around 0.06, 0.15 and 0.3%, respectively, suggesting that the capacity of oil droplet solubilisation was increased with increasing surfactant micelle concentrations. More clearly, in the presence of 0.5% Tween 80 micelles, the mean particle size was increased considerably when the oil concentration was between 0.05% and 0.1%, and then it remained constant again. The samples diluted with 1% Tween 80 micelles gave rise to a similar trend for 0.5% Tween 80 micelle although there was a difference in that the increasing interval was between 0.12% and 0.2% oil. In the presence of 2% Tween 80 micelles, the mean particle size remained constant from 0% to 0.2% oil concentration, and then increased markedly up to 0.5%, which suggested the Tween 80 micelles became fully saturated with 0.5% IPM oil molecules. Any further emulsion droplets added to the system were no longer dissolved. Therefore, there was an increase in the measured mean particle size at higher oil concentration.

Generally, the oil concentration for food application in the final beverage product is very low (< 0.1%) (Ziani et al. 2012b). Hence, the formation and stability of oil droplets smaller than 100 nm in a system produced by a relatively high dilution level are very important. The results obtained in this study imply that the concentration and size of oil droplets in the formation of nano- and microemulsions by dilution of a stock emulsion stabilized by small molecule surfactant into a surfactant solution can be fabricated by modulating the concentration and type of surfactant solution.

### 4.3.5 Effect of oil type on droplet solubilisation

The ability of different types of oils (sunflower oil, tributyrin, lemon oil and Imwitor 308) to form o/w emulsions and their influence on the solubilisation of oil droplets in Tween 80 micelle solutions were examined and compared with the results of systems prepared with IMP oil as already described above. Sunflower oil is a triglyceride oil in which a number of different fatty acids are esterified on glycerol and its density is lower than water. Tributyrin is also a triglyceride oil but it is only esterified with a short chain fatty acid of butyric acid (C4:0) and its density is higher than water. Isopropyl myristate (IPM) is the ester of isopropyl alcohol and myristic acid (C14:0), and it owns low viscosity. Lemon oil is one of the most common flavour oils, which has the lowest viscosity among all the different types of oils used in this study. Imwitor 308 exists in a solid state at ambient temperature and is produced by esterification of glycerol with a short chain fatty acid of caprylic acid (C8:0) (Conley et al. 1998; Edelman et al. 2003; Grompone 2005; Liebert 1982; Raß et al. 2008; Su et al. 2004). The initial mean particle size and size distribution of stock emulsions (10% oil and 1% Tween 80) prepared with each of these oils are shown in Table 4.3 and Figure 4.13, respectively.

**Table 4.3** The particle size of initial stock emulsions (10% w/w oil and 1% w/w Tween 80) which were used to prepare the secondary emulsions.

| Oil type | Z-Average (d.nm) | PdI[a] |
|---|---|---|
| Sunflower oil | 236.13 ± 3.98 | 0.23 ± 0.01 |
| Tributyrin | 831.40 ± 148.38 | 1.00 ± 0.00 |
| IPM[b] | 239.79 ± 1.79 | 0.20 ± 0.03 |
| Lemon oil | 189.24 ± 7.23 | 0.38 ± 0.02 |
| Imwitor 308 | 874.28 ± 169.14 | 0.98 ± 0.04 |

Each value represents the mean ± SD ($n \geq 3$).

[a] PdI, polydispersity index.

[b] IPM, Isopropyl Myristate.

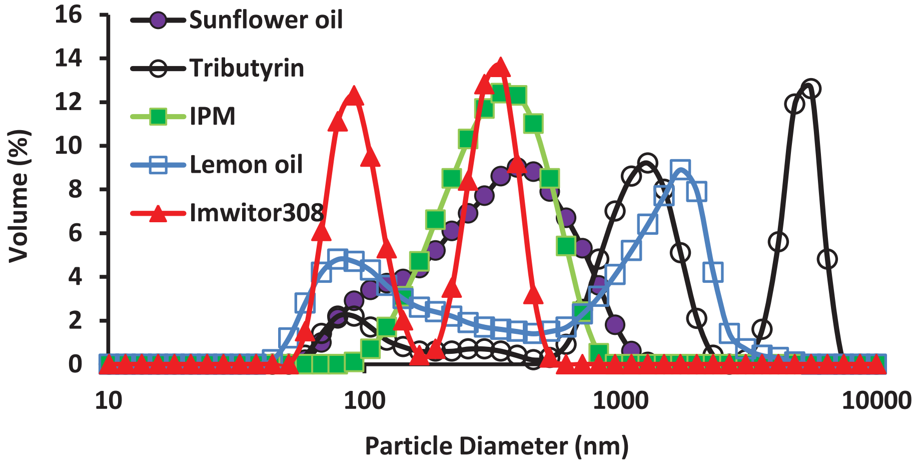


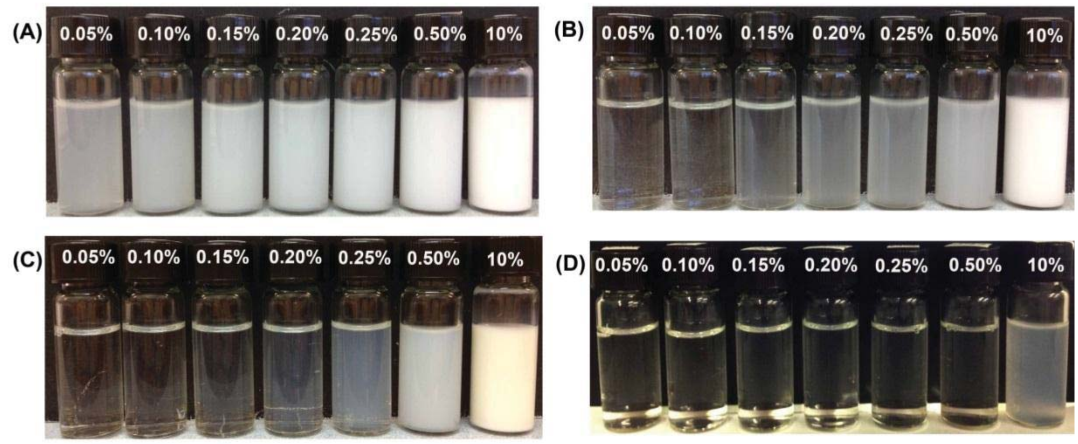
**Figure 4.13** Influence of oil type on the particle size distributions of the initial stock emulsions produced from 10% w/w oil and 1% w/w Tween 80.

The emulsions containing sunflower oil or IPM had a monomodal particle size distribution with a similar mean particle size of around 240 nm in diameter between them but the sunflower emulsion had a broader size distribution than did the IPM emulsion. In cases of the other emulsions containing lemon oil or Imwitor 308, a bimodal particle size distribution was obtained with a mean particle diameter of 189 nm and 874 nm, respectively. Tributyrin containing emulsion had a multimodal particle size distribution with a mean particle size of 831 nm. Some recent studies indicated that o/w emulsions containing lemon oil, Imwitor 308 or tributyrin as the lipid phase were highly unstable to droplet growth due to Ostwald ripening because of their relatively high water solubility (Li, Le Maux, Xiao, & McClements 2009; Rao & McClements 2012a; Ziani et al. 2012a). This means that due to Ostwald ripening, the droplets of emulsions grow rapidly from the small to the large droplets after homogenisation. Consequently, the emulsions have a population of relatively large droplets and a broad distribution. However, it is worth noting that although the lemon oil emulsion had a broad distribution, it had a relatively smaller mean particle size (189 ± 7 nm), which could partly be attributed to its low oil viscosity. This means that a high viscosity of oil phase is difficult to disrupt during the homogenisation process (McClements 2005; Walstra 2003).

Subsequently after the preparation of emulsions, a series of different amounts of the stock emulsions (10% oil and 1% Tween 80) were mixed with 1% Tween 80 solution to determine the behaviour of different oils on the formation of nano- and microemulsions. The effects of oil type and concentration on the appearance, turbidity, and mean particle size of the secondary emulsions were measured after storage overnight at 20$^{o}$C.

The visual observations of samples prepared before and after 1 day storage are shown in Figure 4.14. The sunflower oil systems were all turbid but their turbidity became increasingly opaque with the increasing oil concentration. On the other hand, the other three oil systems were transparent at low oil contents, but became substantially turbid at higher oil levels, which was similar to the IPM systems as shown earlier in the above (Figures 4.2 and 4.4). More specifically, the tributyrin system was transparent when the oil content was under 0.1%, which means from the appearance observation the maximum amount of tributyrin that can form microemulsion system was 0.1%. Concurrently, the IPM system was 0.16% (Figure 4.2), the lemon oil system was 0.25%, and the Imwitor 308 was 0.5%. It should be mentioned that the stock emulsion containing Imwitor 308 was noticeably different in its appearance being slightly turbid even the content of Imwitor 308 was 10%. According to the chemical information, 20% of water in Imwitor 308 forms a clear, low viscous liquid (Sasol 2006). The reason for this phenomenon is not clearly understood and it needs to be further investigated.

**(1) After preparation (day 0)**



**(2) After storage for 24 hours (day 1)**

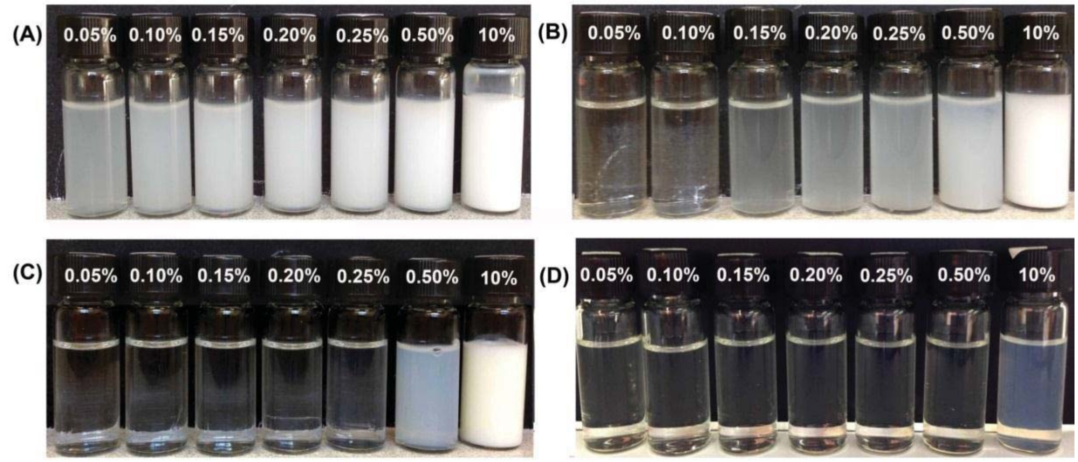


**Figure 4.14** Physical appearances of secondary emulsions prepared by titrating an emulsion (10% w/w oil, 1% w/w Tween 80) into 1% w/w Tween 80 surfactant solutions (A) Sunflower oil; (B) Tributyrin; (C) Lemon oil; (D) Imwitor 308. Numbers refers to oil weight fraction (wt%) in the secondary emulsions. The pictures were taken before (1) and after storage overnight (2) at 20$^{o}$C.

These visual observations were confirmed by turbidity measurements (Figure 4.15). For the sunflower oil system, the turbidity increased rapidly with the increasing oil concentration and then changed slowly when the oil content added was up to 0.15%. For the tributyrin system, the turbidity remained low at oil concentration from 0 to 0.1%, and then increased modestly after an obvious increase when the oil contentment was increased from 0.1 to 0.2%. The IPM system and the lemon oil system showed a similar behaviour that the turbidity remained low up to a critical oil concentration and then increased appreciably. The difference was that the critical oil concentration of the IPM system was around 0.15%, while the lemon oil system was approximately 0.25%. However, the turbidity of the Imwitor 308 system always remained almost 0 cm$^{-1}$ even up to 0.5% oil content.

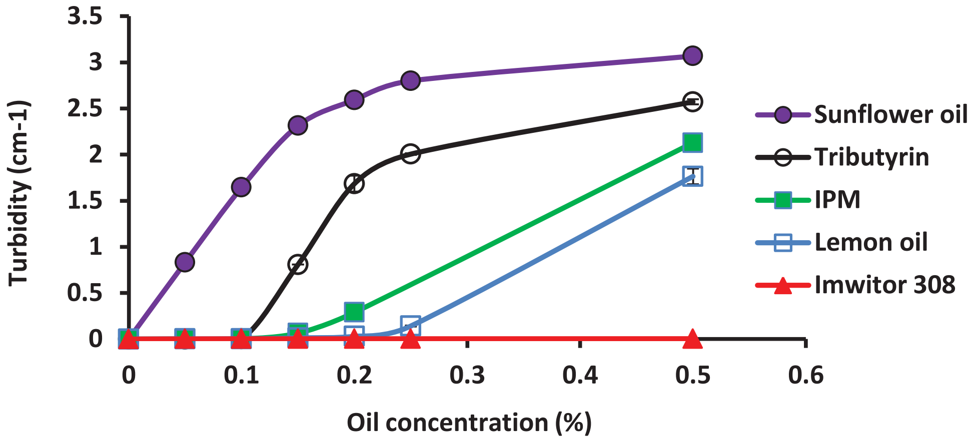


**Figure 4.15** Influence of oil type and concentration on the turbidity of secondary emulsions prepared by titrating a stock emulsion (10% oil and 1% Tween 80) into 1% Tween 80 surfactant solution. The turbidity was measured at 600 nm after overnight storage at ambient temperature.

Figure 4.16 shows that the mean particle size remained constant in the sunflower oil secondary emulsions which were similar to that of the original stock sunflower oil emulsion. This suggested that the sunflower oil droplets were not incorporated into the Tween 80 micelles. The reason can be attributed to its relatively large molecular weight and low water solubility compared to the other types of oil molecules. Surfactant micelles cannot completely accommodate hydrocarbon oil molecules in their hydrophobic interior when the hydrocarbon is significantly longer than the tail of surfactant molecules (Weiss et al. (1997). Moreover, the end of the hydrocarbon molecule may protrude into the more polar palisade layer or even into the aqueous phase. For the Imwitor 308 system, there was no change in the mean particle size when the oil content added was increased, but its particle size value ($d_{max} \approx 20$ nm) was markedly smaller than the mean particle size of its original stock emulsion (d = 874 nm). The emulsion systems made with tributyrin, IPM and lemon oil exhibited similar qualitative behaviour: the mean particle size remained small at relatively low oil concentration, then increased considerably, and later increased modestly.

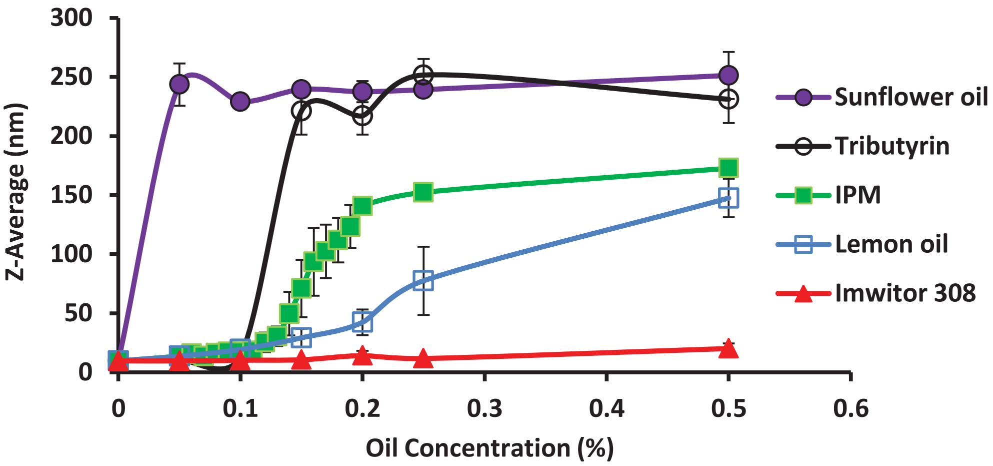


**Figure 4.16** Influence of oil type and concentration on the mean particle diameter of secondary emulsions prepared by titrating a stock emulsion (10% oil and 1% Tween 80) into 1% Tween 80 surfactant solution. The measurement was done after overnight storage at ambient temperature.

All the measurements suggested that tributyrin, IPM, lemon oil and Imwitor 308 could be used to fabricate stable microemulsions with Tween 80 micelles by using the emulsion titration method, while the sunflower oil could only form conventional emulsion due to its relatively high viscosity and large molecule dimensions. The high viscosity oil has a slower rate of solubilisation than the low viscosity oil (Ziani et al., 2012a). Moreover, some small molecules are incorporated into the hydrophobic interior of surfactant micelles (Rao & McClements 2012a; Weiss et al. 1997; Ziani et al. 2012a). In this study, the maximum amount of oil ($C_{max}$) were defined as the concentration of oil that could be incorporated into Tween 80 micelles before the mean particle size exceeded an arbitrary threshold level of 100 nm in diameter. According to the measurements, it was found that the order of $C_{max}$ was Imwitor 308 > lemon oil > IPM > tributyrin.

It should be pointed out that the molecules of Imwitor 308 could be easily incorporated within Tween 80 micelles than the other oils used. In addition, there was another possibility that some of the Imwitor 308 were directly solubilized in the aqueous phase, rather than in the surfactant micelles because 20% of water in Imwitor 308 could form a clear, low viscous liquid, according to the specification of Imwitor 308 provided by the manufacturer (Sasol 2006). This suggested that Imwitor 308 may be able to act as co-surfactants to alter the optimum curvature of the surfactant monolayer and the optimum size of the swollen micelles (Ziani et al. 2012b). With regard to tributyrin, there was a lower fraction of molecules, which were capable of being packed between the tails of surfactant molecules, compared to the IPM and lemon oil, and their present in the system may influence the stability of the emulsion droplets to coalescence and Ostwald ripening (Chanamai, Horn, & McClements 2002; Ziani et al. 2012b).

### 4.3.6 Effect of some environmental factors on droplet solubilisation

The pH and ionic strengths of emulsified foods and beverages vary substantially depending on the nature of products, and the temperatures applied during manufacture, storage, and utilization of emulsion systems can also vary. Therefore, understanding of the influence of some physical and chemical factors on the stability of emulsion oil droplets is important. Owing to its electrically neutral property, non-ionic surfactant could be compatible in any solution (Sirshendu & Sourav 2012). This is because their physicochemical properties are less affected by ionic surfactants and pH. The primary mechanism to prevent aggregation in non-ionic surfactant stabilised emulsions is driven by the short-range repulsive forces, such as steric repulsion, rather than electrostatic repulsive force (Lawrence & Rees 2000; McClements 2005). In this study, the systems stabilised by Tween 80 were investigated for their stability against pH, ionic strength and heat treatment.

#### *4.3.6.1 Influence of pH*

The initial pH of the stock emulsion prepared with 10% w/w IPM and 1% w/w Tween 80 was 5.16. The effect of pH on the stability of secondary emulsions was studied. As described above, different amounts of oil droplets from the stock emulsion were added into 1% Tween 80 solution to prepare secondary emulsions containing 0.05, 0.15, and 0.5% IPM. The pH values of these secondary emulsions were then adjusted to pH 1, 3, 5, 7, 9, and 11. After overnight storage at 20$^{o}$C, the appearance, mean particle size and particle size distribution of the secondary emulsions were analysed.

Visual observations showed that three groups of secondary emulsions remained stable to pH change with no noticeable aggregation and creaming (Figure 4.17). All 0.05% IPM secondary emulsions were clear in their optical properties, and 0.15% IPM secondary emulsions were translucent, whereas all 0.5% IPM secondary emulsions were opaque. No separation or aggregation was found among these samples, regardless of different pHs from 1-11.

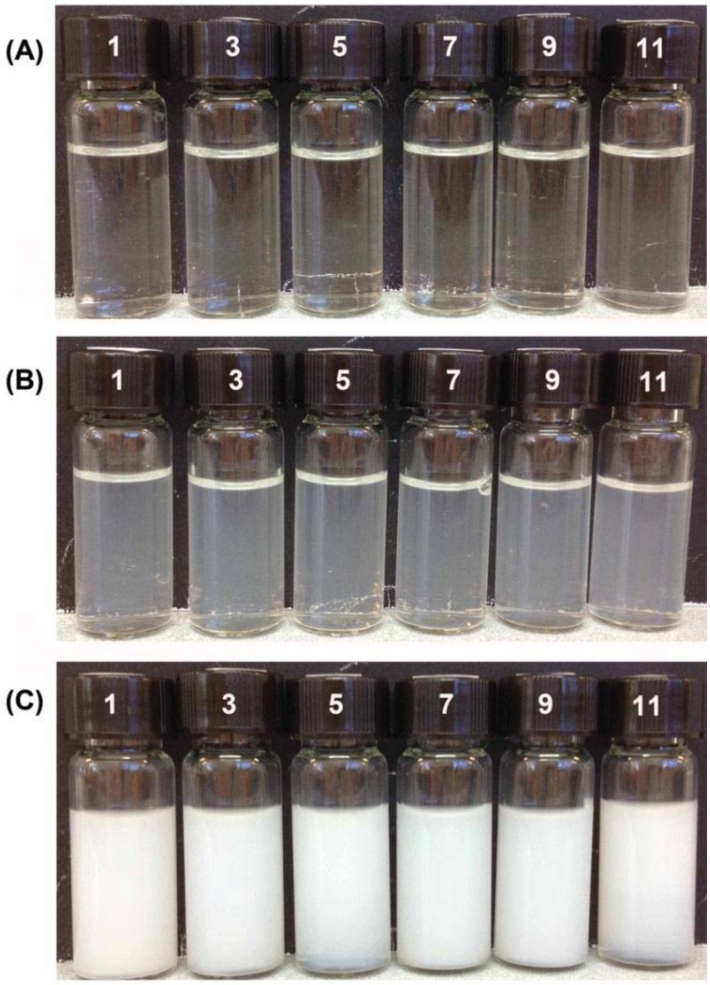


**Figure 4.17** Physical appearances of secondary emulsions (1% w/w Tween 80) with different pH value. (A) 0.05% w/w IPM; (B) 0.15% w/w IPM; (C) 0.5% w/w IPM. Numbers refer to pH values of the secondary emulsions. The pictures were taken after overnight storage at ambient temperature.

The results shown in Figures 4.18 and 4.19 illustrated that there was no significant difference in the mean particle size and size distribution for each group under different pH level. The mean particle diameters of 0.05% IPM secondary emulsions were around 15 nm; that of 0.15% IPM secondary emulsions were around 110 nm; and that of 0.5% IPM secondary emulsions were around 180 nm.

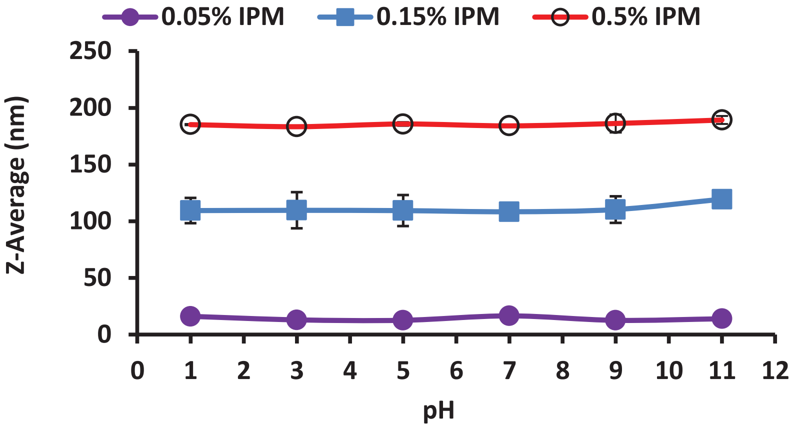


**Figure 4.18** Effect of pH on the mean particle diameter of secondary emulsions (0.05%, 0.15% and 0.5% w/w IPM: 1% w/w Tween 80).

Overall, Tween 80-stabilised secondary emulsions were stable to pH change. These results were in agreement with the other studies on pH stability of non-ionic surfactant-stabilised o/w emulsions (Lawrence & Rees 2000; McClements 2005; Sirshendu & Sourav 2012; Yang, Leser, Sher, & McClements 2013). Due to the relatively large hydrophilic (polyoxyethylene) head group of Tween molecules, steric repulsion is believed to play a main role in preventing them from droplet aggregation (Klang & Valenta 2011).

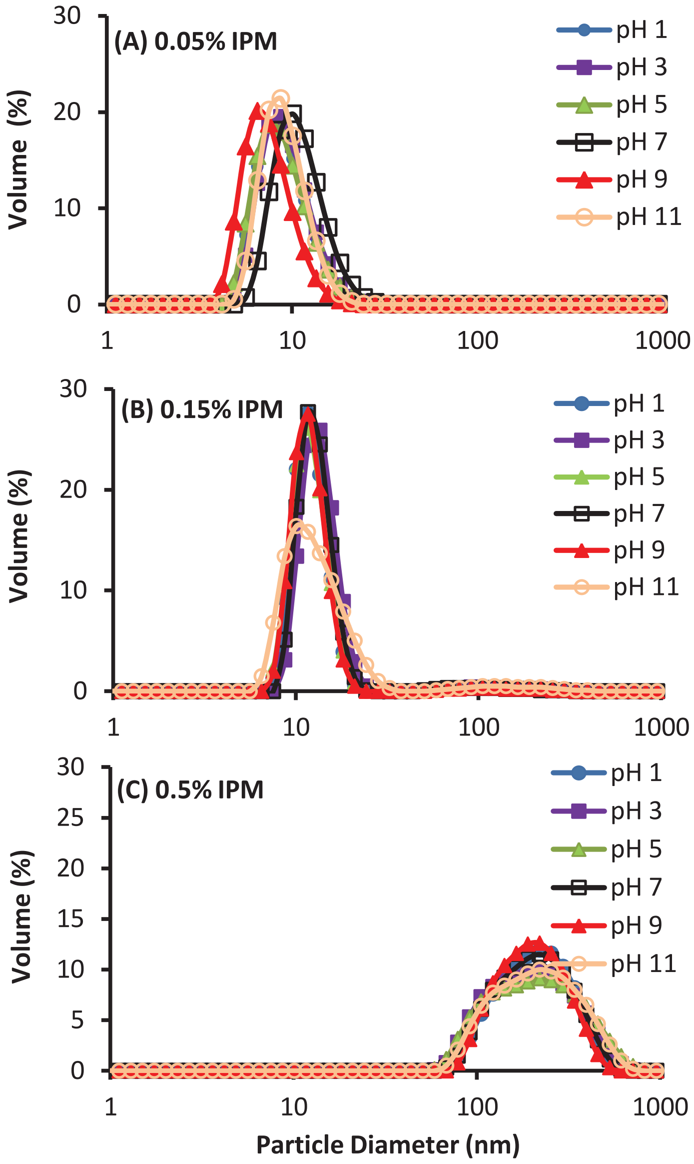


**Figure 4.19** Effect of pH on the particle size distributions of secondary emulsions (1% w/w Tween 80) (A) 0.05% w/w IPM; (B) 0.15% w/w IPM; (C) 0.5% w/w IPM.

#### *4.3.6.2 Influence of ionic strength*

The effect of ionic strength was studied by adding different concentrations of salt (0-500 mM NaCl) to three groups of secondary emulsions containing different IPM concentrations, and then examining their stability after storage overnight at 20$^{o}$C. The results were similar to that of pH influence that all samples were stable at all salt concentrations studied. There was no significant change in mean particle size for three groups (Figure 4.20), and no visible evidence of aggregation (Figure 4.21). The size distributions of each group exhibited their similar trends (Figure 4.21). All these results showed that the stability of non-ionic surfactant stabilized emulsions were not affected by pH and ionic strength and their stability against droplet aggregation is mainly driven by steric repulsion rather than electrostatic repulsion (McClements 2005; Yang et al. 2013).

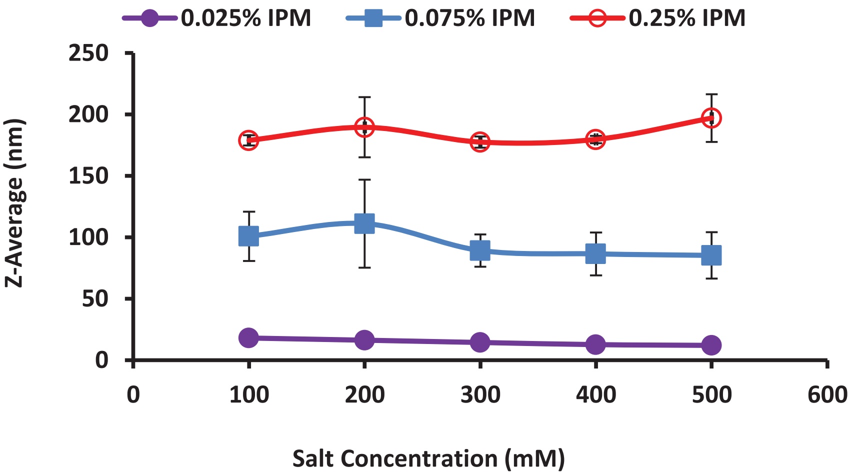


**Figure 4.20** Effect of salt (NaCl) concentration on the mean particle diameter of secondary emulsions (0.025%, 0.075% and 0.25% w/w IPM; 1% w/w Tween 80).

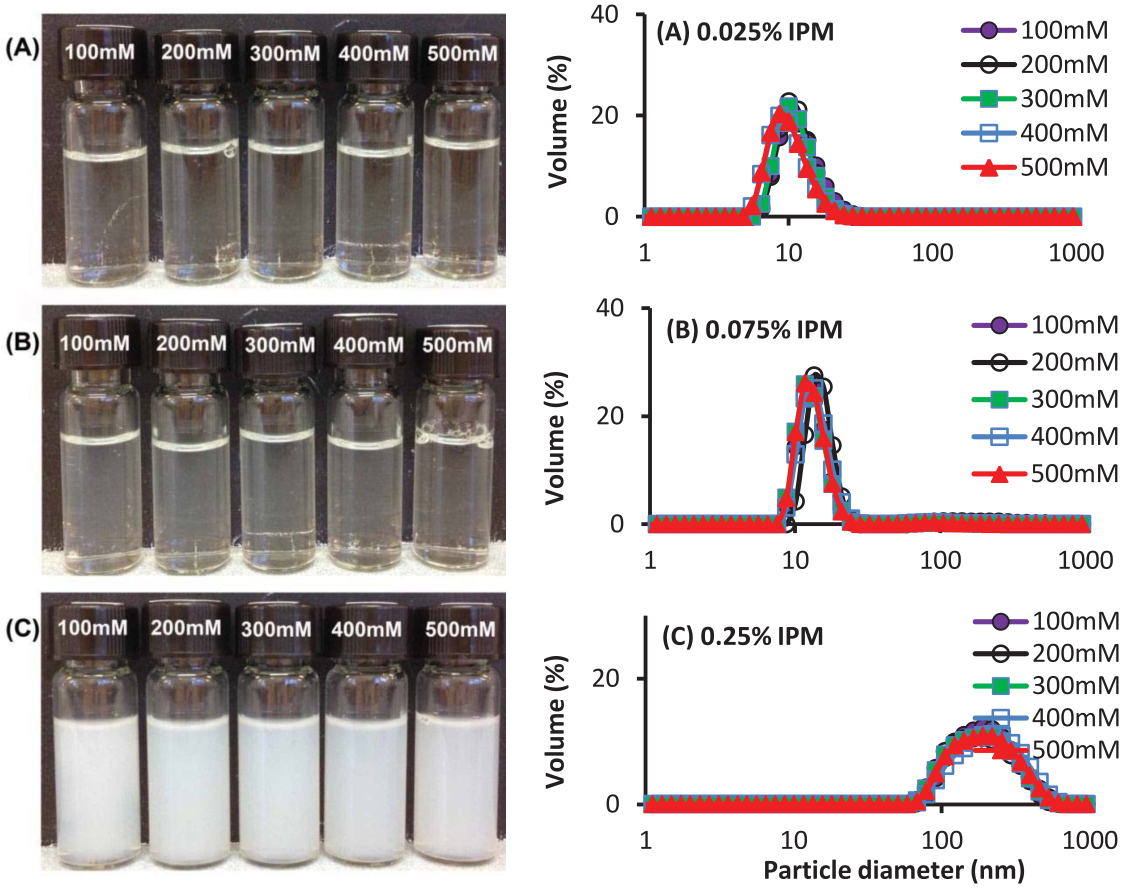


**Figure 4.21** Physical appearances and particle size distributions of secondary emulsions (1% Tween 80) containing different concentration of salt.(A) 0.025% IPM; (B) 0.075% IPM; (C) 0.25% IPM. The size and pictures were taken after overnight storage at 20°C.

#### *4.3.6.3 Influence of thermal treatment*

The influence of heat treatment was examined by heating the secondary emulsions (0.05%, 0.15% and 0.5% w/w IPM, 1% w/w Tween 80) and the stock emulsion (10% w/w IPM and 1% w/w Tween 80) at 50 and 80$^{o}$C each for 15 minutes. The visual appearance and particle size of samples were measured immediately after heat treatment and during storage for 7 days at 20$^{o}$C.

The physical appearance shown in Figure 4.22 indicated that there was not significant change for the batch of the 0.05% w/w IPM secondary emulsions and the stock emulsions (10% w/w IPM). However, appreciable difference was observed when the 0.15% and 0.5% w/w IPM secondary emulsions were heated at 80$^{o}$C. The appearances of samples were changed from opaque (0.15% IPM secondary emulsion) and translucent (0.5% IPM secondary emulsion) into transparent after heating, but as the storage time increased, their appearances were then back to their original state. The mean particle diameter of samples showed that the microemulsions (0.05% IPM secondary emulsion) had the relatively high stability to changes in their particle size over the 1 week period of storage used in this study as compared to the other samples (Figure 4.23).

The reason for these observations may be attributed to the changes in the optimum curvature (molecular geometry) or relative solubility of Tween 80 with changing temperature, as shown in Figure 4.24 (Anton & Vandamme 2009; Gutiérrez et al. 2008; McClements & Rao 2011). At low temperature, the non-ionic surfactant is more soluble in water due to its highly hydrated head group. As the temperature increases, the solubility of surfactant in water is decreased since its head group becomes progressive dehydrated. Moreover, there is a particular temperature point where the solubility of the non-ionic surfactant in the water and oil phases is equal, and when the temperature is higher than this particular temperature, the surfactant becomes more soluble in the oil phase than in the water phase (Anton & Vandamme 2009; McClements & Rao 2011). Hence, this would be a problem for applications in some food and beverage products which require some form of thermal treatment, such as pasteurization, sterilization and cooking (McClements & Rao 2011).

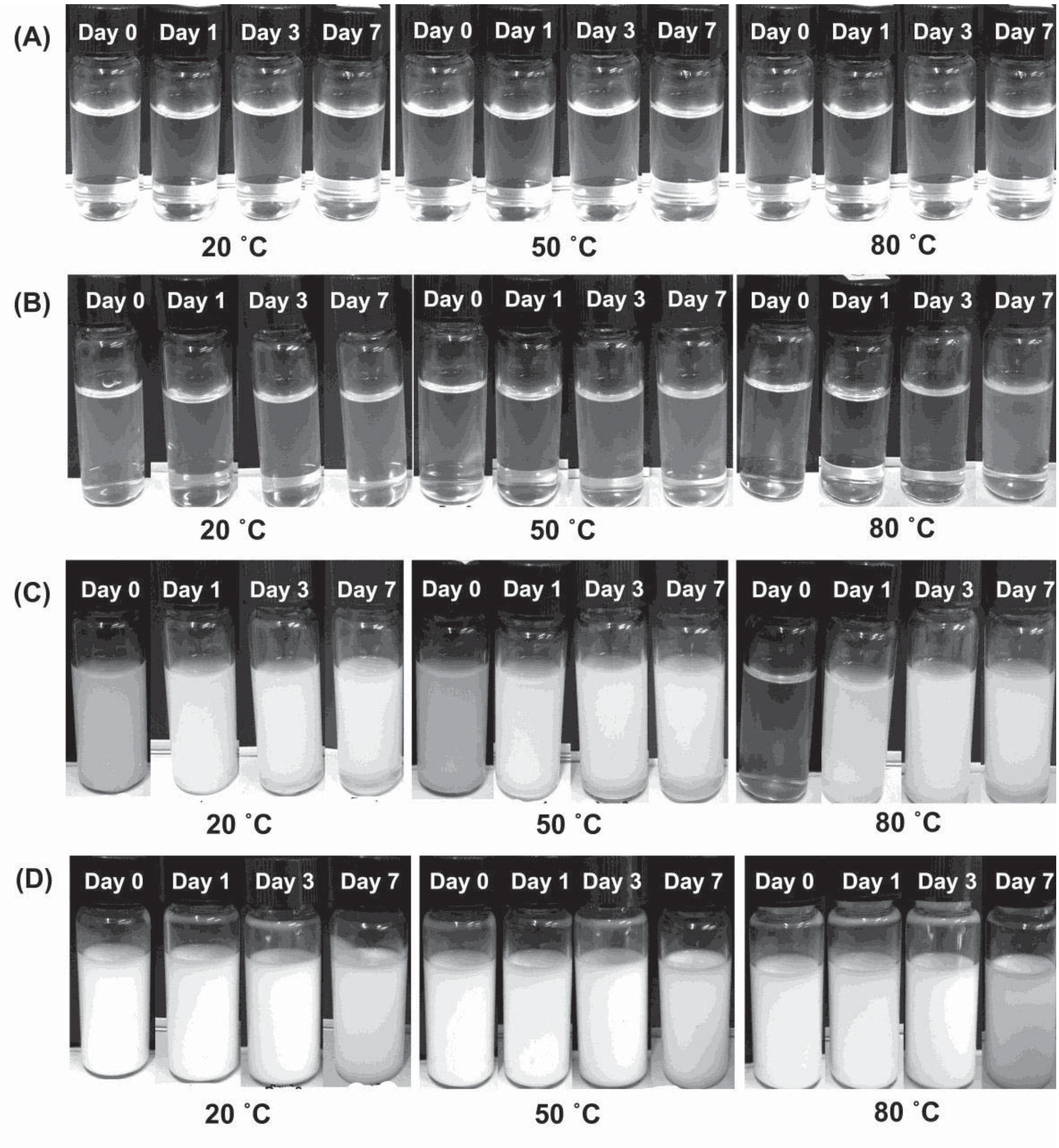


**Figure 4.22** Physical appearances of 1% w/w Tween 80 secondary emulsions or stock emulsions at 20 ˚C (blank), after heating at 50 ˚C, and after heating at 80 ˚C (A) 0.05% w/w IPM; (B) 0.15%; (C) 0.5% w/w IPM; (D) 10% w/w IPM (stock emulsions). Numbers refer to the storage day of emulsions.

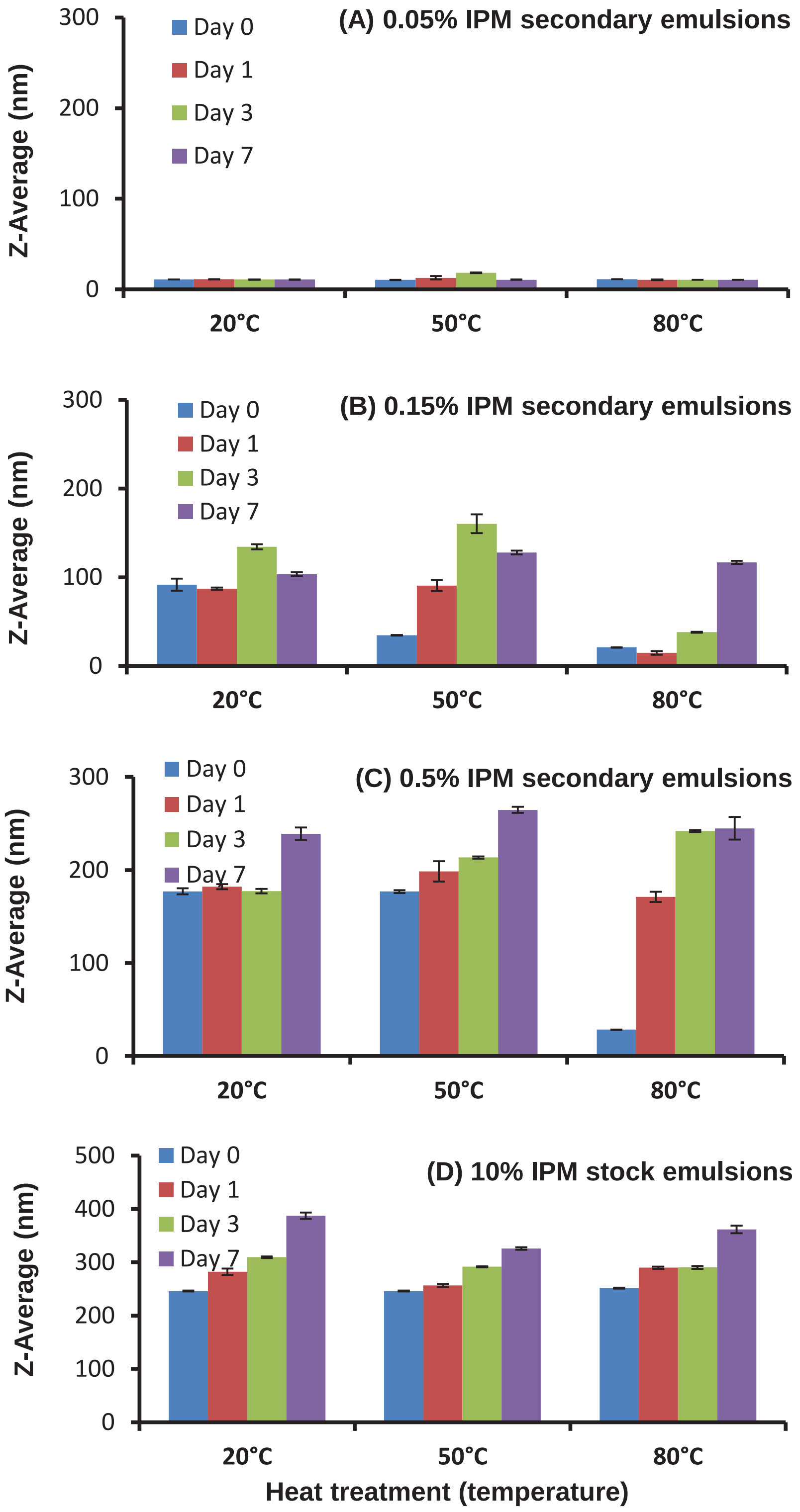


**Figure 4.23** Effect of thermal processing on the mean particle diameter (Z-Average) of 1% w/w Tween 80 secondary emulsions or stock emulsions at 20 ˚C (blank), after heating at 50 ˚C, and after heating at 80 ˚C (A) 0.05% w/w IPM; (B) 0.15%; (C) 0.5% w/w IPM; (D) 10% w/w IPM (stock emulsions).

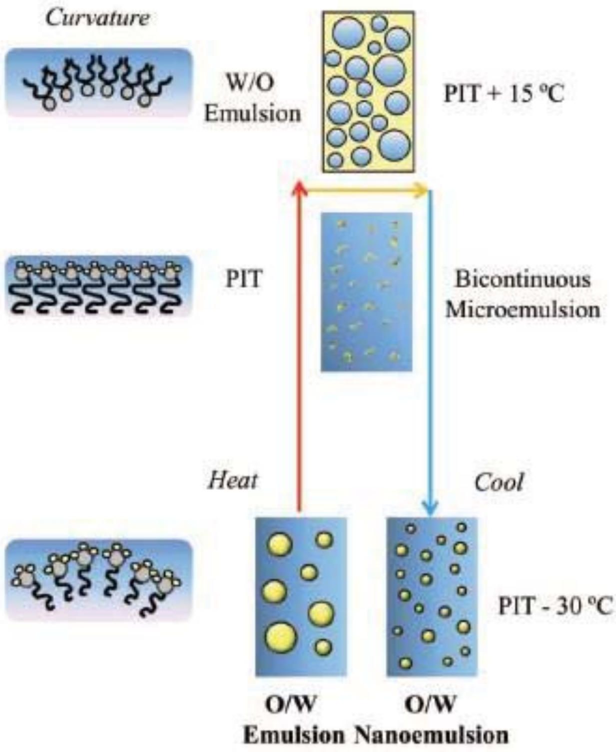


**Figure 4.24** Schematic diagram of the temperature influence on emulsion properties (McClements & Rao 2011).

### 4.3.7 Phase diagram

In order to compare the two different methods used in the preparation of nano- and microemulsions in Chapters 3 and 4, the ternary phase diagram, based on the dilution process of Tween 80-stabilised stock emulsion containing 10% lemon oil into 1% Tween 80 micellar solution by the emulsion titration method, was constructed roughly as shown in Figure 4.25. The stock emulsion (point A) prepared by using the high-pressure homogenization process was an o/w coarse emulsion with 189 nm in its mean particle size. This point in the phase diagram shown in Figure 3.2B was the area designated as multiphase including coarse emulsion. As dilution of the stock emulsion with 1% Tween 80 micelle solution increased, the mean particle size of the resulting secondary emulsions decreased. When the dilution process reached at point B where the secondary emulsion contained 0.05% lemon oil, the system turned a transparent microemulsion. This was the same as the point in Figure 3.2B denoted as the region of o/w microemulsions, meaning the results were in agreement with each other. This implies although the two methods have different principles, they both could be analyzed by the ternary phase diagrams.

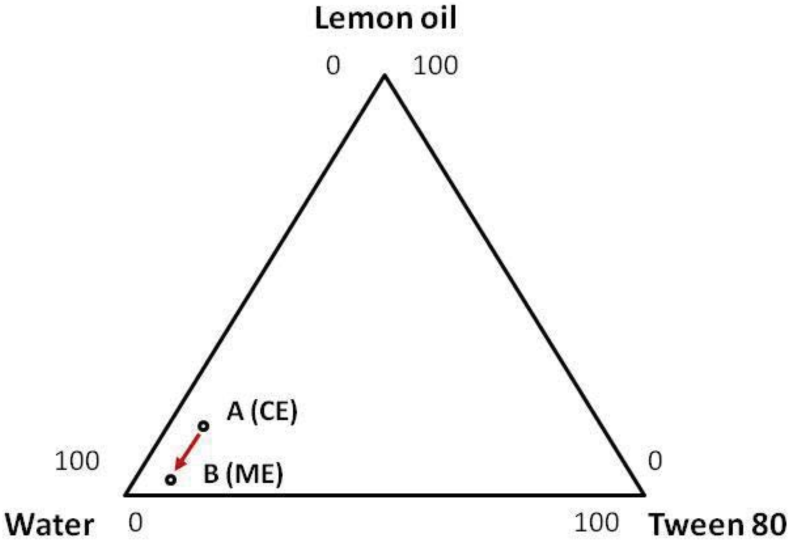


**Figure 4.25** Ternary phase diagram based on the dilution process from point A (stock lemon oil emulsion) to point B by titration of conventional Tween 80-stabilised stock emulsion into 1 wt% Tween 80 micelle solution. CE (coarse emulsion, 10% lemon oil and 1% Tween 80); ME (microemulsion, 0.05% lemon oil and 1% Tween 80).

## 4.4 Conclusions

This study focused on using an emulsion titration method to study the solubilisation of different types of oil droplets prepared by using high pressure homogenisation into different surfactant micelles to form o/w transparent microemulsions. Mass-transport of oil molecules from emulsion droplets to surfactant micelles was a relative rapid process. For all four types of surfactants used, Tween 60 and Tween 80 had the best capacity of droplet solubilisation due to their longer hydrocarbon tails compared to Tween 20 and Tween 40. Moreover, the capacity of droplet solubilisation increased with the increasing surfactant micelles concentration. The ability to form transparent o/w microemulsions also depended on the oil type. Sunflower oil molecules could not form microemulsion and remained as their original stock emulsion droplets rather than moving and solubilisation into surfactant micelles due to relatively large molecular dimensions and high viscosity. The order of the maximum amount of oil solubilisation in surfactant micelles ($C_{max}$) was Imwitor 308 > lemon oil > IPM > tributyrin. This indicated that the high viscosity oil has a lower degree of solubilisation than the low viscosity oil. Due to the systems stabilised by non-ionic surfactants, the nano- and microemulsion systems formed were stable to changes in pH and ionic strength but sensitive to thermal treatment.

# Chapter 5
# Overall Conclusions and Recommendations

The present study showed that non-ionic surfactant-stabilised transparent microemulsions could be prepared by two different approaches, phase inversion and emulsion dilution (titration). In the first approach, the phase behaviours of two different systems, lemon oil/Tween 20/water and lemon oil/Tween 80/water, were analyzed. Based on the analysis results of samples, phase diagrams were created to identify the composition of three components that allowed the formation of o/w and w/o microemulsions. The o/w microemulsions were formed in a small region of the ternary phase diagrams for both ternary systems (Tween 20 and Tween 80). In case of w/o microemulsions, they were found to form more extensively, compared to o/w microemulsion, at the corner of surfactant-rich area along dilution lines 1, 2, 3 in the phase diagrams for both Tween 20 and Tween 80 ternary systems. In addition to o/w or w/o microemulsions, some other types of phase systems were found to form in both systems, including bi- and multiphases, liquid crystals, gel and coarse emulsions.

In the second approach for the preparation of w/o nano- and microemulsions using emulsion titration method, the effects of type and concentration of surfactants (Tween 20, 40, 60 and 80) and oils (sunflower oil, tributyrin, IPM, lemon oil and Imwitor 308) on the solubilisation of oil from a pre-formed surfactant-stabilised emulsion into different types of surfactant micelle solutions were investigated. Tween 60 and Tween 80 had the better capacity of oil droplet solubilisation than that of Tween 20 and Tween 40. For each surfactant, the capacity of droplet solubilisation increased with the surfactant micelles concentration. The low viscosity oil has a faster rate of solubilisation than the high viscosity oil. Due to the relative large molecular dimension and high viscosity, microemulsions could not be formed using sunflower oil.

The two methods used in this study have a high potential for use in preparing transparent nano- and microemulsion systems to delivery lipophilic bioactive compounds for application in food, pharmaceuticals, cosmetics, etc. However, some limitations of their application in food products are that a relatively high ratio of surfactant to oil is required and most of common edible triglyceride oils cannot be used due to their large molecular dimension and high viscosity.

Based on this study, further work is recommended including:

- Use of a wider range of different types of oils and surfactants
- Use of a combination of two or more different types of emulsifiers
- Investigation of the relationship between emulsifier type and microemulsion formation, in terms of hydrophilic-lipophilic balance (HLB) system of emulsifiers,
- Effect of cosurfactant/cosolvent for nano-and microemulsions preparation.
- Encapsulation of lipophilic bioactive compounds in transparent nano- and microemulsion.
- Determination of encapsulation efficiency and compatibility of various types of bioactives in nano- and microemulsions.
- Analysis of oxidative stability of encapsulated lipophilic bioactives during storage of nano- and microemulsions under different conditions.
- Digestibility of nano- and microemulsions and release characteristics of encapsulated bioactives under simulated intestinal and gastric conditions.

# Chapter 6
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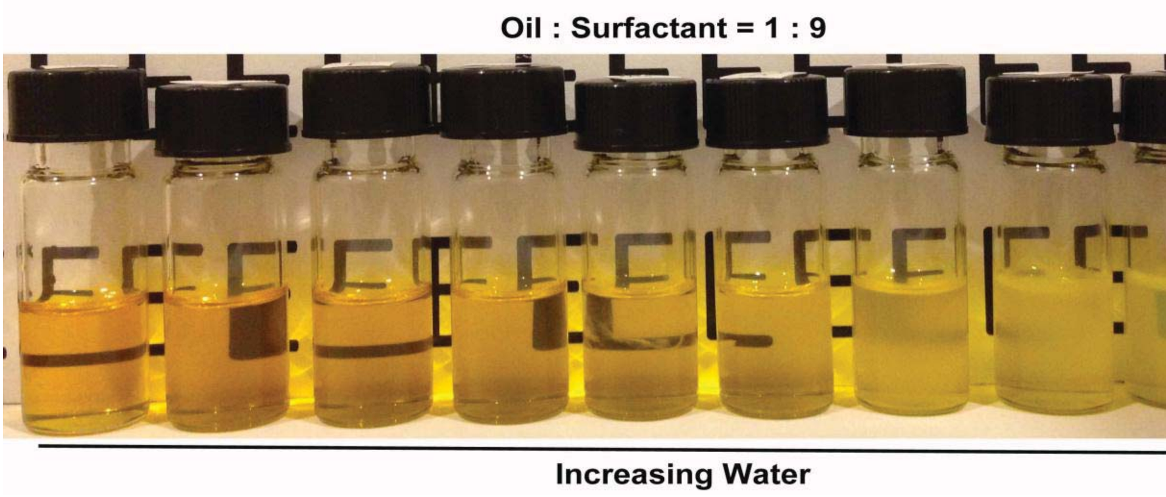
# Appendices

**Appendix 1** Real composition corresponding to Line 1 (1 : 9 dilution line) of Lemon oil/Tween 20/Water systems.

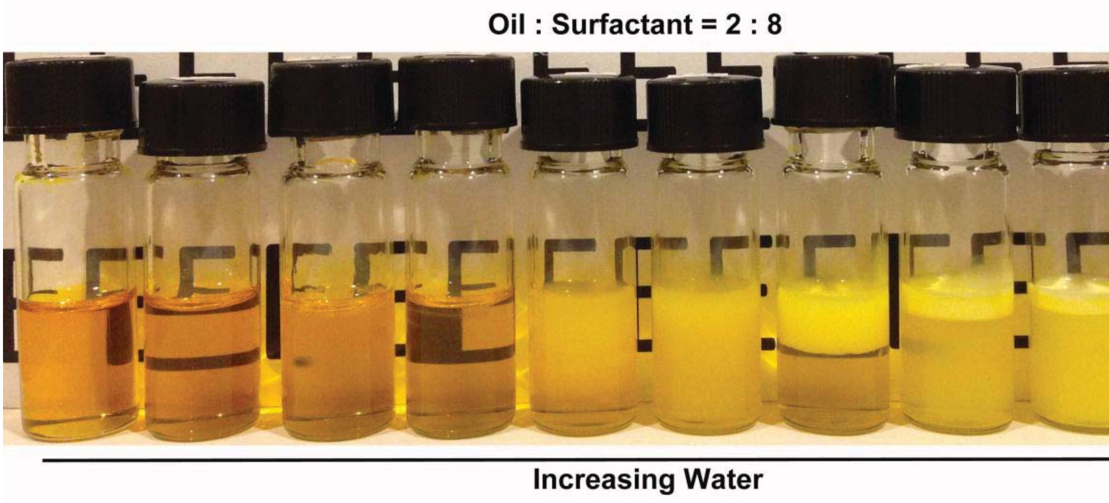
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L100 | 1.80 | 0.20 | 0.00 | 2 | 90 | 10 | 0 | 100 | 1 clear phas |
| L110 | 1.62 | 0.18 | 0.20 | 2 | 81 | 9 | 10 | 100 | 1 clear phas |
| L120 | 1.44 | 0.16 | 0.40 | 2 | 72 | 8 | 20 | 100 | 1 clear phas |
| L130 | 1.26 | 0.14 | 0.60 | 2 | 63 | 7 | 30 | 100 | 1 clear phas |
| L140 | 1.08 | 0.12 | 0.80 | 2 | 54 | 6 | 40 | 100 | 1 clear phase, red-yello |
| L150 | 0.90 | 0.10 | 1.00 | 2 | 45 | 5 | 50 | 100 | 1 clear phase, red-yello |
| L160 | 0.72 | 0.08 | 1.20 | 2 | 36 | 4 | 60 | 100 | 1 phase, tra |
| L170 | 0.54 | 0.06 | 1.40 | 2 | 27 | 3 | 70 | 100 | 1 phase, tra |
| L180 | 0.36 | 0.04 | 1.60 | 2 | 18 | 2 | 80 | 100 | 1 phase, tra |
| L190 | 0.18 | 0.02 | 1.80 | 2 | 9 | 1 | 90 | 100 | 1 cloudy |



**Appendix 2** Visual appearance of Line 1 (1 : 9 dilution line) of Lemon oil/Tween 20/Wat

**Appendix 3** Real composition corresponding to Line 2 (2 : 8 dilution line) of Lemon oil/Tween 20/Water systems.

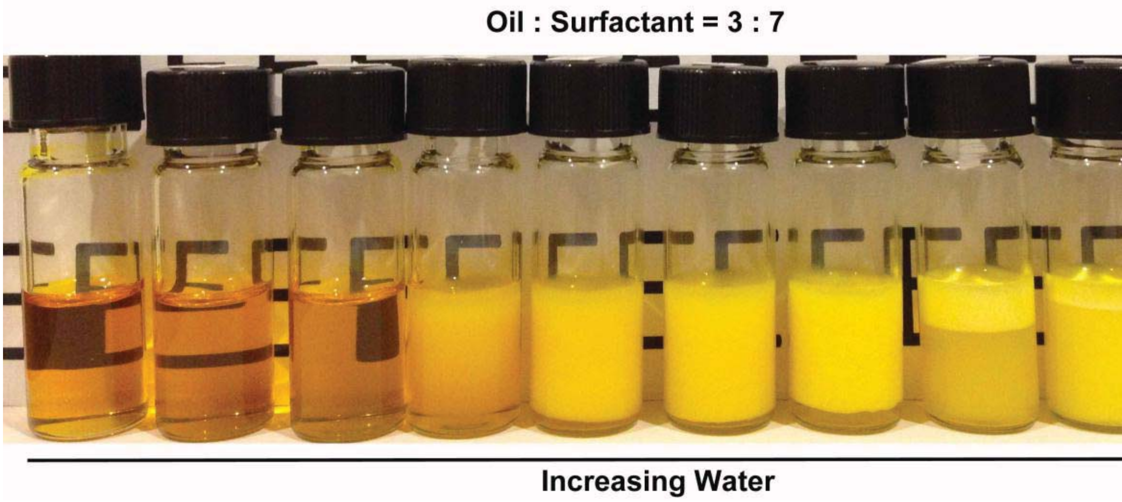
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L200 | 1.60 | 0.40 | 0.00 | 2 | 80 | 20 | 0 | 100 | 1 clear phase, |
| L210 | 1.44 | 0.36 | 0.20 | 2 | 72 | 18 | 10 | 100 | 1 clear phase, |
| L220 | 1.28 | 0.32 | 0.40 | 2 | 64 | 16 | 20 | 100 | 1 clear phase, re |
| L230 | 1.12 | 0.28 | 0.60 | 2 | 56 | 14 | 30 | 100 | 1 clear phase, re |
| L240 | 0.96 | 0.24 | 0.80 | 2 | 48 | 12 | 40 | 100 | 2 phases, upper phase t |
| L250 | 0.80 | 0.20 | 1.00 | 2 | 40 | 10 | 50 | 100 | 2 phases, upper phase tu |
| L260 | 0.64 | 0.16 | 1.20 | 2 | 32 | 8 | 60 | 100 | 2 phases, upper p |
| L270 | 0.48 | 0.12 | 1.40 | 2 | 24 | 6 | 70 | 100 | 2 phases, up |
| L280 | 0.32 | 0.08 | 1.60 | 2 | 16 | 4 | 80 | 100 | 2 phases, up |
| L290 | 0.16 | 0.04 | 1.80 | 2 | 8 | 2 | 90 | 100 | 1 turbid |



**Appendix 4** Visual appearance of Line 2 (2 : 8 dilution line) of Lemon oil/Tween 20/Wat

**Appendix 5** Real composition corresponding to Line 3 (3 : 7 dilution line) of Lemon oil/Tween 20/Water systems.

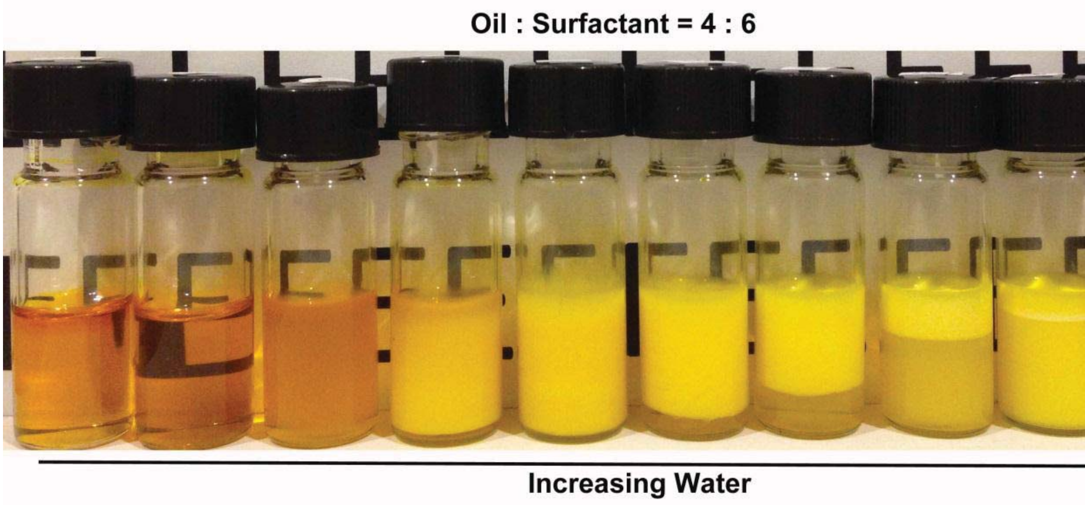
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L300 | 1.40 | 0.60 | 0.00 | 2 | 70 | 30 | 0 | 100 | 1 clear ph |
| L310 | 1.26 | 0.54 | 0.20 | 2 | 63 | 27 | 10 | 100 | 1 clear ph |
| L320 | 1.12 | 0.48 | 0.40 | 2 | 56 | 24 | 20 | 100 | 1 clear phase |
| L330 | 0.98 | 0.42 | 0.60 | 2 | 49 | 21 | 30 | 100 | 2 phases, |
| L340 | 0.84 | 0.36 | 0.80 | 2 | 42 | 18 | 40 | 100 | 2 phases, upper p |
| L350 | 0.70 | 0.30 | 1.00 | 2 | 35 | 15 | 50 | 100 | 2 phases |
| L360 | 0.56 | 0.24 | 1.20 | 2 | 28 | 12 | 60 | 100 | 2 phases |
| L370 | 0.42 | 0.18 | 1.40 | 2 | 21 | 9 | 70 | 100 | 2 phases, |
| L380 | 0.28 | 0.12 | 1.60 | 2 | 14 | 6 | 80 | 100 | 2 t |
| L390 | 0.14 | 0.06 | 1.80 | 2 | 7 | 3 | 90 | 100 | 2 t |



**Appendix 6** Visual appearance of Line 3 (3 : 7 dilution line) of Lemon oil/Tween 20/Wat

**Appendix 7** Real composition corresponding to Line 4 (4 : 6 dilution line) of Lemon oil/Tween 20/Water systems.

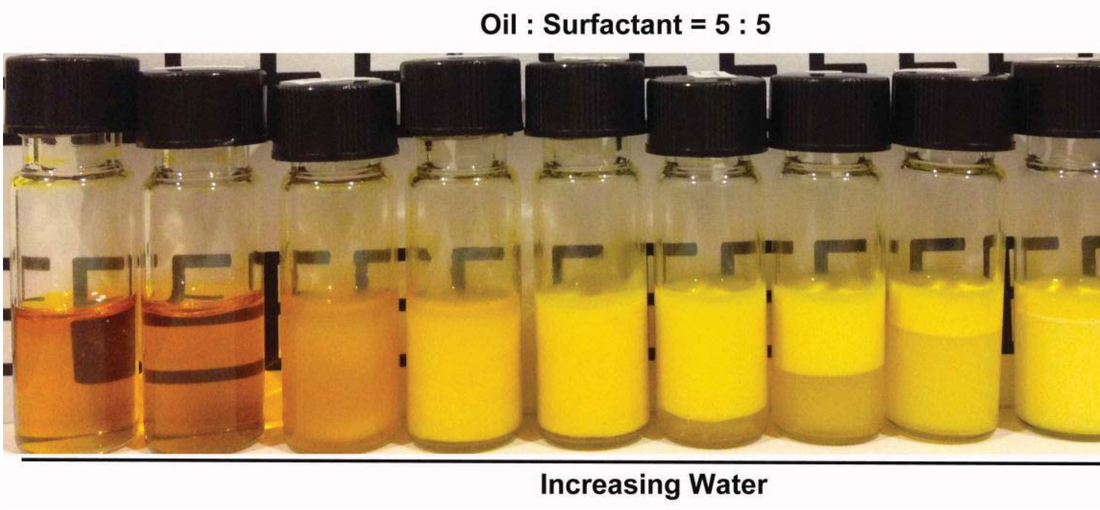
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L400 | 1.20 | 0.80 | 0.00 | 2 | 60 | 40 | 0 | 100 | 1 clear ph |
| L410 | 1.08 | 0.72 | 0.20 | 2 | 54 | 36 | 10 | 100 | 1 clear ph |
| L420 | 0.96 | 0.64 | 0.40 | 2 | 48 | 32 | 20 | 100 | 1 turbid ph |
| L430 | 0.84 | 0.56 | 0.60 | 2 | 42 | 28 | 30 | 100 | 1 turbid p |
| L440 | 0.72 | 0.48 | 0.80 | 2 | 36 | 24 | 40 | 100 | 1 turbid p |
| L450 | 0.60 | 0.40 | 1.00 | 2 | 30 | 20 | 50 | 100 | 2 phase |
| L460 | 0.48 | 0.32 | 1.20 | 2 | 24 | 16 | 60 | 100 | 2 phase |
| L470 | 0.36 | 0.24 | 1.40 | 2 | 18 | 12 | 70 | 100 | 2 phases |
| L480 | 0.24 | 0.16 | 1.60 | 2 | 12 | 8 | 80 | 100 | 2 |
| L490 | 0.12 | 0.08 | 1.80 | 2 | 6 | 4 | 90 | 100 | 2 |



**Appendix 8** Visual appearance of Line 4 (4 : 6 dilution line) of Lemon oil/Tween 20/Wat

**Appendix 9** Real composition corresponding to Line 5 (5 : 5 dilution line) of Lemon oil/Tween 20/Water systems.

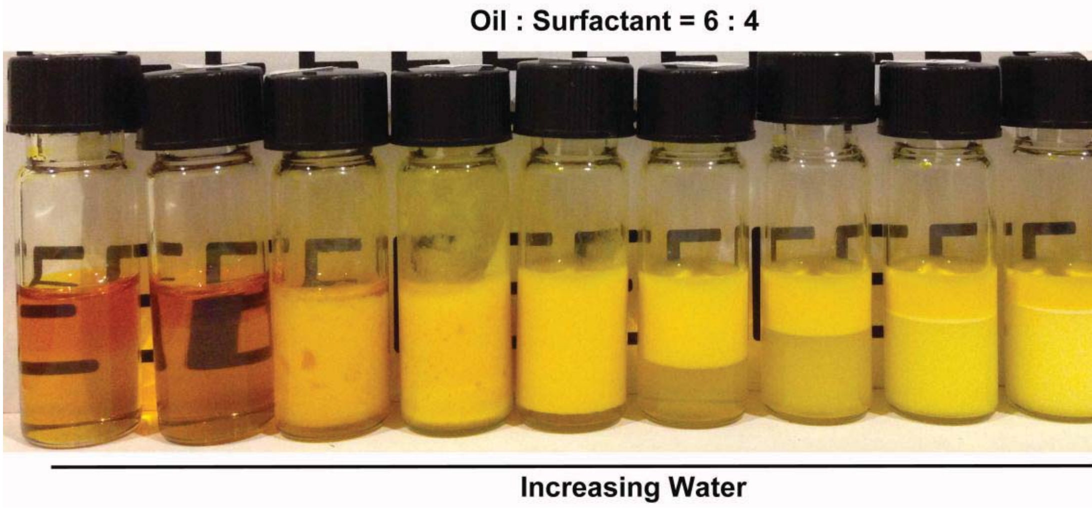
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L500 | 1.00 | 1.00 | 0.00 | 2 | 50 | 50 | 0 | 100 | 1 clea |
| L510 | 0.90 | 0.90 | 0.20 | 2 | 45 | 45 | 10 | 100 | 1 clea |
| L520 | 0.80 | 0.80 | 0.40 | 2 | 40 | 40 | 20 | 100 | 1 turbid |
| L530 | 0.70 | 0.70 | 0.60 | 2 | 35 | 35 | 30 | 100 | 1 turb |
| L540 | 0.60 | 0.60 | 0.80 | 2 | 30 | 30 | 40 | 100 | 1 turb |
| L550 | 0.50 | 0.50 | 1.00 | 2 | 25 | 25 | 50 | 100 | 2 ph |
| L560 | 0.40 | 0.40 | 1.20 | 2 | 20 | 20 | 60 | 100 | 2 ph |
| L570 | 0.30 | 0.30 | 1.40 | 2 | 15 | 15 | 70 | 100 | |
| L580 | 0.20 | 0.20 | 1.60 | 2 | 10 | 10 | 80 | 100 | |
| L590 | 0.10 | 0.10 | 1.80 | 2 | 5 | 5 | 90 | 100 | |



**Appendix 10** Visual appearance of Line 5 (5 : 5 dilution line) of Lemon oil/Tween 20/Wa

**Appendix 11** Real composition corresponding to Line 6 (6 : 4 dilution line) of Lemon oil/Tween 20/Water systems.

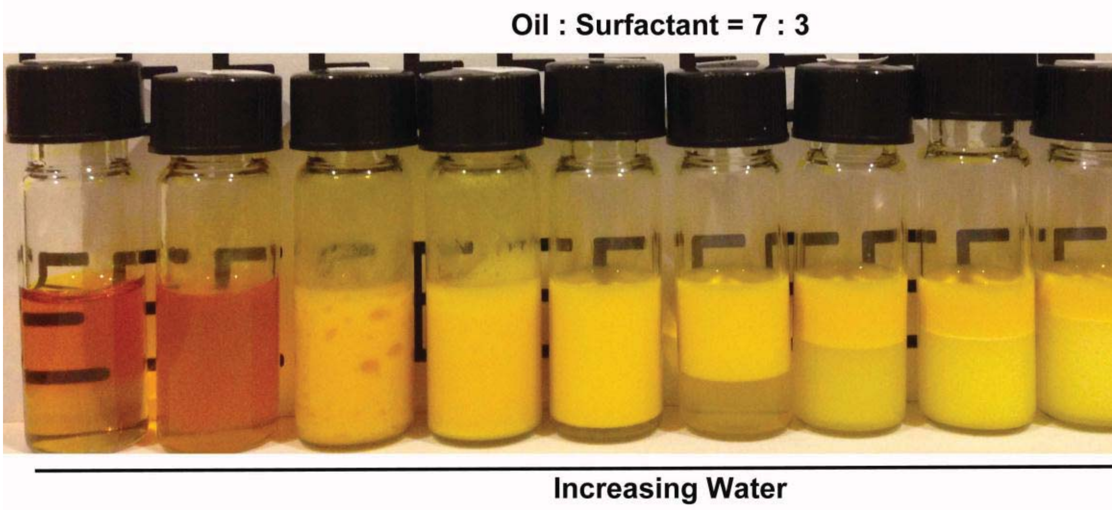
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L600 | 0.80 | 1.20 | 0.00 | 2 | 40 | 60 | 0 | 100 | 2 clear phases, u |
| L610 | 0.72 | 1.08 | 0.20 | 2 | 36 | 54 | 10 | 100 | 2 clear phases, u |
| L620 | 0.64 | 0.96 | 0.40 | 2 | 32 | 48 | 20 | 100 | 1 turbid phase |
| L630 | 0.56 | 0.84 | 0.60 | 2 | 28 | 42 | 30 | 100 | 1 turbid pha |
| L640 | 0.48 | 0.72 | 0.80 | 2 | 24 | 36 | 40 | 100 | 2 phases, up |
| L650 | 0.40 | 0.60 | 1.00 | 2 | 20 | 30 | 50 | 100 | 2 phases, |
| L660 | 0.32 | 0.48 | 1.20 | 2 | 16 | 24 | 60 | 100 | 2 phases |
| L670 | 0.24 | 0.36 | 1.40 | 2 | 12 | 18 | 70 | 100 | 2 tu |
| L680 | 0.16 | 0.24 | 1.60 | 2 | 8 | 12 | 80 | 100 | 2 tu |
| L690 | 0.08 | 0.12 | 1.80 | 2 | 4 | 6 | 90 | 100 | 2 tu |



**Appendix 12** Visual appearance of Line 6 (6 : 4 dilution line) of Lemon oil/Tween 20/Wa

**Appendix 13** Real composition corresponding to Line 7 (7 : 3 dilution line) of Lemon oil/Tween 20/Water systems.

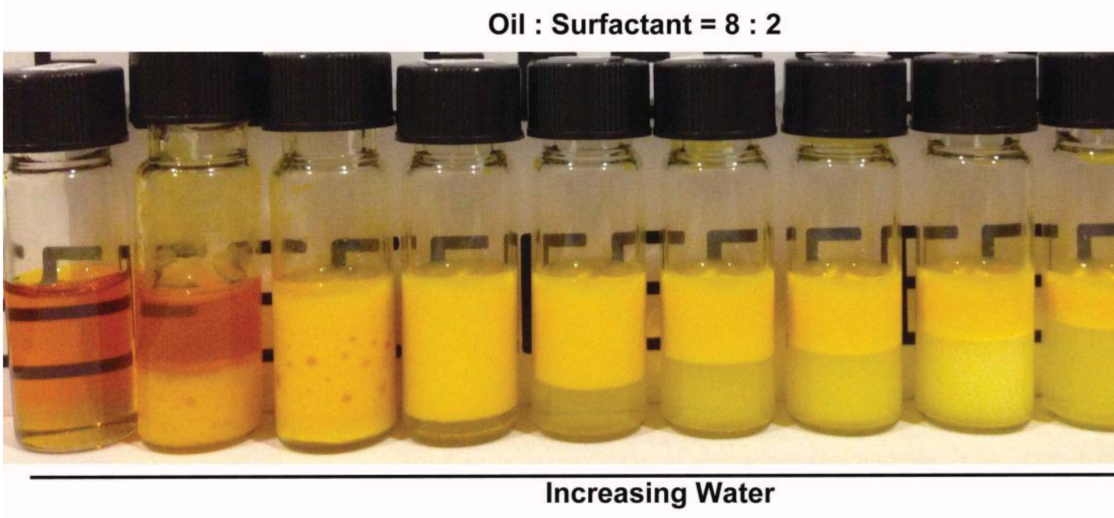
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L700 | 0.60 | 1.40 | 0.00 | 2 | 30 | 70 | 0 | 100 | 2 clear phases, |
| L710 | 0.54 | 1.26 | 0.20 | 2 | 27 | 63 | 10 | 100 | 1 turbid ph |
| L720 | 0.48 | 1.12 | 0.40 | 2 | 24 | 56 | 20 | 100 | 1 turbid ph |
| L730 | 0.42 | 0.98 | 0.60 | 2 | 21 | 49 | 30 | 100 | 1 turbid p |
| L740 | 0.36 | 0.84 | 0.80 | 2 | 18 | 42 | 40 | 100 | 2 phase |
| L750 | 0.30 | 0.70 | 1.00 | 2 | 15 | 35 | 50 | 100 | 2 phase |
| L760 | 0.24 | 0.56 | 1.20 | 2 | 12 | 28 | 60 | 100 | 2 |
| L770 | 0.18 | 0.42 | 1.40 | 2 | 9 | 21 | 70 | 100 | 2 |
| L780 | 0.12 | 0.28 | 1.60 | 2 | 6 | 14 | 80 | 100 | 2 |
| L790 | 0.06 | 0.14 | 1.80 | 2 | 3 | 7 | 90 | 100 | 2 |



**Appendix 14** Visual appearance of Line 7 (7 : 3 dilution line) of Lemon oil/Tween 20/

**Appendix15** Real composition corresponding to Line 8 (8 : 2 dilution line) of Lemon oil/Tween 20/Water systems.

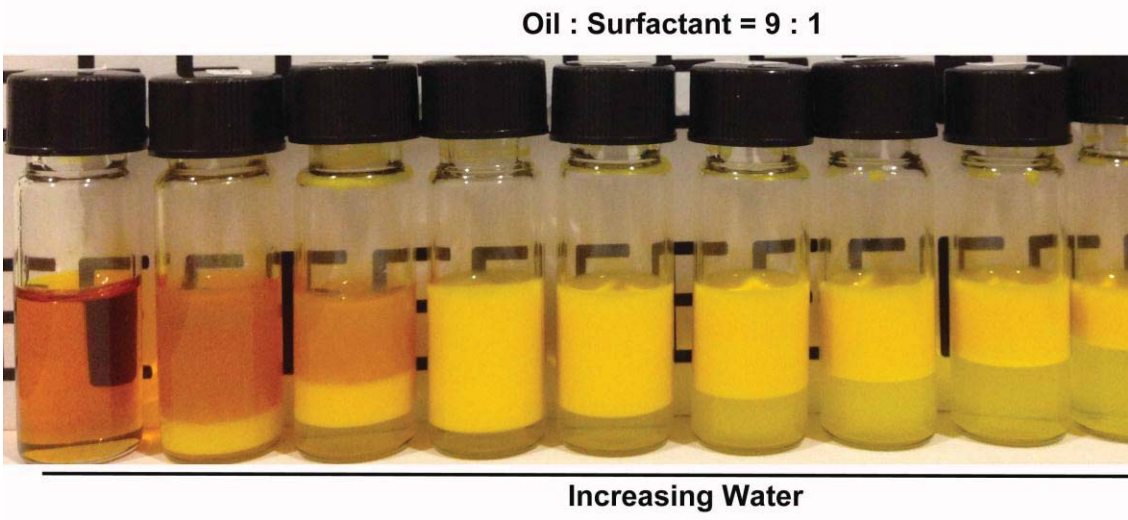
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L800 | 0.40 | 1.60 | 0.00 | 2 | 20 | 80 | 0 | 100 | 2 clear phases, |
| L810 | 0.36 | 1.44 | 0.20 | 2 | 18 | 72 | 10 | 100 | 2 phases |
| L820 | 0.32 | 1.28 | 0.40 | 2 | 16 | 64 | 20 | 100 | 1 turbid ph |
| L830 | 0.28 | 1.12 | 0.60 | 2 | 14 | 56 | 30 | 100 | 2 phases, |
| L840 | 0.24 | 0.96 | 0.80 | 2 | 12 | 48 | 40 | 100 | 2 phases, |
| L850 | 0.20 | 0.80 | 1.00 | 2 | 10 | 40 | 50 | 100 | 2 phase |
| L860 | 0.16 | 0.64 | 1.20 | 2 | 8 | 32 | 60 | 100 | 2 phase |
| L870 | 0.12 | 0.48 | 1.40 | 2 | 6 | 24 | 70 | 100 | 2 |
| L880 | 0.08 | 0.32 | 1.60 | 2 | 4 | 16 | 80 | 100 | 2 |
| L890 | 0.04 | 0.16 | 1.80 | 2 | 2 | 8 | 90 | 100 | 2 |



**Appendix 16** Visual appearance of Line 8 (8 : 2 dilution line) of Lemon oil/Tween 20/

**Appendix 17** Real composition corresponding to Line 9 (9 : 1 dilution line) of Lemon oil/Tween 20/Water systems.

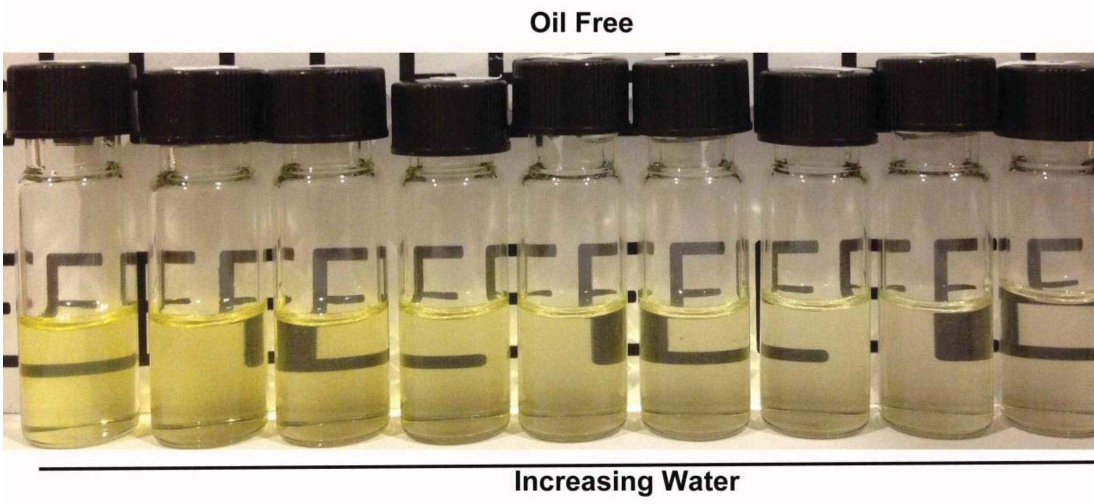
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L900 | 0.20 | 1.80 | 0.00 | 2 | 10 | 90 | 0 | 100 | 2 clear phases |
| L910 | 0.18 | 1.62 | 0.20 | 2 | 9 | 81 | 10 | 100 | 2 phases, l |
| L920 | 0.16 | 1.44 | 0.40 | 2 | 8 | 72 | 20 | 100 | 3 phases, uppe |
| L930 | 0.14 | 1.26 | 0.60 | 2 | 7 | 63 | 30 | 100 | 2 phas |
| L940 | 0.12 | 1.08 | 0.80 | 2 | 6 | 54 | 40 | 100 | 2 phas |
| L950 | 0.10 | 0.90 | 1.00 | 2 | 5 | 45 | 50 | 100 | 2 phases, lowe |
| L960 | 0.08 | 0.72 | 1.20 | 2 | 4 | 36 | 60 | 100 | 2 phases, lowe |
| L970 | 0.06 | 0.54 | 1.40 | 2 | 3 | 27 | 70 | 100 | 2 phases, lowe |
| L980 | 0.04 | 0.36 | 1.60 | 2 | 2 | 18 | 80 | 100 | 2 phases, lowe |
| L990 | 0.02 | 0.18 | 1.80 | 2 | 1 | 9 | 90 | 100 | 2 phases, lowe |



**Appendix 18** Visual appearance of Line 9 (9 : 1 dilution line) of Lemon oil/Tween 20/

**Appendix 19** Real composition corresponding to Line 10 (Oil free) of Lemon oil/Tween 20/Water systems.

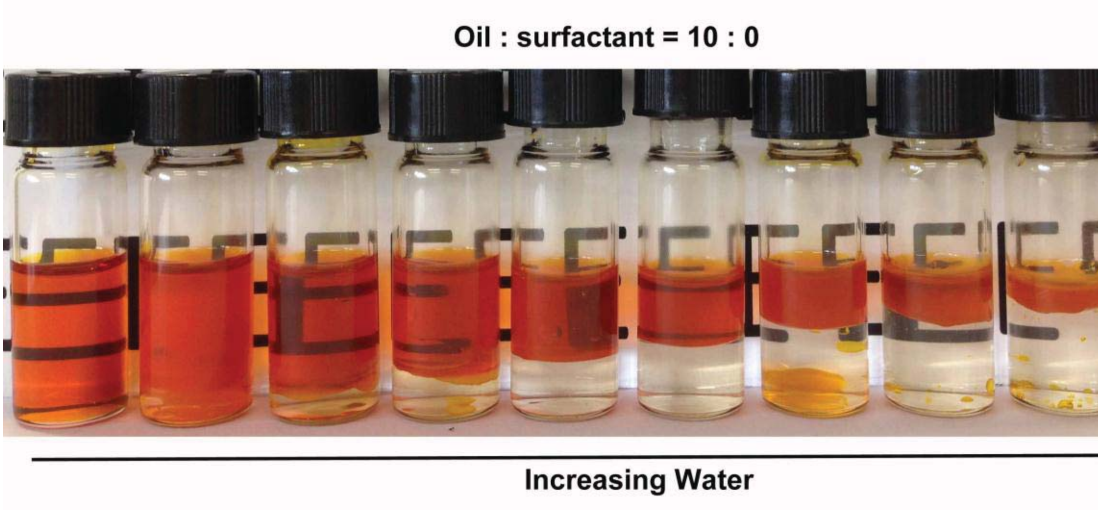
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L1000 | 2.00 | 0.00 | 0.00 | 2 | 100 | 0 | 0 | 100 | 1 clear phas |
| L1010 | 1.80 | 0.00 | 0.20 | 2 | 90 | 0 | 10 | 100 | 1 clear phas |
| L1020 | 1.60 | 0.00 | 0.40 | 2 | 80 | 0 | 20 | 100 | 1 clear phas |
| L1030 | 1.40 | 0.00 | 0.60 | 2 | 70 | 0 | 30 | 100 | 1 clear phas |
| L1040 | 1.20 | 0.00 | 0.80 | 2 | 60 | 0 | 40 | 100 | 1 clear phas |
| L1050 | 1.00 | 0.00 | 1.00 | 2 | 50 | 0 | 50 | 100 | 1 clear phas |
| L1060 | 0.80 | 0.00 | 1.20 | 2 | 40 | 0 | 60 | 100 | 1 clear |
| L1070 | 0.60 | 0.00 | 1.40 | 2 | 30 | 0 | 70 | 100 | |
| L1080 | 0.40 | 0.00 | 1.60 | 2 | 20 | 0 | 80 | 100 | |
| L1090 | 0.20 | 0.00 | 1.80 | 2 | 10 | 0 | 90 | 100 | |



**Appendix 20** Visual appearance of Line 10 (Oil free) of Lemon oil/Tween 20/Wat

**Appendix 21** Real composition corresponding to Line 11 (surfactant free) of Lemon oil/Tween 20/Water systems.

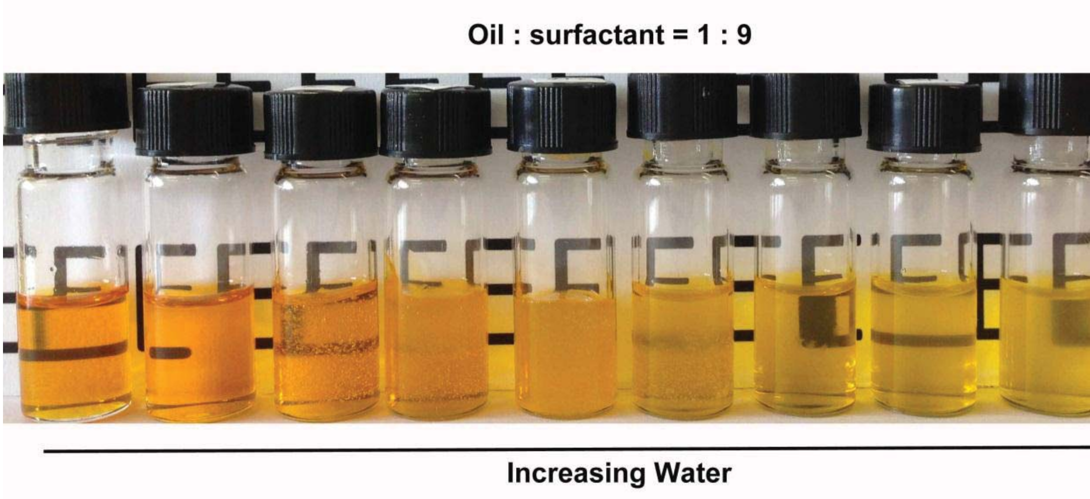
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Surfactant | Oil | Water | Total | Surfactant | Oil | Water | Total | |
| L1100 | 2.00 | 0.00 | 0.00 | 2 | 100 | 0 | 0 | 100 | 1 c |
| L1110 | 1.80 | 0.00 | 0.20 | 2 | 90 | 0 | 10 | 100 | 2 phase, upper p |
| L1120 | 1.60 | 0.00 | 0.40 | 2 | 80 | 0 | 20 | 100 | 2 phase, upper p |
| L1130 | 1.40 | 0.00 | 0.60 | 2 | 70 | 0 | 30 | 100 | 2 phase, upper p |
| L1140 | 1.20 | 0.00 | 0.80 | 2 | 60 | 0 | 40 | 100 | 2 phase, upper p |
| L1150 | 1.00 | 0.00 | 1.00 | 2 | 50 | 0 | 50 | 100 | 2 phase, upper p |
| L1160 | 0.80 | 0.00 | 1.20 | 2 | 40 | 0 | 60 | 100 | 2 phase, upper p |
| L1170 | 0.60 | 0.00 | 1.40 | 2 | 30 | 0 | 70 | 100 | 2 phase, upper p |
| L1180 | 0.40 | 0.00 | 1.60 | 2 | 20 | 0 | 80 | 100 | 2 phase, upper p |
| L1190 | 0.20 | 0.00 | 1.80 | 2 | 10 | 0 | 90 | 100 | 2 phase, upper p |



**Appendix 22** Visual appearance of Line 11 (surfactant free) of Lemon oil/Tween 20/

**Appendix 23** Real composition corresponding to Line 1 (1 : 9 dilution line) of Lemon oil/Tween 80/Water systems.

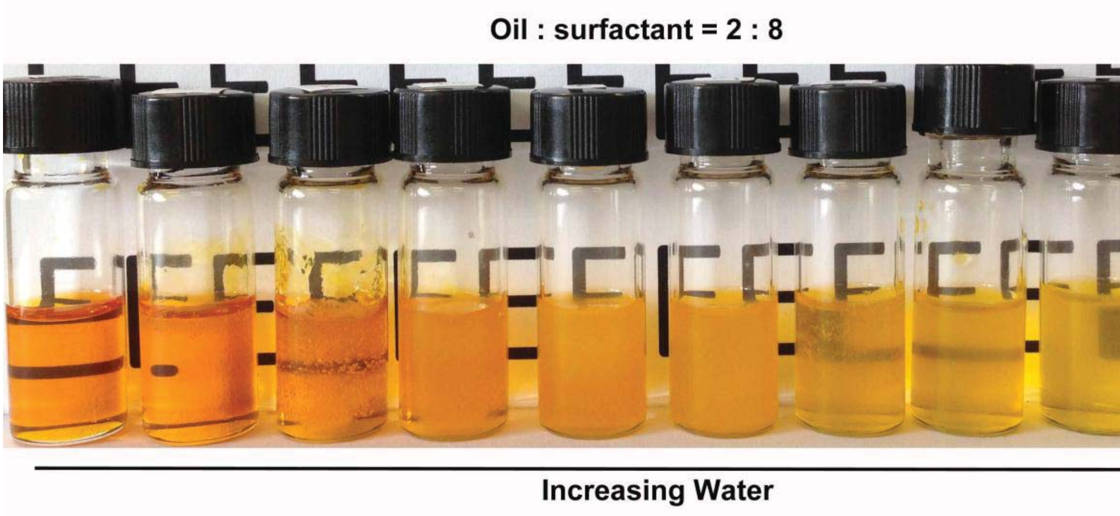
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L100 | 0.20 | 1.80 | 0.00 | 2 | 10 | 90 | 0 | 100 | 1 cle |
| L110 | 0.18 | 1.62 | 0.20 | 2 | 9 | 81 | 10 | 100 | 1 clear phase, |
| L120 | 0.16 | 1.44 | 0.40 | 2 | 8 | 72 | 20 | 100 | 1 clear phase, re |
| L130 | 0.14 | 1.26 | 0.60 | 2 | 7 | 63 | 30 | 100 | 2 phase, upper |
| L140 | 0.12 | 1.08 | 0.80 | 2 | 6 | 54 | 40 | 100 | 1 |
| L150 | 0.10 | 0.90 | 1.00 | 2 | 5 | 45 | 50 | 100 | 2 clear phas |
| L160 | 0.08 | 0.72 | 1.20 | 2 | 4 | 36 | 60 | 100 | 1 clear |
| L170 | 0.06 | 0.54 | 1.40 | 2 | 3 | 27 | 70 | 100 | 1 c |
| L180 | 0.04 | 0.36 | 1.60 | 2 | 2 | 18 | 80 | 100 | 1 c |
| L190 | 0.02 | 0.18 | 1.80 | 2 | 1 | 9 | 90 | 100 | 1 c |



**Appendix 24** Visual appearance of Line 1 (1 : 9 dilution line) of Lemon oil/Tween 80/

**Appendix 25** Real composition corresponding to Line 2 (2 : 8 dilution line) of Lemon oil/Tween 80/Water systems.

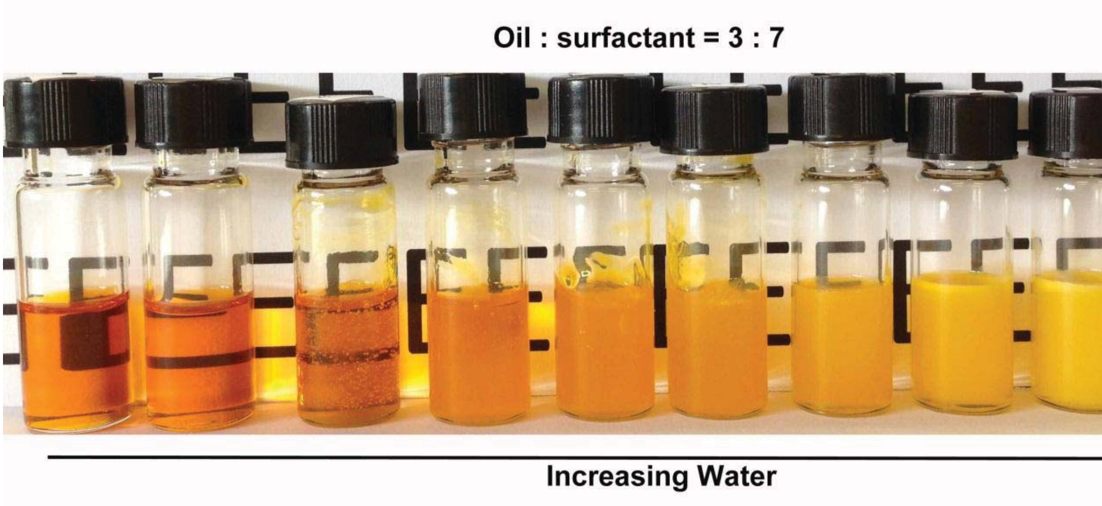
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L200 | 0.40 | 1.60 | 0.00 | 2 | 20 | 80 | 0 | 100 | 1 clea |
| L210 | 0.36 | 1.44 | 0.20 | 2 | 18 | 72 | 10 | 100 | 1 clear pha |
| L220 | 0.32 | 1.28 | 0.40 | 2 | 16 | 64 | 20 | 100 | 1 clear phas |
| L230 | 0.28 | 1.12 | 0.60 | 2 | 14 | 56 | 30 | 100 | 1 turbid ph |
| L240 | 0.24 | 0.96 | 0.80 | 2 | 12 | 48 | 40 | 100 | 1 turbid ph |
| L250 | 0.20 | 0.80 | 1.00 | 2 | 10 | 40 | 50 | 100 | 1 turbid ph |
| L260 | 0.16 | 0.64 | 1.20 | 2 | 8 | 32 | 60 | 100 | 1 cl |
| L270 | 0.12 | 0.48 | 1.40 | 2 | 6 | 24 | 70 | 100 | 1 cl |
| L280 | 0.08 | 0.32 | 1.60 | 2 | 4 | 16 | 80 | 100 | 1 cl |
| L290 | 0.04 | 0.16 | 1.80 | 2 | 2 | 8 | 90 | 100 | 1 cl |



**Appendix 26** Visual appearance of Line 2 (2 : 8 dilution line) of Lemon oil/Tween 80/

**Appendix 27** Real composition corresponding to Line 3 (3 : 7 dilution line) of Lemon oil/Tween 80/Water systems.

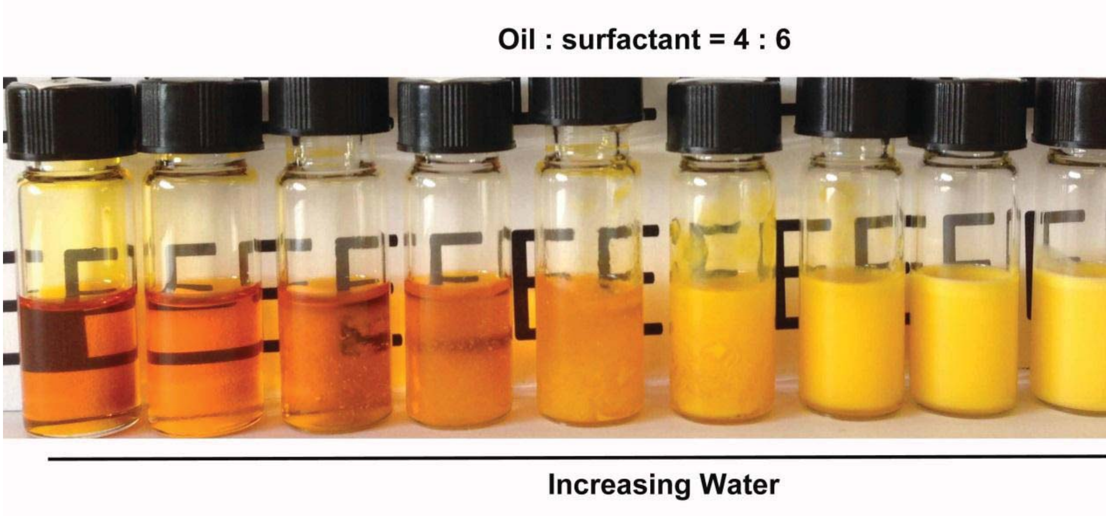
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L300 | 0.60 | 1.40 | 0.00 | 2 | 30 | 70 | 0 | 100 | 1 cle |
| L310 | 0.54 | 1.26 | 0.20 | 2 | 27 | 63 | 10 | 100 | 1 clear p |
| L320 | 0.48 | 1.12 | 0.40 | 2 | 24 | 56 | 20 | 100 | 1 clear pha |
| L330 | 0.42 | 0.98 | 0.60 | 2 | 21 | 49 | 30 | 100 | 2 phases, low |
| L340 | 0.36 | 0.84 | 0.80 | 2 | 18 | 42 | 40 | 100 | 1 turbid |
| L350 | 0.30 | 0.70 | 1.00 | 2 | 15 | 35 | 50 | 100 | 1 turbid |
| L360 | 0.24 | 0.56 | 1.20 | 2 | 12 | 28 | 60 | 100 | 1 turbi |
| L370 | 0.18 | 0.42 | 1.40 | 2 | 9 | 21 | 70 | 100 | 2 turbid phase |
| L380 | 0.12 | 0.28 | 1.60 | 2 | 6 | 14 | 80 | 100 | 2 turbid phase |
| L390 | 0.06 | 0.14 | 1.80 | 2 | 3 | 7 | 90 | 100 | 1 t |



**Appendix 28** Visual appearance of Line 3 (3 : 7 dilution line) of Lemon oil/Tween 80/

**Appendix 29** Real composition corresponding to Line 4 (4 : 6 dilution line) of Lemon oil/Tween 80/Water systems.

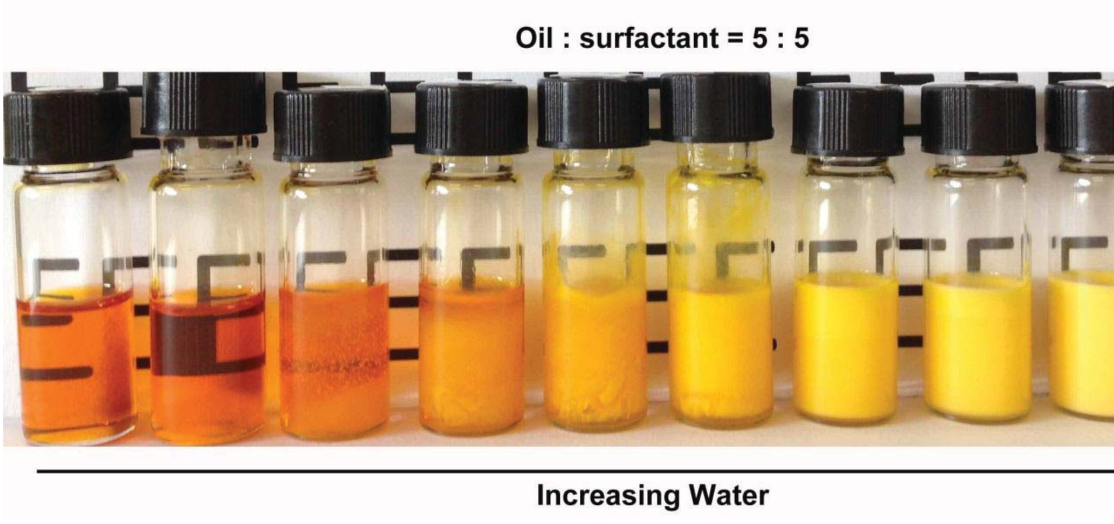
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L400 | 0.80 | 1.20 | 0.00 | 2 | 40 | 60 | 0 | 100 | 1 cle |
| L410 | 0.72 | 1.08 | 0.20 | 2 | 36 | 54 | 10 | 100 | 1 clear ph |
| L420 | 0.64 | 0.96 | 0.40 | 2 | 32 | 48 | 20 | 100 | 1 phase, red-yello |
| L430 | 0.56 | 0.84 | 0.60 | 2 | 28 | 42 | 30 | 100 | 2 phases, lower |
| L440 | 0.48 | 0.72 | 0.80 | 2 | 24 | 36 | 40 | 100 | 1 turbid p |
| L450 | 0.40 | 0.60 | 1.00 | 2 | 20 | 30 | 50 | 100 | 1 turbid p |
| L460 | 0.32 | 0.48 | 1.20 | 2 | 16 | 24 | 60 | 100 | 1 turbid |
| L470 | 0.24 | 0.36 | 1.40 | 2 | 12 | 18 | 70 | 100 | 2 turbid phase |
| L480 | 0.16 | 0.24 | 1.60 | 2 | 8 | 12 | 80 | 100 | 2 turbid phase |
| L490 | 0.08 | 0.12 | 1.80 | 2 | 4 | 6 | 90 | 100 | 1 tu |



**Appendix 30** Visual appearance of Line 4 (4 : 6 dilution line) of Lemon oil/Tween 80/

**Appendix 31** Real composition corresponding to Line 5 (5 : 5 dilution line) of Lemon oil/Tween 80/Water systems.

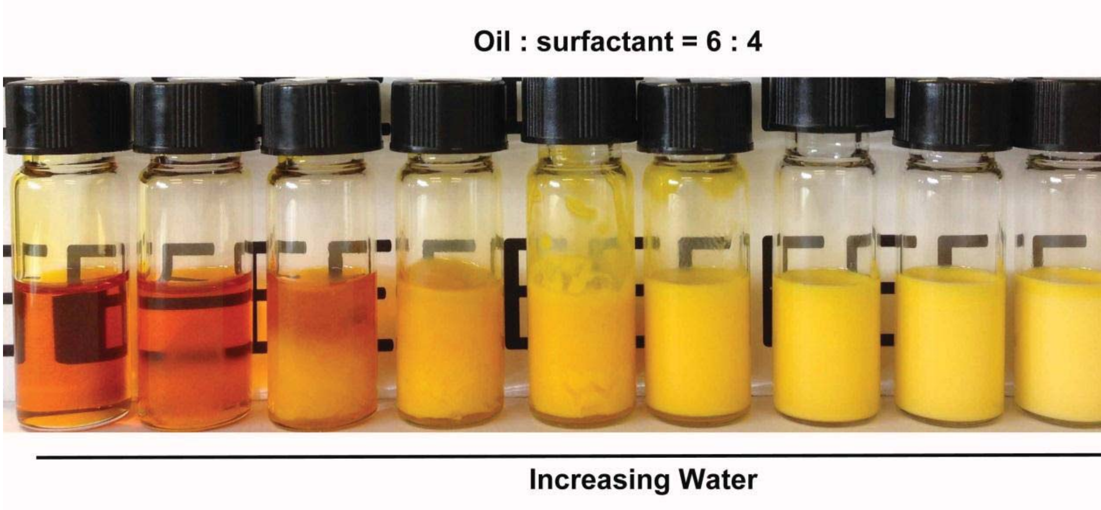
| Tube NO. | Weight (g) | | | | Percentag (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L500 | 1.00 | 1.00 | 0.00 | 2 | 50 | 50 | 0 | 100 | |
| L510 | 0.90 | 0.90 | 0.20 | 2 | 45 | 45 | 10 | 100 | 2 phase |
| L520 | 0.80 | 0.80 | 0.40 | 2 | 40 | 40 | 20 | 100 | 3 phases, u |
| L530 | 0.70 | 0.70 | 0.60 | 2 | 35 | 35 | 30 | 100 | 3 phases, upp |
| L540 | 0.60 | 0.60 | 0.80 | 2 | 30 | 30 | 40 | 100 | 1 turbid |
| L550 | 0.50 | 0.50 | 1.00 | 2 | 25 | 25 | 50 | 100 | 1 turbid p |
| L560 | 0.40 | 0.40 | 1.20 | 2 | 20 | 20 | 60 | 100 | 2 turbid phase |
| L570 | 0.30 | 0.30 | 1.40 | 2 | 15 | 15 | 70 | 100 | 2 turbid phase |
| L580 | 0.20 | 0.20 | 1.60 | 2 | 10 | 10 | 80 | 100 | 2 turbid phase |
| L590 | 0.10 | 0.10 | 1.80 | 2 | 5 | 5 | 90 | 100 | 2 turbid phase |



**Appendix 32** Visual appearance of Line 5 (5 : 5 dilution line) of Lemon oil/Tween 80/

**Appendix 33** Real composition corresponding to Line 6 (6 : 4 dilution line) of Lemon oil/Tween 80/Water sy

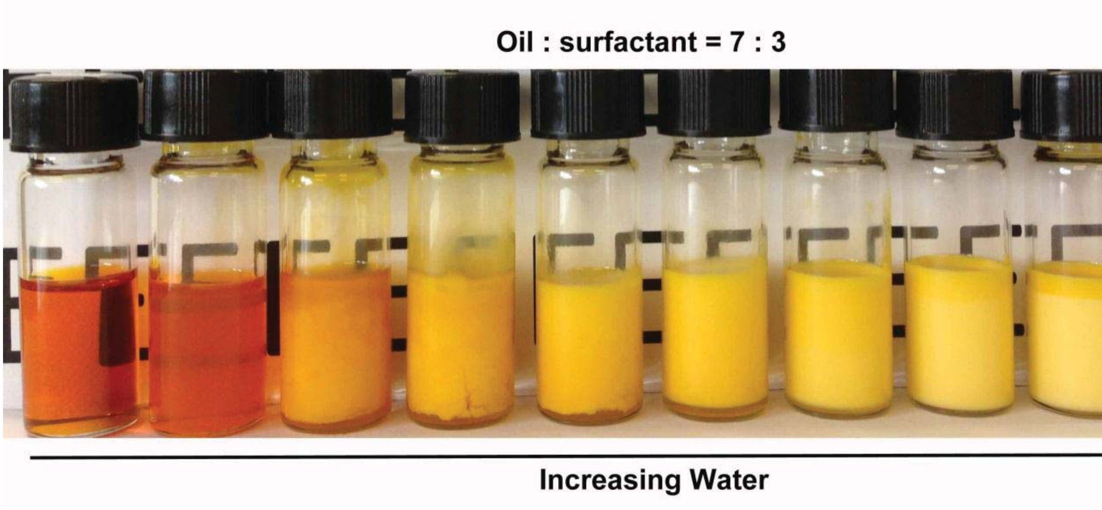
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L600 | 1.20 | 0.80 | 0.00 | 2 | 60 | 40 | 0 | 100 | 1 c |
| L610 | 1.08 | 0.72 | 0.20 | 2 | 54 | 36 | 10 | 100 | 2 phases, uppe |
| L620 | 0.96 | 0.64 | 0.40 | 2 | 48 | 32 | 20 | 100 | 3 phases, upper a |
| L630 | 0.84 | 0.56 | 0.60 | 2 | 42 | 28 | 30 | 100 | 1 turbid pha |
| L640 | 0.72 | 0.48 | 0.80 | 2 | 36 | 24 | 40 | 100 | 1 turbid pha |
| L650 | 0.60 | 0.40 | 1.00 | 2 | 30 | 20 | 50 | 100 | 1 turbid pha |
| L660 | 0.48 | 0.32 | 1.20 | 2 | 24 | 16 | 60 | 100 | 2 turbid phases, |
| L670 | 0.36 | 0.24 | 1.40 | 2 | 18 | 12 | 70 | 100 | 2 turbid phases, |
| L680 | 0.24 | 0.16 | 1.60 | 2 | 12 | 8 | 80 | 100 | 2 turbid phases, |
| L690 | 0.12 | 0.08 | 1.80 | 2 | 6 | 4 | 90 | 100 | 2 turbid phases, |



**Appendix 34** Visual appearance of Line 6 (6 : 4 dilution line) of Lemon oil/Tween 80/

**Appendix 35** Real composition corresponding to Line 7 (7 : 3 dilution line) of Lemon oil/Tween 80/Water systems.

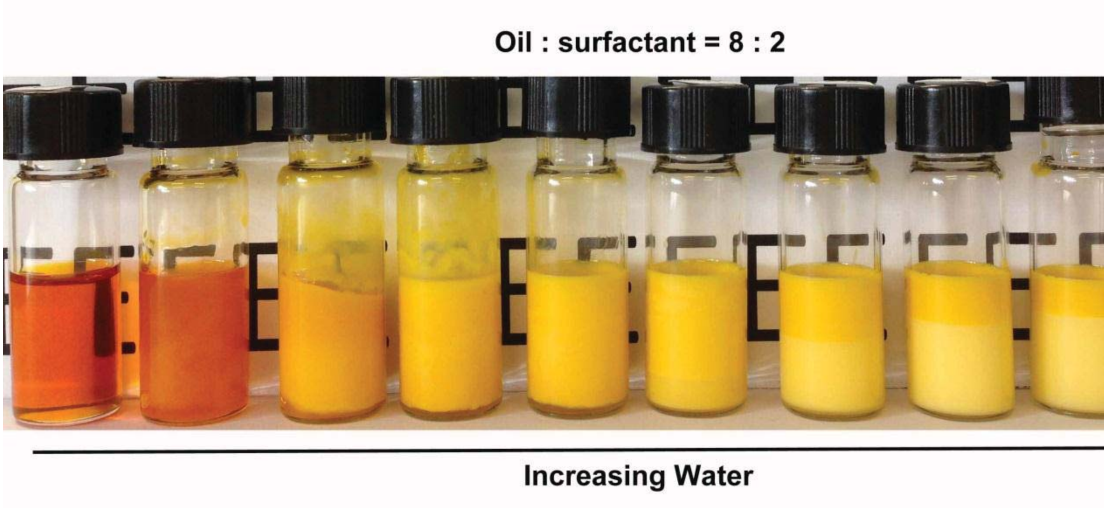
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L700 | 1.40 | 0.60 | 0.00 | 2 | 70 | 30 | 0 | 100 | 1 c |
| L710 | 1.26 | 0.54 | 0.20 | 2 | 63 | 27 | 10 | 100 | 1 phase, |
| L720 | 1.12 | 0.48 | 0.40 | 2 | 56 | 24 | 20 | 100 | 1 turbid ph |
| L730 | 0.98 | 0.42 | 0.60 | 2 | 49 | 21 | 30 | 100 | 1 turbid ph |
| L740 | 0.84 | 0.36 | 0.80 | 2 | 42 | 18 | 40 | 100 | 1 turbid ph |
| L750 | 0.70 | 0.30 | 1.00 | 2 | 35 | 15 | 50 | 100 | 1 turbid pha |
| L760 | 0.56 | 0.24 | 1.20 | 2 | 28 | 12 | 60 | 100 | 2 turbid phases, |
| L770 | 0.42 | 0.18 | 1.40 | 2 | 21 | 9 | 70 | 100 | 2 turbid phases, |
| L780 | 0.28 | 0.12 | 1.60 | 2 | 14 | 6 | 80 | 100 | 2 turbid phases, |
| L790 | 0.14 | 0.06 | 1.80 | 2 | 7 | 3 | 90 | 100 | 2 turbid phases, |



**Appendix 36** Visual appearance of Line 7 (7 : 3 dilution line) of Lemon oil/Tween 80/

**Appendix 37** Real composition corresponding to Line 8 (8 : 2 dilution line) of Lemon oil/Tween 80/Water systems.

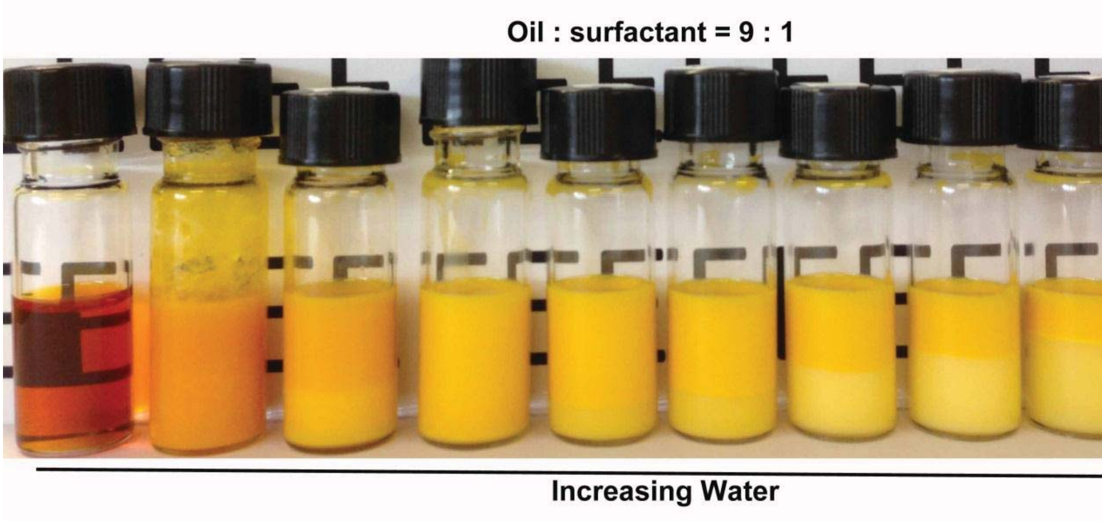
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L800 | 1.60 | 0.40 | 0.00 | 2 | 80 | 20 | 0 | 100 | 1 c |
| L810 | 1.44 | 0.36 | 0.20 | 2 | 72 | 18 | 10 | 100 | 1 phase, tu |
| L820 | 1.28 | 0.32 | 0.40 | 2 | 64 | 16 | 20 | 100 | 1 turbid pha |
| L830 | 1.12 | 0.28 | 0.60 | 2 | 56 | 14 | 30 | 100 | 1 turbid pha |
| L840 | 0.96 | 0.24 | 0.80 | 2 | 48 | 12 | 40 | 100 | 1 turbid pha |
| L850 | 0.80 | 0.20 | 1.00 | 2 | 40 | 10 | 50 | 100 | 2 turbid phases, |
| L860 | 0.64 | 0.16 | 1.20 | 2 | 32 | 8 | 60 | 100 | 2 turbid phases, |
| L870 | 0.48 | 0.12 | 1.40 | 2 | 24 | 6 | 70 | 100 | 2 turbid phases, |
| L880 | 0.32 | 0.08 | 1.60 | 2 | 16 | 4 | 80 | 100 | 2 turbid phases, |
| L890 | 0.16 | 0.04 | 1.80 | 2 | 8 | 2 | 90 | 100 | 2 turbid phases, |



**Appendix 38** Visual appearance of Line 8 (8 : 2 dilution line) of Lemon oil/Tween 80/

**Appendix 39** Real composition corresponding to Line 9 (9 : 1 dilution line) of Lemon oil/Tween 80/Water systems.

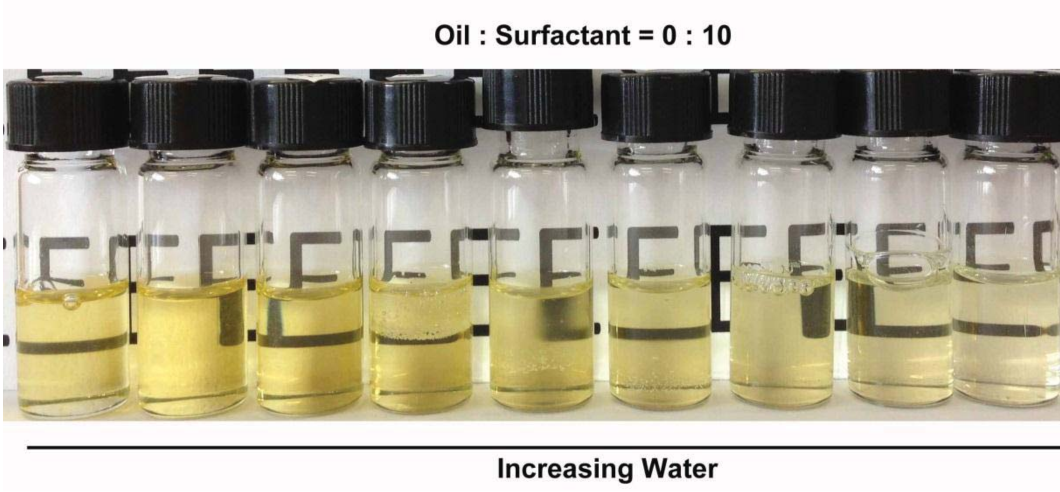
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L900 | 1.80 | 0.20 | 0.00 | 2 | 90 | 10 | 0 | 100 | 1 c |
| L910 | 1.62 | 0.18 | 0.20 | 2 | 81 | 9 | 10 | 100 | 2 turbid phase |
| L920 | 1.44 | 0.16 | 0.40 | 2 | 72 | 8 | 20 | 100 | 2 turbid phases, |
| L930 | 1.26 | 0.14 | 0.60 | 2 | 63 | 7 | 30 | 100 | 1 turbid p |
| L940 | 1.08 | 0.12 | 0.80 | 2 | 54 | 6 | 40 | 100 | 2 turbid phases, |
| L950 | 0.90 | 0.10 | 1.00 | 2 | 45 | 5 | 50 | 100 | 2 turbid phases, |
| L960 | 0.72 | 0.08 | 1.20 | 2 | 36 | 4 | 60 | 100 | 2 turbid phases, |
| L970 | 0.54 | 0.06 | 1.40 | 2 | 27 | 3 | 70 | 100 | 2 turbid phases, |
| L980 | 0.36 | 0.04 | 1.60 | 2 | 18 | 2 | 80 | 100 | 2 turbid phases, |
| L990 | 0.18 | 0.02 | 1.80 | 2 | 9 | 1 | 90 | 100 | 2 turbid phases, |



**Appendix 40** Visual appearance of Line 9 (9 : 1 dilution line) of Lemon oil/Tween 80/

**Appendix 41** Real composition corresponding to Line 10 (oil free) of Lemon oil/Tween 80/Water systems.

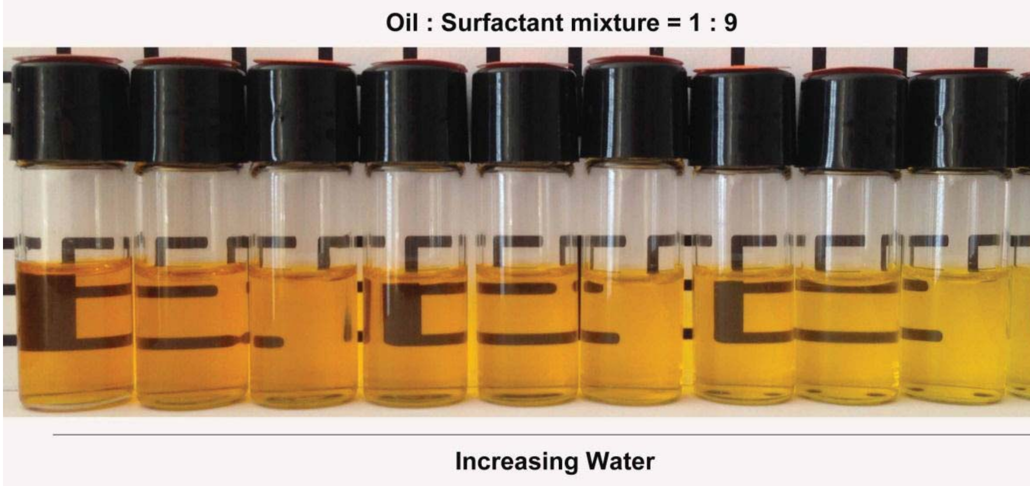
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Oil | Surfactant | Water | Total | Oil | Surfactant | Water | Total | |
| L1000 | 0.00 | 2.00 | 0.00 | 2 | 0 | 100 | 0 | 100 | 1 clear ph |
| L1010 | 0.00 | 1.80 | 0.20 | 2 | 0 | 90 | 10 | 100 | 1 clear ph |
| L1020 | 0.00 | 1.60 | 0.40 | 2 | 0 | 80 | 20 | 100 | 1 clear phas |
| L1030 | 0.00 | 1.40 | 0.60 | 2 | 0 | 70 | 30 | 100 | 1clear phas |
| L1040 | 0.00 | 1.20 | 0.80 | 2 | 0 | 60 | 40 | 100 | 1 phase, |
| L1050 | 0.00 | 1.00 | 1.00 | 2 | 0 | 50 | 50 | 100 | 1clear phas |
| L1060 | 0.00 | 0.80 | 1.20 | 2 | 0 | 40 | 60 | 100 | 1 clear ph |
| L1070 | 0.00 | 0.60 | 1.40 | 2 | 0 | 30 | 70 | 100 | 1 clear ph |
| L1080 | 0.00 | 0.40 | 1.60 | 2 | 0 | 20 | 80 | 100 | |
| L1090 | 0.00 | 0.20 | 1.80 | 2 | 0 | 10 | 90 | 100 | |



**Appendix 42** Visual appearance of Line 10 (oil free) of Lemon oil/Tween 80/Wate

**Appendix 43** Real composition corresponding to Line 1 (1 : 9 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

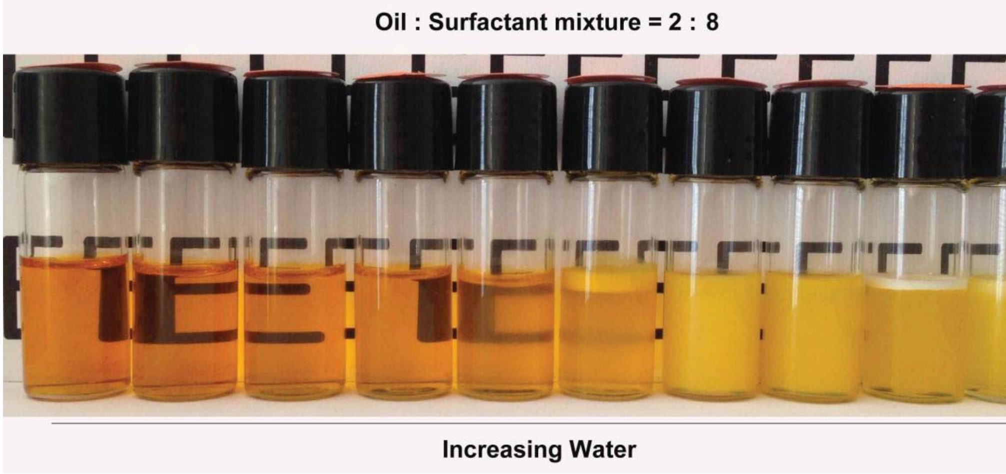
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L100 | 1.80 | 0.20 | 0.00 | 2 | 45 | 45 | 10 | 0 | 100 | S |
| L110 | 1.62 | 0.18 | 0.20 | 2 | 40.5 | 40.5 | 9 | 10 | 100 | S |
| L120 | 1.44 | 0.16 | 0.40 | 2 | 36 | 36 | 8 | 20 | 100 | S |
| L130 | 1.26 | 0.14 | 0.60 | 2 | 31.5 | 31.5 | 7 | 30 | 100 | S |
| L140 | 1.08 | 0.12 | 0.80 | 2 | 27 | 27 | 6 | 40 | 100 | S |
| L150 | 0.90 | 0.10 | 1.00 | 2 | 22.5 | 22.5 | 5 | 50 | 100 | S |
| L160 | 0.72 | 0.08 | 1.20 | 2 | 18 | 18 | 4 | 60 | 100 | S |
| L170 | 0.54 | 0.06 | 1.40 | 2 | 13.5 | 13.5 | 3 | 70 | 100 | S |
| L180 | 0.36 | 0.04 | 1.60 | 2 | 9 | 9 | 2 | 80 | 100 | S |
| L190 | 0.18 | 0.02 | 1.80 | 2 | 4.5 | 4.5 | 1 | 90 | 100 | S |



**Appendix 44** Visual appearance of Line 1 (1 : 9 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 45** Real composition corresponding to Line 2 (2 : 8 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

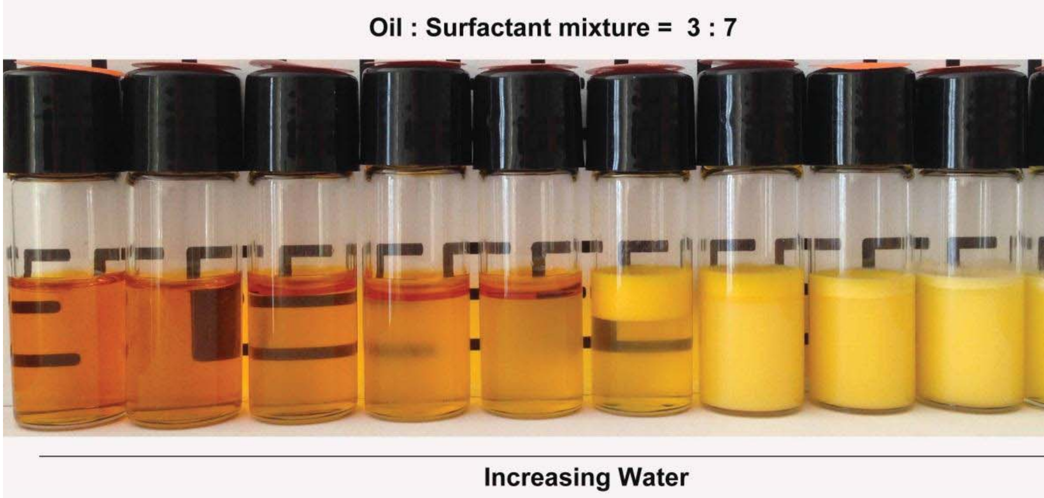
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L200 | 1.60 | 0.40 | 0.00 | 2 | 40 | 40 | 20 | 0 | 100 | Singl |
| L210 | 1.44 | 0.36 | 0.20 | 2 | 36 | 36 | 18 | 10 | 100 | Singl |
| L220 | 1.28 | 0.32 | 0.40 | 2 | 32 | 32 | 16 | 20 | 100 | Singl |
| L230 | 1.12 | 0.28 | 0.60 | 2 | 28 | 28 | 14 | 30 | 100 | Singl |
| L240 | 0.96 | 0.24 | 0.80 | 2 | 24 | 24 | 12 | 40 | 100 | Singl |
| L250 | 0.80 | 0.20 | 1.00 | 2 | 20 | 20 | 10 | 50 | 100 | Two |
| L260 | 0.64 | 0.16 | 1.20 | 2 | 16 | 16 | 8 | 60 | 100 | Si |
| L270 | 0.48 | 0.12 | 1.40 | 2 | 12 | 12 | 6 | 70 | 100 | Si |
| L280 | 0.32 | 0.08 | 1.60 | 2 | 8 | 8 | 4 | 80 | 100 | Two |
| L290 | 0.16 | 0.04 | 1.80 | 2 | 4 | 4 | 2 | 90 | 100 | Two |



**Appendix 46** Visual appearance of Line 2 (2 : 8 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 47** Real composition corresponding to Line 3 (3 : 7 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

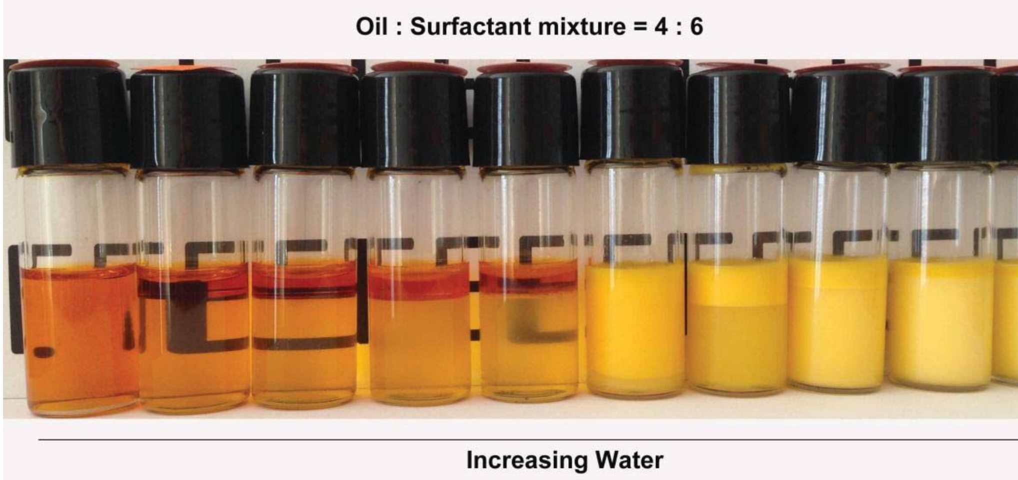
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L300 | 1.40 | 0.60 | 0.00 | 2 | 35 | 35 | 30 | 0 | 100 | Single |
| L310 | 1.26 | 0.54 | 0.20 | 2 | 31.5 | 31.5 | 27 | 10 | 100 | Single |
| L320 | 1.12 | 0.48 | 0.40 | 2 | 28 | 28 | 24 | 20 | 100 | Two |
| L330 | 0.98 | 0.42 | 0.60 | 2 | 24.5 | 24.5 | 21 | 30 | 100 | Two |
| L340 | 0.84 | 0.36 | 0.80 | 2 | 21 | 21 | 18 | 40 | 100 | Two |
| L350 | 0.70 | 0.30 | 1.00 | 2 | 17.5 | 17.5 | 15 | 50 | 100 | Two p |
| L360 | 0.56 | 0.24 | 1.20 | 2 | 14 | 14 | 12 | 60 | 100 | Two phas |
| L370 | 0.42 | 0.18 | 1.40 | 2 | 10.5 | 10.5 | 9 | 70 | 100 | Two phas |
| L380 | 0.28 | 0.12 | 1.60 | 2 | 7 | 7 | 6 | 80 | 100 | Two phas |
| L390 | 0.14 | 0.06 | 1.80 | 2 | 3.5 | 3.5 | 3 | 90 | 100 | Two phas |



**Appendix 48** Visual appearance of Line 3 (3 : 7 dilution line) of Lemon oil/Tween 80/Etha

**Appendix49** Real composition corresponding to Line 4 (4 : 6 dilution line) of Lemon oil/Tween 80/Ethanol/

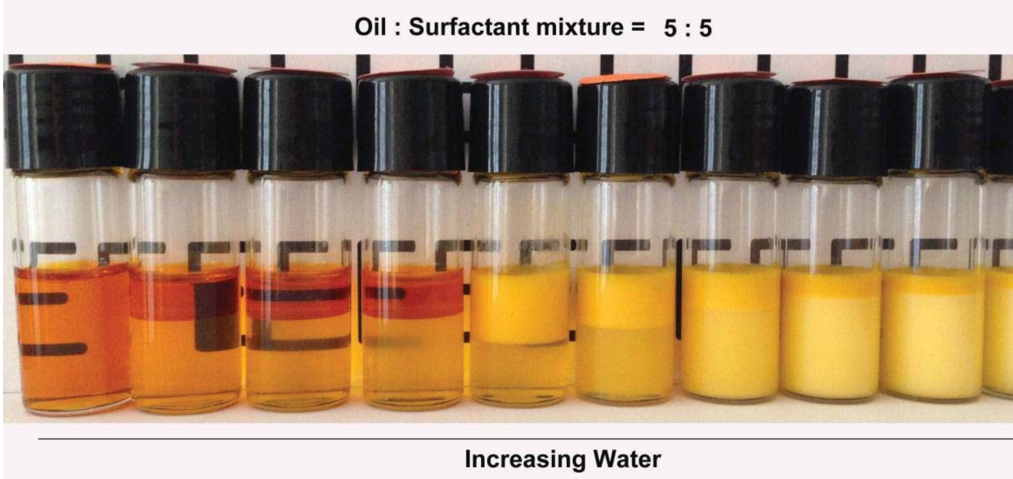
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L400 | 1.20 | 0.80 | 0.00 | 2 | 30 | 30 | 40 | 0 | 100 | Singl |
| L410 | 1.08 | 0.72 | 0.20 | 2 | 27 | 27 | 36 | 10 | 100 | Tw |
| L420 | 0.96 | 0.64 | 0.40 | 2 | 24 | 24 | 32 | 20 | 100 | Tw |
| L430 | 0.84 | 0.56 | 0.60 | 2 | 21 | 21 | 28 | 30 | 100 | Tw |
| L440 | 0.72 | 0.48 | 0.80 | 2 | 18 | 18 | 24 | 40 | 100 | Tw |
| L450 | 0.60 | 0.40 | 1.00 | 2 | 15 | 15 | 20 | 50 | 100 | T |
| L460 | 0.48 | 0.32 | 1.20 | 2 | 12 | 12 | 16 | 60 | 100 | T |
| L470 | 0.36 | 0.24 | 1.40 | 2 | 9 | 9 | 12 | 70 | 100 | T |
| L480 | 0.24 | 0.16 | 1.60 | 2 | 6 | 6 | 8 | 80 | 100 | T |
| L490 | 0.12 | 0.08 | 1.80 | 2 | 3 | 3 | 4 | 90 | 100 | T |



**Appendix 50** Visual appearance of Line 4 (4 : 6 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 51** Real composition corresponding to Line 5 (5 : 5 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

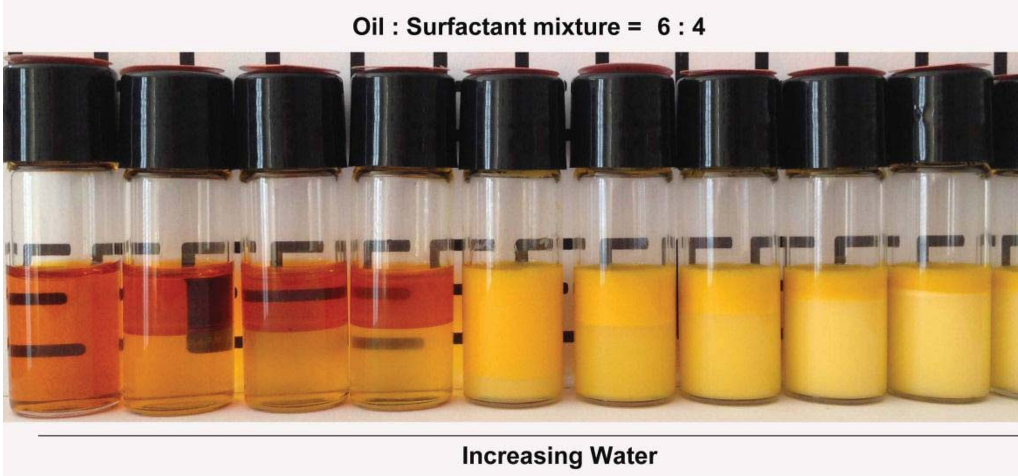
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L500 | 1.00 | 1.00 | 0.00 | 2 | 25 | 25 | 50 | 0 | 100 | Sing |
| L510 | 0.90 | 0.90 | 0.20 | 2 | 22.5 | 22.5 | 45 | 10 | 100 | Tw |
| L520 | 0.80 | 0.80 | 0.40 | 2 | 20 | 20 | 40 | 20 | 100 | Tw |
| L530 | 0.70 | 0.70 | 0.60 | 2 | 17.5 | 17.5 | 35 | 30 | 100 | Tw |
| L540 | 0.60 | 0.60 | 0.80 | 2 | 15 | 15 | 30 | 40 | 100 | Two |
| L550 | 0.50 | 0.50 | 1.00 | 2 | 12.5 | 12.5 | 25 | 50 | 100 | Two ph |
| L560 | 0.40 | 0.40 | 1.20 | 2 | 10 | 10 | 20 | 60 | 100 | Two ph |
| L570 | 0.30 | 0.30 | 1.40 | 2 | 7.5 | 7.5 | 15 | 70 | 100 | Two ph |
| L580 | 0.20 | 0.20 | 1.60 | 2 | 5 | 5 | 10 | 80 | 100 | Two ph |
| L590 | 0.10 | 0.10 | 1.80 | 2 | 2.5 | 2.5 | 5 | 90 | 100 | Two ph |



**Appendix 52** Visual appearance of Line 5 (5 : 5 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 53** Real composition corresponding to Line 6 (6 : 4 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

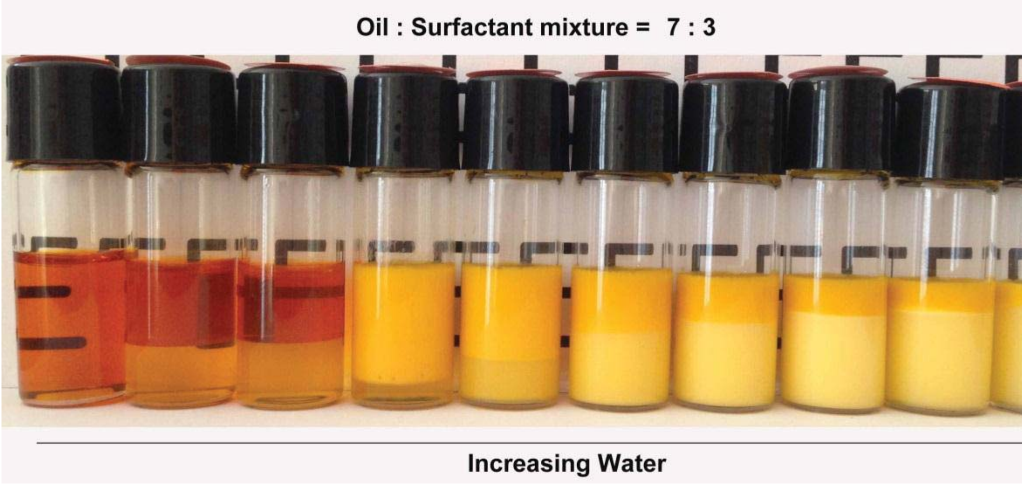
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L600 | 0.80 | 1.20 | 0.00 | 2 | 20 | 20 | 60 | 0 | 100 | Sin |
| L610 | 0.72 | 1.08 | 0.20 | 2 | 18 | 18 | 54 | 10 | 100 | T |
| L620 | 0.64 | 0.96 | 0.40 | 2 | 16 | 16 | 48 | 20 | 100 | T |
| L630 | 0.56 | 0.84 | 0.60 | 2 | 14 | 14 | 42 | 30 | 100 | T |
| L640 | 0.48 | 0.72 | 0.80 | 2 | 12 | 12 | 36 | 40 | 100 | Two p |
| L650 | 0.40 | 0.60 | 1.00 | 2 | 10 | 10 | 30 | 50 | 100 | Two p |
| L660 | 0.32 | 0.48 | 1.20 | 2 | 8 | 8 | 24 | 60 | 100 | Two p |
| L670 | 0.24 | 0.36 | 1.40 | 2 | 6 | 6 | 18 | 70 | 100 | Two p |
| L680 | 0.16 | 0.24 | 1.60 | 2 | 4 | 4 | 12 | 80 | 100 | Two p |
| L690 | 0.08 | 0.12 | 1.80 | 2 | 2 | 2 | 6 | 90 | 100 | Two p |



**Appendix 54** Visual appearance of Line 6 (6 : 4 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 55** Real composition corresponding to Line 7 (7 : 3 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

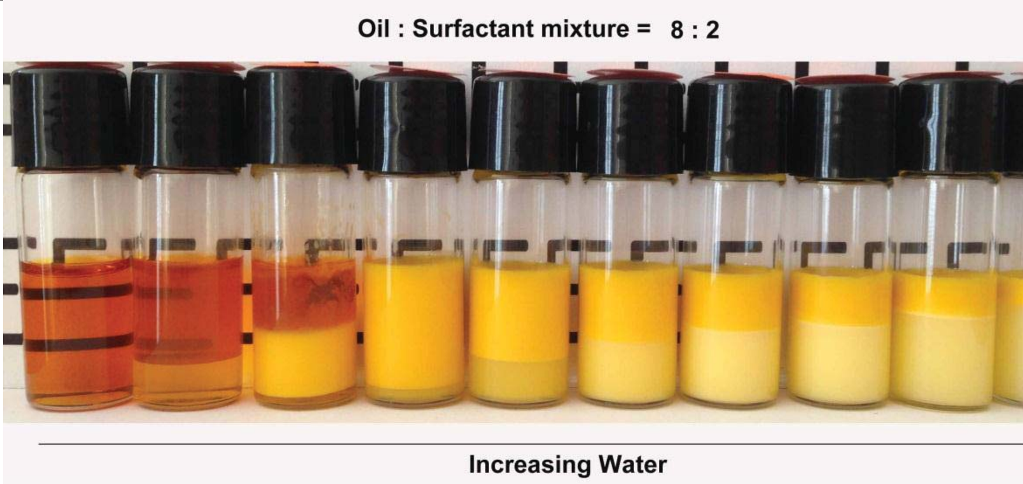
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L700 | 0.60 | 1.40 | 0.00 | 2 | 15 | 15 | 70 | 0 | 100 | Sing |
| L710 | 0.54 | 1.26 | 0.20 | 2 | 13.5 | 13.5 | 63 | 10 | 100 | Tw |
| L720 | 0.48 | 1.12 | 0.40 | 2 | 12 | 12 | 56 | 20 | 100 | Tw |
| L730 | 0.42 | 0.98 | 0.60 | 2 | 10.5 | 10.5 | 49 | 30 | 100 | Two |
| L740 | 0.36 | 0.84 | 0.80 | 2 | 9 | 9 | 42 | 40 | 100 | Two ph |
| L750 | 0.30 | 0.70 | 1.00 | 2 | 7.5 | 7.5 | 35 | 50 | 100 | Two ph |
| L760 | 0.24 | 0.56 | 1.20 | 2 | 6 | 6 | 28 | 60 | 100 | Two ph |
| L770 | 0.18 | 0.42 | 1.40 | 2 | 4.5 | 4.5 | 21 | 70 | 100 | Two ph |
| L780 | 0.12 | 0.28 | 1.60 | 2 | 3 | 3 | 14 | 80 | 100 | Two ph |
| L790 | 0.06 | 0.14 | 1.80 | 2 | 1.5 | 1.5 | 7 | 90 | 100 | Two ph |



**Appendix 56** Visual appearance of Line 7 (7 : 3 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 57** Real composition corresponding to Line 8 (8 : 2 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

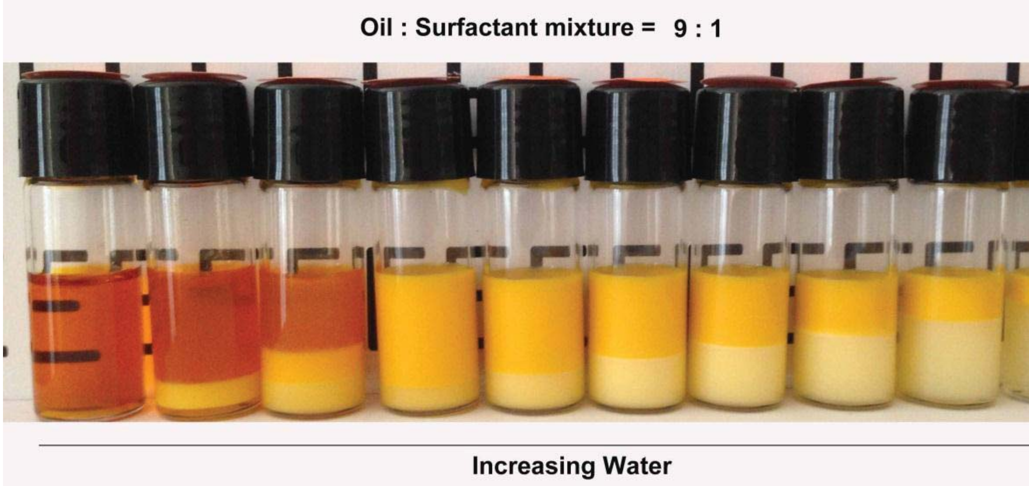
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L800 | 0.40 | 1.60 | 0.00 | 2 | 10 | 10 | 80 | 0 | 100 | Sing |
| L810 | 0.36 | 1.44 | 0.20 | 2 | 9 | 9 | 72 | 10 | 100 | Tw |
| L820 | 0.32 | 1.28 | 0.40 | 2 | 8 | 8 | 64 | 20 | 100 | Tw |
| L830 | 0.28 | 1.12 | 0.60 | 2 | 7 | 7 | 56 | 30 | 100 | Two ph |
| L840 | 0.24 | 0.96 | 0.80 | 2 | 6 | 6 | 48 | 40 | 100 | Two ph |
| L850 | 0.20 | 0.80 | 1.00 | 2 | 5 | 5 | 40 | 50 | 100 | Two ph |
| L860 | 0.16 | 0.64 | 1.20 | 2 | 4 | 4 | 32 | 60 | 100 | Two ph |
| L870 | 0.12 | 0.48 | 1.40 | 2 | 3 | 3 | 24 | 70 | 100 | Two ph |
| L880 | 0.08 | 0.32 | 1.60 | 2 | 2 | 2 | 16 | 80 | 100 | Two ph |
| L890 | 0.04 | 0.16 | 1.80 | 2 | 1 | 1 | 8 | 90 | 100 | Two ph |



**Appendix 58** Visual appearance of Line 8 (8 : 2 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 59** Real composition corresponding to Line 9 (9 : 1 dilution line) of Lemon oil/Tween 80/Ethanol/Water syst

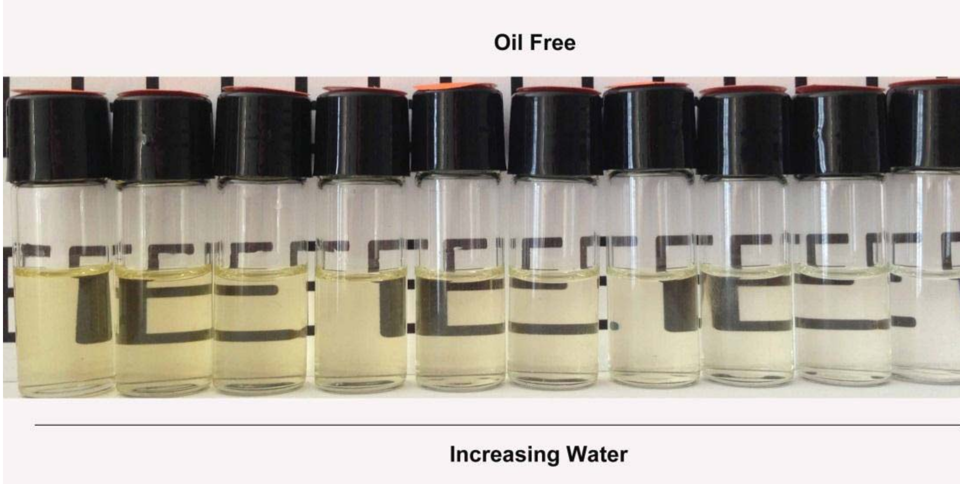
| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L900 | 0.20 | 1.80 | 0.00 | 2 | 5 | 5 | 90 | 0 | 100 | Singl |
| L910 | 0.18 | 1.62 | 0.20 | 2 | 4.5 | 4.5 | 81 | 10 | 100 | Three p |
| L920 | 0.16 | 1.44 | 0.40 | 2 | 4 | 4 | 72 | 20 | 100 | Three p |
| L930 | 0.14 | 1.26 | 0.60 | 2 | 3.5 | 3.5 | 63 | 30 | 100 | Two ph |
| L940 | 0.12 | 1.08 | 0.80 | 2 | 3 | 3 | 54 | 40 | 100 | Two ph |
| L950 | 0.10 | 0.90 | 1.00 | 2 | 2.5 | 2.5 | 45 | 50 | 100 | Two ph |
| L960 | 0.08 | 0.72 | 1.20 | 2 | 2 | 2 | 36 | 60 | 100 | Two ph |
| L970 | 0.06 | 0.54 | 1.40 | 2 | 1.5 | 1.5 | 27 | 70 | 100 | Two ph |
| L980 | 0.04 | 0.36 | 1.60 | 2 | 1 | 1 | 18 | 80 | 100 | Two ph |
| L990 | 0.02 | 0.18 | 1.80 | 2 | 0.5 | 0.5 | 9 | 90 | 100 | Two ph |



**Appendix 60** Visual appearance of Line 9 (9 : 1 dilution line) of Lemon oil/Tween 80/Etha

**Appendix 61** Real composition corresponding to Line 10 (oil free) of Lemon oil/Tween 80/Ethanol /Water systems.

| Tube NO. | Weight (g) | | | | Percentage (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Surfactant and Ethanol | Oil | Water | Total | Surfactant | Ethanol | Oil | Water | Total | |
| L1000 | 2.00 | 0.00 | 0.00 | 2 | 50 | 50 | 0 | 0 | 100 | Single clea |
| L1010 | 1.80 | 0.00 | 0.20 | 2 | 45 | 45 | 0 | 10 | 100 | Single clea |
| L1020 | 1.60 | 0.00 | 0.40 | 2 | 40 | 40 | 0 | 20 | 100 | Single clea |
| L1030 | 1.40 | 0.00 | 0.60 | 2 | 35 | 35 | 0 | 30 | 100 | Single clea |
| L1040 | 1.20 | 0.00 | 0.80 | 2 | 30 | 30 | 0 | 40 | 100 | Single clea |
| L1050 | 1.00 | 0.00 | 1.00 | 2 | 25 | 25 | 0 | 50 | 100 | Single clea |
| L1060 | 0.80 | 0.00 | 1.20 | 2 | 20 | 20 | 0 | 60 | 100 | Single clea |
| L1070 | 0.60 | 0.00 | 1.40 | 2 | 15 | 15 | 0 | 70 | 100 | Single clea |
| L1080 | 0.40 | 0.00 | 1.60 | 2 | 10 | 10 | 0 | 80 | 100 | Single |
| L1090 | 0.20 | 0.00 | 1.80 | 2 | 5 | 5 | 0 | 90 | 100 | Single |



**Appendix 62** Visual appearance of Line 10 (oil free) of Lemon oil/Tween 80/Ethanol /