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# Preparation, characterization, and in vitro protein release studies in pharmaceutically relevant lecithin microemulsions

Khushboo Parekh
*The University of Toledo*

A Thesis

entitled

# Preparation, Characterization, and *In Vitro* Protein Release Studies in Pharmaceutically relevant Lecithin Microemulsions

By

Khushboo Parekh

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Pharmaceutical Sciences with the Industrial Pharmacy option

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Dr. Jerry Nesamony, Committee Chair

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Dr. Curtis Black, Committee Member

---

Dr. Kenneth Alexander, Committee Member

---

Dr. Patricia Komuniecki, Dean

College of Graduate Studies

**The University of Toledo**

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An Abstract of

Preparation, Characterization, and *In Vitro* Protein Release Studies in Pharmaceutically Relevant Lecithin Microemulsions

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The objective of this research project was to prepare and characterize pharmaceutically relevant microemulsions, using lecithin as the surfactant and isopropyl alcohol as the co-surfactant. Visually transparent microemulsions were obtained, by titrating mixtures of lecithin, isopropyl alcohol, and oil with water. The quantity of isopropyl alcohol and lecithin was modulated in the formulations, by changing the Km ratio [Km = (surfactant concentration)/ (co-surfactant concentration)] and using various surfactant-oil mixtures. Pseudoternary phase diagrams were plotted, to identify microemulsion forming compositions. The microemulsions, which incorporated appropriate amounts of water, were selected for further characterization. Samples of the formulations were subjected to polarized light microscopy, to verify the formation of the microemulsions. The electrical conductivity of lecithin/Isopropyl Myristate and lecithin/Ethyl Oleate microemulsions, was measured. The state of the water droplets in the lecithin/Isopropyl Myristate microemulsions, was characterized via subambient differential scanning calorimetry.

Microemulsions, were further evaluated using dynamic light scattering experiments, to estimate the particle size distribution. Comparison between poloxamer and isopropyl alcohol as co-surfactants was made by comparing the amount of water incorporated into the microemulsions. Bovine serum albumin was incorporated into these water in oil lecithin microemulsions, anticipating the use of these microemulsions in biocatalysis of proteins/enzymes, thereby preventing their denaturation. Release studies were performed on the microemulsions, containing bovine serum albumin, to evaluate the release profile of the protein from the microemulsion system. The Hartree Lowry assay was used for the determination of albumin, in phosphate buffer of pH 7.4.The pseudo Ternary phase diagrams indicated that stable and clear microemulsions, formed when the Km ratio ranged from 0.5 to 2.0 and the water concentration varied between 3% and 10%. The absence of optical birefringence in the clear formulation samples indicated the production of a water-in-oil microemulsion. The electrical conductivity of the formulations demonstrated a composition dependant change with low conductivity values of 0.0 to 0.4 μSiemens/cm observed in microemulsions. Formulations that exhibited turbidity possessed high conductivity values that ranged from 1.6 to 3.0 μSiemens/cm. The conductivity studies, exhibited a "percolation phenomenon", in the formulations. Sub-ambient DSC established the existence of different types of water in the formulations. A microemulsion containing 3% water did not show any peak attributable to the freezing of water indicating that all the water present in the microemulsion is tightly bound to the surfactant and remains non-freezable down to –100°C. However, thermal events observed at ~ -70 °C in formulations containing a larger quantity of water confirmed the presence of bound freezable water. The composition of the formulations affected the particle size

distribution with larger droplets observed as the percentage of water increased. The estimated droplet diameter in the formulations varied between 5 to 800 nm. Isopropyl alcohol was able to reduce the interfacial tension to a greater extent than the poloxamer in use, and hence was able to incorporate a greater quantity of water than the poloxamer. The presence of albumin did not influence the stability of the microemulsion formulation, as almost similar percentages of albumin solution were incorporated into the lecithin/Ethyl Oleate and lecithin/Isopropyl Myristate mixtures, as compared to that of R.O water. The *invitro* protein release studies were carried out. Lecithin is capable of forming stable microemulsions in Isopropyl Myristate and Ethyl Oleate in the presence of Isopropyl alcohol. The influence of various formulation parameters such as Km ratio, surfactant/oil ratio, and percentage of water in the system on the type of microemulsion formed was evaluated. These microemulsions are currently being assessed for use as a drug delivery system.

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## 1.0 Microemulsions

## 1.1 Introduction:

Water and oil are not miscible. When mixed together, they separate into two phases. The interfacial tension between oil and water is as high as 30-50 dynes/cm. A large amount of energy is required in order to reduce the interfacial tension between oil and water. Since surfactants can reduce the interfacial tension, they can be used to aid the mixing of water and oil by adsorbing at the liquid-liquid interface. Depending on the type of surfactant used in the system, we can determine the type of microemulsion formed. If the surfactant is lipophilic in nature, water will be emulsified throughout the continuous oil phase. On the other hand, if the surfactant is hydrophilic in nature, oil will be emulsified throughout the continuous water phase [1].

Mixtures of two or more immiscible liquids are known as emulsions. A continuous phase and a dispersed phase make up an emulsion. Emulsions show instability problems such as coalescence and creaming [2]. Coalescence is the form of instability in which smaller droplets merge to form larger droplets. In the case of creaming, migration of two phases occurs to the top or bottom, depending on the densities of the two phases [3].

Microemulsions are isotropic mixtures of a lipophilic, a hydrophilic, and an amphiphilic component. Microemulsions differ from macroemulsions, in two ways. Microemulsions are thermodynamically stable whereas macroemulsions are unstable systems. Microemulsions are nanostructured systems and transparent, whereas macroemulsions are turbid and milky white with droplet sizes of up to several microns in diameter [4].

Water in oil microemulsions are also known as reverse micelles, they have been used for biological studies and applied biotechnology. These systems have the ability to solubilize both hydrophilic and hydrophobic substances. Microemulsions containing enzymes are currently of interest [5].

### 1.2 Characteristics of Microemulsions:

Optically transparent, thermodynamically stable, isotropic colloidal dispersion of oil, water, and a surfactant is known as a microemulsion. Microemulsions can be formulated with or without co-surfactant, but in the case of lecithin microemulsions, the addition of a co-surfactant is required to form a stable microemulsion. Microemulsions are different from emulsions, in a number of different ways. Emulsions are thermodynamically unstable and their phases separate over time, whereas microemulsions are thermodynamically stable and do not undergo phase separation [1, 6]. Microemulsions form spontaneously at room temperature and require very little energy, as compared to emulsions which require a large amount of energy. Microemulsions also posses very low interfacial tension. The particle size of the droplets in microemulsions, are in the nanometer range. Microemulsions are also transparent in nature, whereas emulsions have a cloudy appearance. A large number of small droplets are produced, when microemulsions form. Due to the small size of the droplets in a microemulsion, they

possess a large interfacial surface area, from which transport of the drug can occur [7]. Generally, the formation of microemulsions involves the achievement of a negative free energy of formation, resulting in a spontaneous reduction in surface tension [8]. Hence, microemulsification is spontaneous and the resulting colloidal dispersion is thermodynamically stable. Water- in- oil microemulsions, are formed by a surfactant with a low HLB (3-6), whereas surfactants with a high HLB of (8-18), are used for the formation of oil in water microemulsions [8]. The surfactant and the co-surfactant mixture typically generate very low free energies per unit of the interfacial area between the dispersed phase and the dispersion medium. This extremely low interfacial energy of $10^{-3}$mN/m, causes an equilibrium between the hydrophilicity and lipophilicity of the surfactant and the co-surfactant, which ultimately produces an increased surface area and enhanced drug delivery. Microemulsions have a flexible interfacial film, and the droplet sizes are less than 200 nm in diameter, which makes the microemulsions optically transparent and appear just like solutions. Due to the large number of microstructures present in microemulsions, and optical transparency, microemulsions are homogenous on a macroscopic scale, but on a molecular scale, they are heterogenous [7,9] . Microemulsions generally show Newtonian flow properties, since their viscosity is low. They exhibit a constant flow rate when subjected to various shear rates, except for bicontinuous microemulsions, which exhibit non-Newtonian flow characteristics. The viscosity of microemulsions is similar to that of water, even at high droplet concentrations in the case of o/w microemulsions. In microemulsions, the interface which is comprised of the surfactant/co-surfactant monolayer is of very small, nanometer sized

droplets. The interfacial rigidity of the droplets in a microemulsion also plays a major role in the flux of drugs from the droplets to the cells and tissues of the body. [1, 7, 10].

## 1.3 Types of Microemulsions

There are three types of microemulsions, which are differentiated based on the nature of the dispersed phase and the dispersion medium:

Type I microemulsion is an oil in water (o/w) type microemulsion.

Type II microemulsion is a water in oil (w/o) type microemulsion.

Type III microemulsion is a bicontinuous microemulsion

### a) Type I microemulsion

In this type of microemulsion, droplets of oil (the internal phase) are surrounded by the continuous phase comprised of water. The surfactant or the co-surfactant generally surrounds the oil droplets as a film. The surfactant is present in the form of monomers solubilized in water. The surfactant monolayer in this type of microemulsion is oriented such that the polar head groups of the surfactant are oriented towards the hydrophilic or water phase, and the lipophilic tails are oriented towards the oil phase. Oil in water microemulsions, can solubilize a hydrophobic drug in the internal oil phase, which is then dispersed in a stable manner in the continuous aqueous phase. The advantage of using oil in water microemulsions, for drug delivery is that it retains its structure as a microemulsion even upon dilution in the aqueous environment present at the tissue or cellular levels within the human body [11, 12].

**b) Type II microemulsion**

Water in oil microemulsions, are made up of droplets of water dispersed in a continuous oil phase. In these microemulsions, the polar heads of the surfactant are oriented towards the water droplets, whereas the long chain fatty acid tails of the surfactant are embedded in the oil phase. Water in oil microemulsions, are also known as reverse micelles. This droplet type of microemulsion is formed when the volume of water in the microemulsion is lower than the continuous phase, or the oil. Generally enzyme containing reverse micelles, show maximum activity between the $w_o$ or the [water]/ [surfactant] molar ratio of 5 to 15 [15]. The properties of water inside the reverse micelles are different from bulk water. This difference decreases, as the $w_o$ value increases. The water loading capacity of reverse micelles also varies with temperature, since water is shed from the reverse micelles at low temperature [14]. A water in oil microemulsion upon dilution with aqueous medium does not remain stable. This is due to an increase in the internal phase volume which eventually leads to phase separation. An increase in the aqueous phase volume in water in oil microemulsions which causes a percolation phenomenon to take place, wherein the microemulsion undergoes phase inversion to form a macroemulsion. Many peptides as well as proteins, have been delivered in water in oil microemulsions. For example, insulin has been delivered in water in oil microemulsions formulated using lecithin as a surfactant [13]. The advantage of solubilization of hydrophilic peptides and enzymes in water in oil microemulsions is that they are less susceptible to enzymatic degradation and denaturation or loss of activity, especially when given orally or parenterally because they remain protected in the internal aqueous phase. Generally, water in oil microemulsions are also used as treatment for dry skin conditions

and as an emollient for the skin. Water in oil microemulsions can also be administered intravenously, as lipid nutrients, provided the surfactant and co-surfactant are safe enough to be given parenterally. [14, 15].

**c) Type III microemulsion**

A bicontinuous microemulsion, is one in which both the water and oil domains are present in equal quantities. It consists of a surfactant film separating the water and oil. The surfactant film has a spontaneous curvature that is close to zero, and it has no tendancy to curve either towards water or oil. Both water and oil exist as a continuous phase in these microemulsions. When a water in oil microemulsion, is converted into an oil in water microemulsion, it passes through the 'bicontinuous' state. Bicontinuous microemulsions show non-Newtonian flow and plasticity. In bicontinuous microemulsions, there is a balance of steric, electrostatic and dispersion type forces on the two sides of the film. The microstructure of the microemulsion leads to a homogenous spontaneous mean curvature, over the dividing surface. These microemulsions are generally useful for topical delivery of drugs, or for intravenous administration, where it forms an oil in water microemulsion upon dilution [16, 17]

## 1.4 Microemulsion Formulation:

### 1.4.1 Formulation considerations:

The properties of the surfactant-oil-water are important in determining the formation of microemulsions. Deviations from the actual formulation may cause the breaking of the microemulsion and formation of an unstable macroemulsion.

A microemulsion generally consists of four different components, a lipophilic phase, a hydrophilic phase, surfactant and co-surfactant [16]. The nature of the components like the oil, surfactant, co-surfactant and water, as well as temperature and pressure which affect the microemulsion systems are known as the formulation variables. The quantities of different substances present, are also likely to change the properties, and are referred to as composition variables which can be expressed as weight, percentage or proportion [18].

In order that the microemulsions attain low interfacial tension and good solubilization ability, it is necessary that the microemulsions be formulated accurately.

The formation of a microemulsion depends on factors such as : (1) oil/surfactant and surfactant/co-surfactant ratio; (2) nature and concentration of the oil, surfactant, co-surfactant and aqueous phase; (3) pH; (4) temperature; and (5) hydrophilicity/lipophilicity of the API, its pKa and polarity [17,18].

All these factors must be considered during the formulation of microemulsions. Moreover, it is important to consider the compatibility of the oil, surfactant or co-surfactant for the desired route of administration, especially for the ocular and parenteral route, since very few excipients can be used for the ocular and parenteral route.

**1.4.1.2 <u>Oil phase:</u>**

The oil phase should be selected appropriately, since it governs the selection of the other ingredients for the microemulsion (mainly in the case of o/w microemulsions). There are two main factors to be considered before selecting the appropriate oil phase. First, to see the solubilizing potential of the oil for the selected drug, in order to assure maximum

solubilization of the drug. Second, the oil is chosen, such that the microemulsion forming region is enhanced. Oils with shorter hydrocarbon chains, such as medium-chain triglycerides (MCTs) or medium chain mono- and di-glycerides are easier to microemulsify, as compared to oils with long hydrocarbon chains. An oils ability to solubilize lipophilic groups is directly propotional to the chain length of the oil. Thus, the selected oil should be such that it is capable of solubilizing the API; and facilitating the formation of microemulsions with desired characteristics [19, 20].

#### 1.4.1.3 Surfactants:

Surfactant choice plays a crucial role in the formulation of microemulsions. The selected surfactants should posses the following two characteristics; it must microemulsify the selected oil and should possess good solubilizing potential for the API. Moreover, the surfactant should be acceptable for the desired route of administration. A high surfactant concentration can irritate the gastric mucosa and skin, or cause hemolysis upon parenteral administration. Natural surfactants are generally preferred over synthetic surfactants (e.g. phospholipids over Tweens or Spans [13]. Non-ionic surfactants possess certain advantages over cationic and anionic surfactants. The use of ionic surfactants may result in membrane perturbation and skin irritation [20, 21]. It is always safe to keep the surfactant concentration in microemulsions as low as possible irrespective of its nature, origin and type. The choice of the surfactant also depends on the type of microemulsion to be formulated. Low hydrophile-lipophile balance (HLB) surfactants are preferred for w/o microemulsion, whereas high HLB surfactants are preferred for o/w microemulsion [31]. Among the various surfactants, polysorbate 80, phospholipids, and poloxamer 188 have good acceptability for oral, dermal and parenteral delivery of APIs. Another class of

surfactants, commonly used in oral and dermal delivery are the Polyglycolysed glycerides[21].

#### 1.4.1.3 a) Thermodynamic Considerations

The thermodynamics of microemulsion formation can be explained by the following equation:

Equation 1: $\Delta G_f = \sigma \Delta A - T\Delta S$

Where,

$\Delta G_f$ is the free energy of Microemulsion formation.

$\sigma$ is the interfacial tension at the oil-water interface.

$\Delta A$ is the change in the interfacial area (associated with reducing droplet size).

S is the system entropy.

T is the absolute temperature

An increase in entropy $\Delta$ S occurs, when oil or water is dispersed in the continuous phase. This increase in entropy leads to the formation of a microemulsion. The interfacial tension is lowered when the surfactant molecules migrate to the oil-water interface. A further reduction in interfacial tension can be achieved, by adding a second surfactant. Droplet size reduction, during microemulsion formation, is associated with an increase in the interfacial area. In case of microemulsions, when the interfacial tension becomes very low, the free energy of the microemulsions becomes less than the entropy of dispersion.

Hence, the free energy of formation of the system becomes zero or negative. This explains the thermodynamic stability of microemulsions [22].

**1.4.1.3b) Role of Surfactants:**

The "Self-assembly theory of micelle and bilayer forming surfactant molecules", explains the formation of microemulsion systems, where the volume of the surfactant is denoted as v, its head group surface area α, and its length l defines the critical packing parameter (CPP):

Equation 2: $(CPP=v/\alpha l)$

If the critical packing parameter (CPP) in Equation 2, has values greater than one; a w/o microemulsion forms, whereas an o/w microemulsion forms when the CPP has values between zero and one. A bicontinuous structure is produced when the critical packing parameter is close to one. The hydrophilic-lipophilic balance (HLB) is another criterion to be considered when choosing surfactants to formulate microemulsions. It is desirable that the HLB of the surfactant blend matches the HLB of the oily component, for any system [22, 23].

**1.4.1.3 c) Nonionic surfactants:**

Nonionic surfactants, such as sorbitan esters and ethoxylated alkyl ethers, as well as nonionic block co-polymers [eg, poly (ethylene oxide) - block-poly (propylene oxide)] are generally less irritating and toxic than ionic surfactants [21]. Another advantage of nonionic surfactants over ionic surfactants is that, they can form microemulsions, even without the use of co-surfactants. There are two main reasons for using nonionic surfactants in topical formulations. One is that they have a good biological acceptance,

and secondly they donot require the use of co-surfactants. [20]. The most commonly used nonionic surfactants, are the Ethoxylated alcohols. Other examples of nonionic surfactants include, sorbitan monoleate (Span 80 R), polyoxyethylene sorbitan monoleate (Tween 80 R), polyoxyethylene sorbitan monolaurate (Tween 20 R) and other polyoxyehtlyene surfactants such as (Brij 35 R). These surfactants are considered safe for parenteral use [24]. The hydrophilic nonionic surfactants, which are used in many cosmetic products are polysorbates, which are series of polyoxyethylenated sorbitan esters.Polysorbates are oily liquids derived from Polyethylene glycolated sorbitan (a derivative of sorbitol) esterified with fatty acids. Surfactants that are esters of plain (non-PEG-ylated) sorbitan with fatty acids are usually referred to by the name Span. Nitrogen based surfactants that are used in microemulsion formulations include, alkanol amides and polyamines. Nonionic surfactants that contain sugar hydrophilic groups, such as alkylpolyglucoside surfactants, and sucrose ester surfactants are very hydrophilic and, in the presence of an alcohol, form temperature-insensitive microemulsions. Sorbitans are a class of lipophilc nonionic surfactants that are partial esters of sorbitol and its mono and dianhydrides with fatty acids [23].

**1.4.1.3 d) Ionic surfactants:**

Ionic surfactants find limited use in pharmaceutical formulations due to their toxicity. Most ionic surfactants have to be combined with co-surfactants in order to form stable micro emulsion formulations. There are three types of ionic surfactants namely, cationic, anionic and zwitterionic [3].

When the hydrophile, consists of a positively charged group, the molecule is known as a cationic surfactant. Cationic surfactants are based on quaternary ammonium cations. Quaternary ammonium cations retain their cationic character at any pH. A few examples of cationic surfactants are cetyltrimethyl ammonium bromide (CTAB), distearyldimethyl ammonium bromide (DDAB) and dialkylmethylimidazolium methylsulfate. Cationic surfactants have unique bactericidal properties [25].

Alkylbenzene sulfonates, ester sulfonates, sulfate esters, phosphate esters, fluorinated surfactants, fatty acid isethionates and sulfosuccinate esters are the most widely used anionic surfactants. Dioctyl sodium sulfosuccinate (DOSS), is a good surfactant to produce water in oil microemulsions. Sodium laureth sulfate, or sodium lauryl ether sulfate (SLES), is a detergent and surfactant found in many personal care products (soaps, shampoos, toothpaste, etc.). It is an inexpensive and very effective foaming agent [26].

Zwitterionic surfactants include phospholipids, particularly lecithin. These surfactants are biocompatible in nature. Lecithin is a naturally occurring, non-toxic and generally regarded as a safe material. When used as a sole surfactant, it is not able to produce isotropic solutions of water and oil over a wide range of compositions. Hence, co-surfactants such as short chain alcohols are used with lecithin in order to produce isotropic microemulsions. Lecithin is a constituent of membrane phospholipids and it contains phosphotidylcholine as a major component [27].

#### 1.4.1.3 e) Mixtures of surfactants.

When combining a nonionic surfactant with an ionic surfactant, the composition range over which microemulsions form in a particular oil-water mixture is greater. Mixtures of ionic and non-ionic surfactants are more resistant to temperature changes than non-ionic surfactants alone [3, 25].

#### 1.4.1.3 f) Co-surfactants:

When a surfactant alone cannot lower the oil-water interfacial tension sufficiently to form a microemulsion, a co-surfactant is usually needed. Co-surfactants affect the packing of surfactant molecules at the interface, because of their short chain amphiphilic nature (with a length of the carbon chain ranging from $C_2$ to $C_{10}$ ). The microemulsion structure and the curvature of the interface, is influenced by the co-surfactant chain length. Long chain alcohols swell the tail region more than the head region (negative curvature), whereas short chain alcohols swell the head region more than the tail region (positive curvature) [26]. The fluidity of the interfacial film has been found to be modified by co-surfactants. Liquid crystalline phases are formed when the surfactant film is too rigid. When co-surfactant molecules penetrate into the surfactant monolayer additional flexibility is provided to the interfacial film. This prevents the formation of liquid crystalline phases. Co-surfactants can alter the hydrophilicity/lipophilicity of solvents by their arrangement in the interfacial layer between the aqueous and oil phases. Transcutol$^{R}$ is a popular co-surfactant for oral and dermal delivery due to its good solubilization capacity and permeation enhancement [27].

#### 1.4.1.3 g) Poloxamers

Poloxamers are nonionic triblock co-polymers, containing a central hydrophobic chain of polyoxypropylene (poly propylene oxide), attached by two hydrophilic chains of polyoxyethylene (poly ethylene oxide). These molecules behave as surfactants, since they are amphiphilic in nature; and are useful in a variety of industrial applications. They can be used to increase the water miscibility of hydrophobic substances and oils and increase the miscibility of two substances with different hydrophobicities. These polymers are commonly used in cosmetics, pharmaceuticals, and many drug delivery applications [28].

### 1.4.2 Aqueous Phase:

Water is used as an aqueous phase in most microemulsions, especially for dermal delivery. The nature of the aqueous phase is important, mainly in the case of w/o microemulsions. For parenteral and ocular microemulsions the aqueous phase must be isoosmotic with blood and tear fluids. In order to achieve isotonicity, additives such as sorbitol, dextrose, glycerol and sodium chloride, may be added to water. These substances can affect the phase behavior of the microemulsions. Hence, the microemulsions must be characterized in the presence of these tonicity modifying agents, along with other constituents of the microemulsion [29]. Phase behavior of microemulsions containing ionic surfactants are influenced by salinity and temperature. In order to predict the behavior of microemulsions *in vivo,* Ringers Solution USP may be used as an aqueous phase. Another important factor that can influence the phase behavior of microemulsions, is pH. On oral delivery, the pH of the microemulsions can change in the presence of gastric and intestinal fluids. Hence, simulated intestinal fluid (pH 6.8) or simulated gastric fluid (pH 1.2) may be used as the aqueous phase. The stability of

lecithin microemulsions depends on pH. Therefore when formulating lecithin based microemulsions, the aqueous pH should be adjusted to between 7-8 to prevent hydrolysis of phospholipids [29, 30].

## 1.5 Uses of Microemulsions:

### 1.5.1 Advantages of Microemulsions:

Microemulsions possess several advantages that make them suitable for drug delivery. These include the following factors.

- **Ease of Preparation:**

Microemulsions form spontaneously at room temperature, and are easy to manufacture, when compared to liposomes and macroemulsions which require high pressure homogenization during preparation [18].

- **Thermodynamic Stability:**

The stability and shelf life of the formulation is improved due to the thermodynamic stability of the microemulsions.

- **Ability to incorporate both hydrophilic and lipophilic therapeutic agents:**

Microemulsions can form diverse microstructures which enables them to solubilize both hydrophilic and hydrophobic drugs, either alone or in combination [31].

- **Improved oral bioavailability:**

Microemulsions have been formulated that resist pH influenced structural changes particularly in alternating acidic and alkaline environments in the gastro-intestinal tract. A commercial example is Cyclosporin A (Neoral$^{R}$)[31].

- **As a template for the synthesis of nanoparticles:**

Microemulsions are thermodynamically stable, and consist of small droplets which possess large interfacial area. These characteristics facilitate their use in nanoparticle synthesis. [32].

- **Improved dermal and mucosal delivery:**

Dermal and mucosal transport of drugs is significantly improved due to enhanced penetrability of microemulsions into the skin [33].

- **Improved drug stability**

Microemulsions have been known to improve the photochemical, chemical; and enzymatic stability of these various therapeutic agents. Some examples include chloramphenicol (chemical stability), peptides (enzymatic stability), and arbutin (Photostability) [34].

### 1.5.2Disadvantages of microemulsions:

Microemulsions require a large quantity of surfactants and co-surfactants during their formulation. Some of these surfactants and co-surfactants are irritants and/or toxic to biological systems. Microemulsion stability may be sensitive to variations in pH and temperature [35].

### 1.5.3 Applications of Microemulsions:

#### 1.5.3 a)Transdermal and Dermal Drug Delivery

Human skin is considered to be the largest organ of the body. It functions as a barrier against penetration of toxic agents, loss of water and essential compounds from the body. Additionally, it also serves as a medium for absorption of drugs locally or systemically. Due to the structure, physiology and barrier properties of the skin, there are a number of opportunities and obstacles for drug delivery across the skin. The skin is made up of four distinct layers, namely the stratum corneum, the epidermis, the dermis and the subcutaneous tissue. The stratum corneum is 10-15 cell layers thick, is made up of dead cells or corneocytes and represents the main barrier to the delivery of most drugs. The intercellular spaces between corneocytes are generally filled with sheets of lipid bilayer membranes that are water impermeable; lipid lamellae within the stratum corneum, functions as an epidermal permeability barrier to water and other penetrants. Dermal and transdermal drug delivery requires overcoming this epidermal barrier without affecting the skin functions. There is a vast difference between dermal and transdermal drug delivery, in terms of their therapeutic requirements and advantages. Dermal delivery is targeted towards various skin infections such as psoriasis, eczema, skin cancer, acne and other fungal or microbial infections. In the case of dermal delivery, systemic absorption is not important, instead delivery of drugs to the pathological sites, is of major concern [33]. Transdermal drug delivery is targeted towards achievement of systemic levels of drugs. The drug, passes through the various layers of the skin, and reaches the systemic circulation, to produce its therapeutic effect. Transdermal Drug Delivery (TDD) is beneficial; for drugs which have a high first pass metabolism and for drugs which show

adverse side effects in the gastrointestinal tract such as gastrointestinal ulcerations. Calcium channel blockers such as felodipine and nifedipine are good candidates for transdermal drug delivery. The local anesthetic agent, lidocaine, has been commercialized as a therapeutic agent for dermal and transdermal drug delivery, and is marketed by the name Tropicaine R [33, 36].

### 1.5.3 b)Delivery of Peptides in Microemulsions:

Oral delivery of peptides is challenging due to their hydrophilicity, poor permeability, and poor stability in the gastrointestinal environment. Water- in- oil microemulsions can encapsulate hydrophilic molecules in the internal phase, and hence protect them from enzymatic degradation. Water-in-oil microemulsions are also known as reverse micelles. They can be used for biological studies, especially basic biochemical research and applied biotechnology. Enzyme containing microemulsions are of great interest, because the biomolecule in the microemulsions can, at times, show superactivity or catalyze unusual reactions. There have been studies showing that medium chain triglycerides and certain surfactants have the ability to resist hydrolysis of certain drugs in the gastrointestinal tract [37]. The nature of oil phase can improve permeability of the API, which may again be of advantage in oral delivery of peptides. Water-in-oil microemulsions were first explored, for their potential in delivery of peptides; such as vasopressin and insulin. The absorption of these peptides was found to be two times higher than their respective aqueous solutions from rat intestine. The bioavailability of these peptides was higher in the presence of straight chain fatty acids [34]. Insulin microemulsions with and without aprotinin; was compared with that of insulin solution.

The microemulsions were much more effective in reducing blood glucose levels when compared to the solution [5, 37].

### 1.5.4 Other Pharmaceutical Uses of microemulsions:

Microemulsions, are used in oral drug delivery and parenteral drug delivery. Most of the drugs formulated in microemulsions are poorly water soluble and their therapeutic efficacy is reduced. Some problems associated with such drugs, include poor oral bioavailability, poor absorption profile, and high intra- and- inter subject variability. Also a number of drugs belonging to BCS class III (high water solubility and poor permeability), show poor bioavailability and hence poor therapeutic efficacy. Since microemulsions possess good solubilizing potential for a variety of drugs, they can be used to improve the oral bioavailability of hydrophilic as well as lipophilic drugs. Self-microemulsifying drug delivery systems have been developed as an anhydrous form of microemulsion. These formulations are isotropic mixtures of oil, surfactant and co-surfactant, which when introduced into an aqueous environment with gentle mixing, spontaneously leads to the formulation of oil- in- water (o/w) microemulsions [38].

### 1.5.5 Other Uses of Microemulsions:

Microemulsions are used in the cosmetics industry due to their transparent nature, low viscosity and absorbability. Their use is advantageous in cosmetics because of their ability to solubilize both hydrophilic and lipophilic substances. Microemulsions also find use in enhanced oil recovery process, soil decontamination, for leather degreasing or washing processes. Microemulsions have also been used in hair products, cleaners, perfumes, gels and skin care products. Microemulsions can be used as artificial blood substitutes in the biological system. [36, 39].

# Chapter 2

## 2.0 Instrumentation:

## 2.1 Introduction

Microemulsions are colloidal drug delivery systems, and hence they can be characterized, by techniques used to characterize colloids. Visual evaluation helps to differentiate between microemulsions and other two phase systems such as emulsions. Microemulsions appear to be translucent or transparent, whereas emulsions are turbid [6,40].

To investigate the microstructure and phase behavior of microemulsions and to differentiate between liquid crystalline systems and various microemulsion types such as o/w , w/o droplet, bicontinuous or solution type systems, a variety of techniques are used. Conductivity experiments can be used to determine the nature of the continuous phase in a microemulsion. Microemulsions containing water in the continuous phase possess larger conductivity values when compared to w/o microemulsions [43]. Differential Scanning Calorimetry (DSC) has been a widely used approach to characterize microemulsion. Thermal behavior helps to understand the phase behavior of

the microemulsion. The freezing of water helps to identify the presence of interfacial and bound water in w/o microemulsions. If water is the continuous phase as in the case of o/wmicroemulsions; it is expected to show a freezing behavior similar to that of pure water [46].

A variety of other techniques can be utilized to study the microstructure of microemulsions. Microemulsions are isotropic and nonbirefringent in nature whereas lamellar liquid crystals are anisotropic and birefringent in nature. Polarizing light microscopy is a technique that can be used to distinguish between pure microemulsions and microemulsions in equilibrium with lamellar liquid crystals [41]. Particle size analysis can be performed using Dynamic Light Scattering (DLS).

**2.1 a) Visual Evaluation:**

Boundaries in a phase diagram can be established by visual evaluation of ternary or pseudoternary systems. Microemulsions, when first formulated, are monitored for transparency, signs of phase separation, and birefringence with the aid of visual evaluation and are then made to equilibrate at room temperature for 24 hours and then reevaluated. Visually microemulsions are transparent or translucent, and can be easily differentiated from emulsions. The transparency of microemulsions is attributed to their small droplet size of (10-100 nm), due to which it does not reflect visible light when compared to the bigger droplet size of turbid emulsions [6].

**2.1 b) Construction of Ternary Phase Diagrams:**

Ternary phase diagrams are used to study the phase behavior of simple microemulsion systems comprising of surfactant, oil and water at fixed pressure and temperature. Each corner of the ternary phase diagram represents 100% concentrations of a particular component. When four or more components are used pseuoternary phase diagrams are used to depict these systems in which each corner represents binary mixtures of two components such as surfactant/co-surfactant, surfactant/water, oil/drug, and water/drug mixtures. A typical ternary phase diagram is shown in figure 1

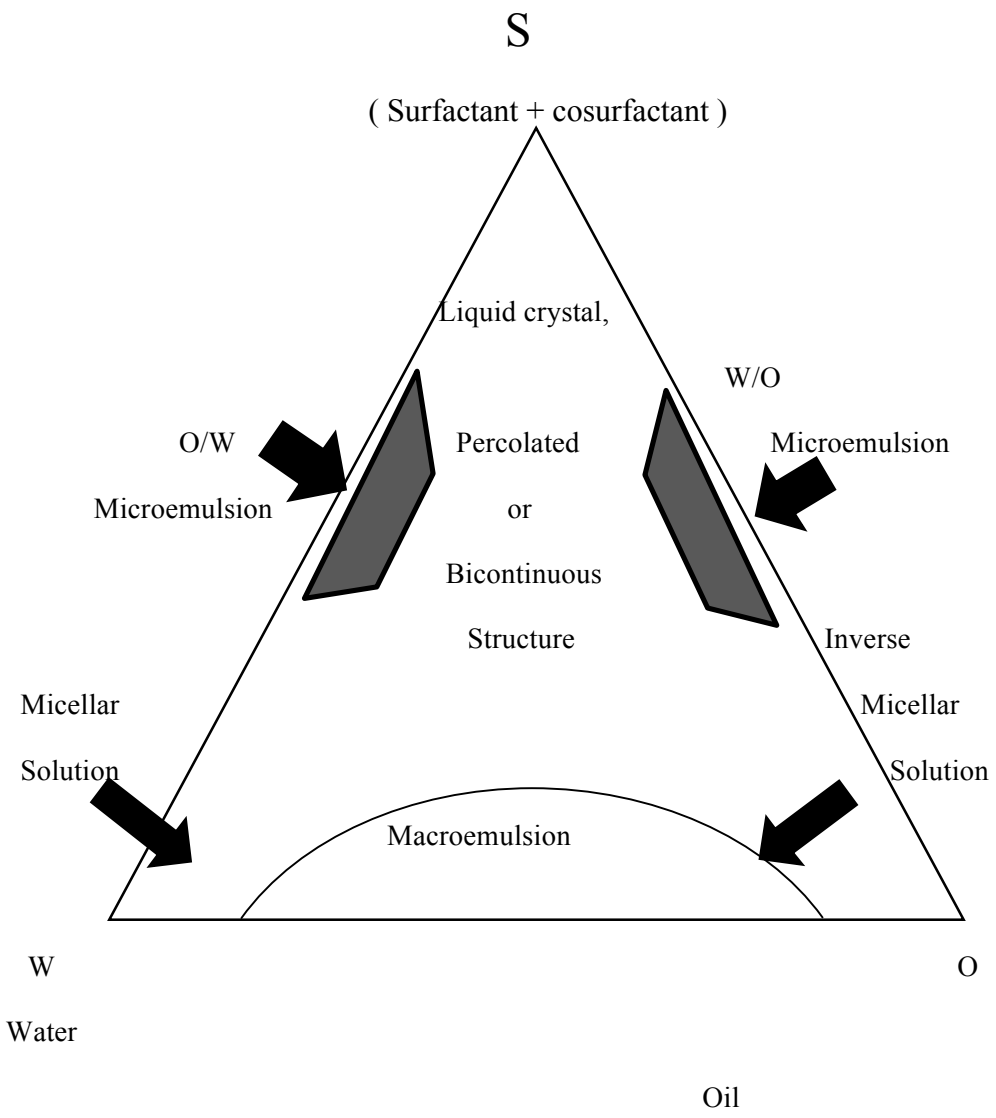



**Figure 2.1 Ternary Phase Diagram showing different phases [40]**

## 2.2 Polarized Light Microscopy:

Microemulsions are isotropic and can be differentiated from the liquid crystalline systems, which are anisotropic and show birefringence. Isotropic materials have optical properties, independent of the direction of incident light, whereas the optical properties of anisotropic materials vary with the direction of incident light. To observe the effects of birefringence and isotropy, one needs linearly polarized light. Common light, is referred to as one in which light waves are vibrating at right angles to the direction of light travel. Linearly polarized light consists of light waves which vibrate only in one direction [41]. Above the objective, the analyzer is usually positioned at $90^0$ to the polarizer, in a crossed position. Plane polarized light is produced when light from any light source is shone onto a polarizer, through which only light propagating into a certain direction can pass. When the plane-polarized light travels through the sample specimen and, if the specimen is anisotropic in nature and shows birefringence (such as lamellar phases, or liquid crystals), the plane polarized light is then split into ordinary and extraordinary light beams. If the sample is an isotropic microemulsion, the light beam will travel undisturbed through the sample. Polarizing light microscopy has the unique ability to identify submicroscopic structures of the samples being examined. Hence this technique is useful in differentiating between pure microemulsions and microemulsions containing liquid crystals [41, 42].

## 2.3 Electrical Conductivity:

Since microemulsions exist as w/o or o/w systems they can be characterized by electrical conductivity experiments. The type of microemulsion formed can be determined from its ionic conductance. Generally, the conductance of an o/w microemulsion (Winsor I) system is similar to the conductance of pure water, whereas the conductance of a w/o microemulsion (Winsor II) system is very low, and the conductance of bicontinuous microemulsion (Winsor III) system is very large. A percolation phenomenon is characterized by a dramatic increase in conductance depending on the composition and temperature of the microemulsion [43].

The low conductance of w/o microemulsions is due to the small quantities of water isolated in droplets and due to the migration of charged droplets in a non-conducting oil phase.

Percolation transition occurs at intermediate water concentrations when there is a gradual increase in the conductivity. For every microemulsion mixture, there is a corresponding critical water volume /concentration ratio (θc) at which percolation occurs. The increase in conductivity of the microemulsions up to ($\theta_c$) is due to an increase in the number of water droplets. The conductivity measurement above (θc) is due to the formation of water channels or droplet clusters, and the droplets fail to exist at this point.

Then there is another transition from the bicontinuous system into an o/w system which causes a sharp increase in the conductivity due to the presence of water as the continuous phase.

In alcohol based systems, the initial increase in conductance has been attributed to the ionization of the surfactant, caused by the alcohol. The decrease in the conductance is due to a change in the shape of the aggregates, or the formation of reverse micelles.

The ease of use, data interpretation and the low cost of the equipment makes; electrical conductivity an important characterization tool in the study of colloidal systems [43, 44].

## 2.4 Differential Scanning Calorimetry:

The Differential Scanning Calorimeter is thermal analysis equipment, which measures the energy required to establish a nearly zero temperature difference between the inert reference material and the substance. When both the sample and the reference are subjected to identical temperature conditions, in an environment heated or cooled at a controlled rate, this difference is then measured.

The mechanism by which DSC works, can be classified into two types [45],

(1) Heat- Flux DSC

(2) Power Compensated DSC.

### 2.4 a) Power- Compensation DSC:

A power compensated DSC contains sample and reference pan that are enclosed in two separate furnaces; heated with two separate heaters. Both the sample and reference are maintained at a constant temperature, and the thermal energy required to maintain the sample and reference at a constant temperature, is plotted as a function of time or temperature [45].

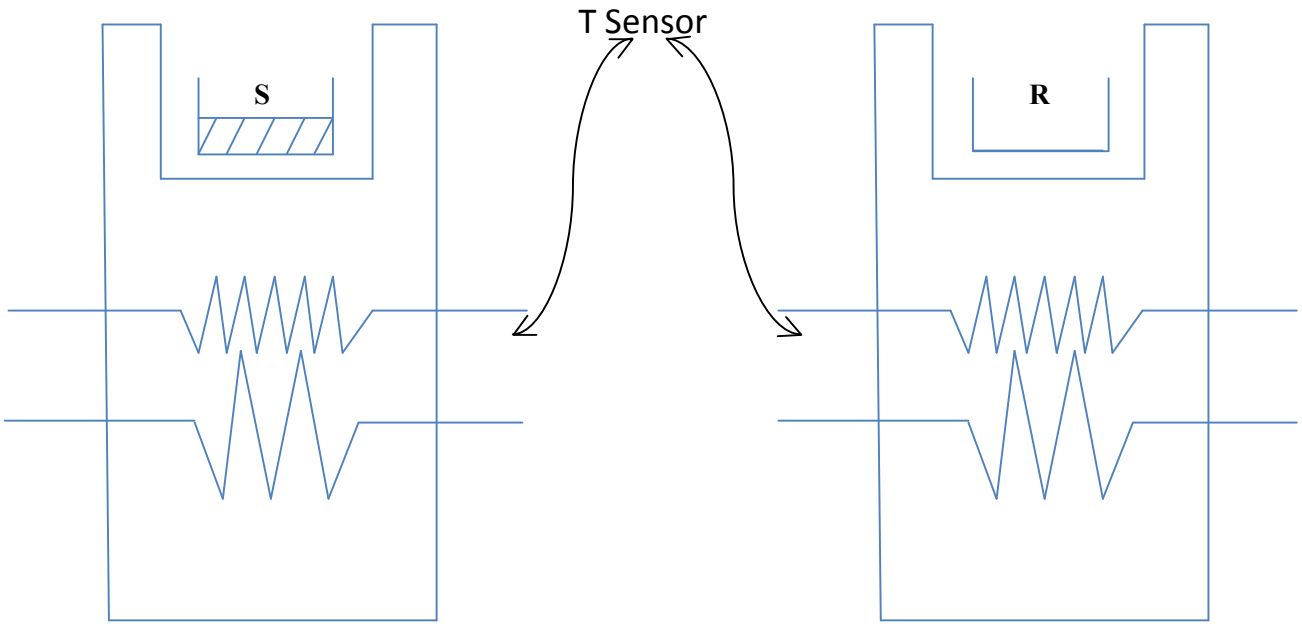



**Figure 2.2-Schematic of a Power Compensation Type ofDSC**

**2.4 b) Components of a DSC.**

The DSC consists of four basic components,

The furnace, autosampler, cooling system and computer.

**The Furnace**: This is the unit, where the sample and the reference are heated, according to a preprogrammed temperature regime.

**The Autosampler:** This feature may not be present in all DSC's. Its function is to load and unload the samples automatically.

**Cooling system:** This is used to achieve the temperature needed for the subambient thermal experiments.

**Computer:** This helps to automatically control the DSC instrument via a software capable of modulating the temperature regime in the furnaces.

DSC has been used in the pharmaceutical industry to study the solid state chemistry of drugs and excipients by determining glass transitions, heat of fusion, melting points, crystallization, oxidative stability, polymorphism, and chemical reactivity.

Differential Scanning Calorimetry has been widely used to characterize microemulsions. DSC depicts the thermal behavior of a pharmaceutical sample as a function of the temperature. Various endothermic and exothermic thermal events can be recorded in a DSC thermogram when samples are heated or cooled. Endothermic events that can be characterized include; sublimation, melting and chemical degradation. Exothermic events include crystallization. The DSC curves represent differential rates of heating, generally expressed in units of calories/second or joules/second, as a function of temperature. The peak areas in the DSC curves correspond to the heat absorbed by or emitted by the sample and is usually expressed as joules/gram. Freezing or melting behavior of water can provide a better understanding of the phase behavior of microemulsions [45].

### 2.4 c) Sub Ambient Differential Scanning Calorimetry:

Low temperature behavior of surfactant based microemulsions has been widely characterized by sub ambient Differential Scanning Calorimetry. A constant heat/cool/heat cycle is often used to understand the thermal behavior of microemulsions at sub-ambient temperatures. The thermal program initially freezes the sample at a constant rate and, subsequently, heats the previously frozen samples at a constant rate. These cycles help to study different types of water present within the microemulsion

system, and also give an insight into the microstructure of the microemulsions. A number of factors influence the freezing behavior of water such as the presence of nuclei, the cooling rate, the volume of water present and the size of microstructures [46].

Different types of water are generally detected in the microemulsions. These include bulk water, interphasal water, and bound water.

Bulk water is very similar to pure water. Hence, it has a heat of fusion close to that of the freezing point of water. Bulk water freezes around $0^{o}$C. At low temperatures it behaves similar to that of ice and at a temperature above $0^{\circ}$C, it behaves similar to that of water.

Water which is influenced by the surface of a substrate is generally referred to as bound water. Bound water is also called as the 'hydration shell'. The thermodynamic, kinetic, and hydrodynamic properties of water are generally altered by the presence of a nearby surface.

The distinction between bulk and bound water is valuable because it helps us to understand many biochemical reactions which can occur within the interfacial surfactant layers. This information is also important to understand the formulation of industrial microemulsions, and the rates of evaporation of water from the microstructure of these systems [46, 47].

## 2.5 Dynamic Light Scattering:

Dynamic Light Scattering (DLS), also known as Photon correlation spectroscopy (PCS) or Quasi Elastic Light Scattering (QELS), is a method for measuring the size of the particles in the submicron range DLS relates the size of the particles to the Brownian

motion experienced by the particles within a solvent system. Brownian motion is defined as the random movement of particles when they undergo bombardment by solvent molecules that surround them.

**2.5 a) <u>Dynamic Light Scattering Principle:</u>**

Emulsions and suspensions contain small particles that undergo Brownian motion induced by the particles bombarding with molecules of the medium..

The size of the particles within a formulation governs the rate at which the intensity of the scattered light fluctuates, When particles present in a liquid medium are illuminated using a laser, the scattered laser light undergoes a particle size dependant fluctuation in intensity. Generally, small particles are accelerated more by the solvent molecules and they start moving faster than large particles present in the medium. These intensity fluctuations when analyzed, yields the translational diffusion coefficient. The diffusion co-efficient is related to the hydrodynamic diameter via the Stokes-Einstein relationship [48, 49].

Equation 3 $d(H) = KT/3\pi\eta D$

where,

d (H) = hydrodynamic diameter

K = Boltzmann's constant

D = translational diffusion coefficient

T = absolute temperature

$\eta$ = viscosity.

The diffusion coefficient of a particle has been found to depend on the following factors,

1. Size of the particle.
2. Surface structure of the particle.
3. Concentration and type of the ions in the medium.

The DLS instrument generally consists of a monochromatic, coherent Helium-Neon laser with a wavelength fixed at 633nm as the light source. The laser is converged into the sample using a focusing lens. The particles scatter light at different angles. The DLS instrument consists of one detector, which detects the scattered light at $90^0$. The fluctuations of the scattered light are collected by the detector and converted into electrical pulses, which are then processed through a digital correlator. The auto-correlation function is then generated. Appropriate data analysis yields the particle size distribution [48].

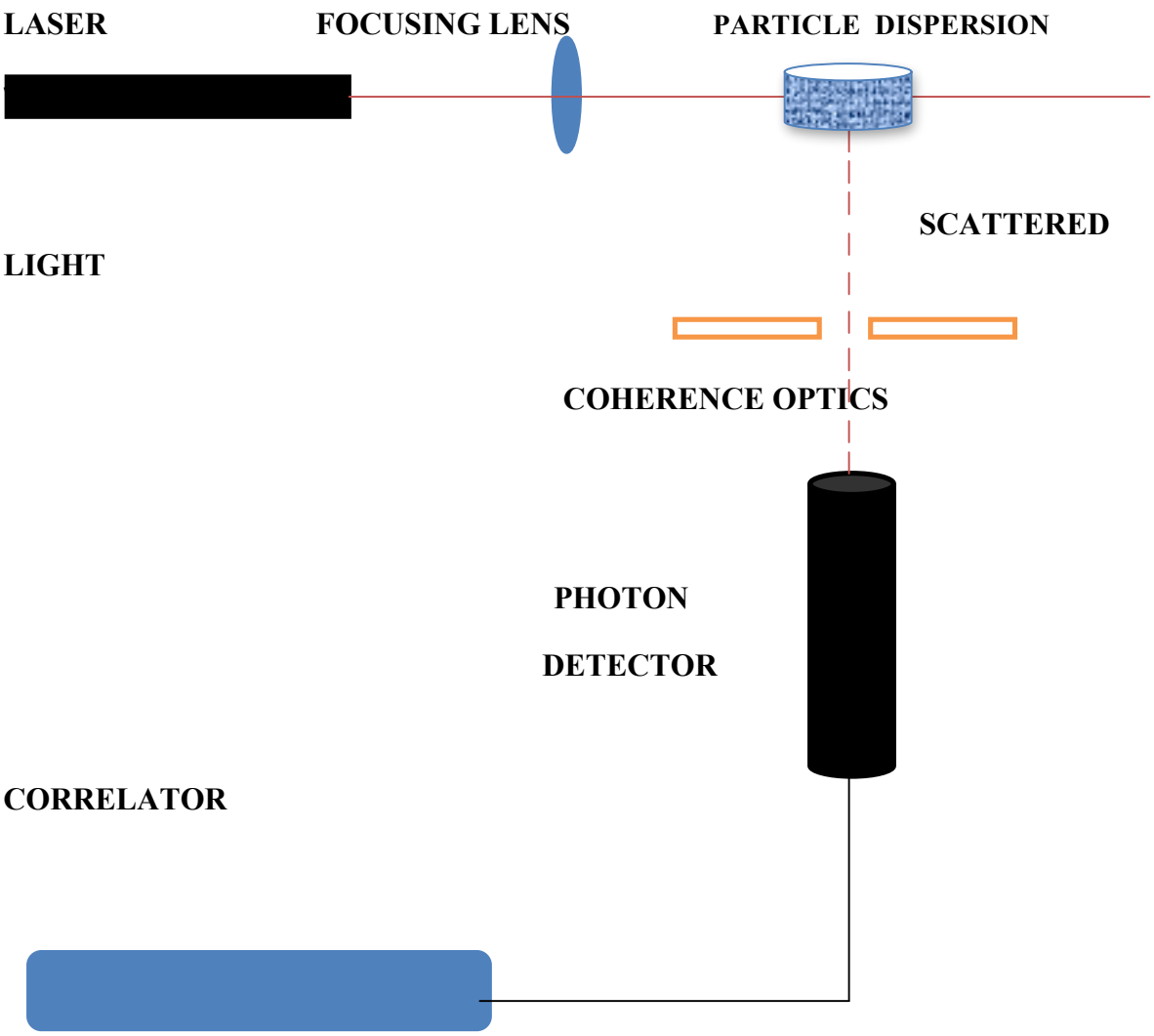



**Figure2. 3- Schematic of the Dynamic Light Scattering Instrument [51]**

## 2.5 d) Advantages of DLS

1. The set up of the instrument is simple, and the particle size measurement is performed automatically.
2. Sizes of less than 1nanometer can also be measured.
3. It permits accurate, reliable and repeatable particle size measurement in a few minutes.
4. It allows measurement, in the native environment of the material.
5. Highly concentrated or turbid samples can be measured directly, and it does not require any sample preparation.
6. It can measure the size of molecules with molecular weight less than 1000 Daltons [49, 50].

## 2.5 e) Applications of DLS

DLS is used in the particle size characterization of proteins, colloidal dispersions, polymers, micelles, carbohydrates, nanoparticles and microemulsions.

It is also used to measure zeta potential [51].

# Chapter 3

## 3. Materials and Methods:

### 3.1Materials:

#### 3.1.1. Isopropyl Myristate:

**Description:**

The oil used in the lecithin microemulsions was isopropyl myristate. Isopropyl myristate (IPM) is the ester of isopropanol and myristic acid. Isopropyl myristate is used in cosmetic and topical medicinal preparations; when good absorption through the skin is desired. Other names of isopropyl myristate are tetradecanoic acid, 1-methylethyl ester, myristic acid isopropyl ester [52, 53].

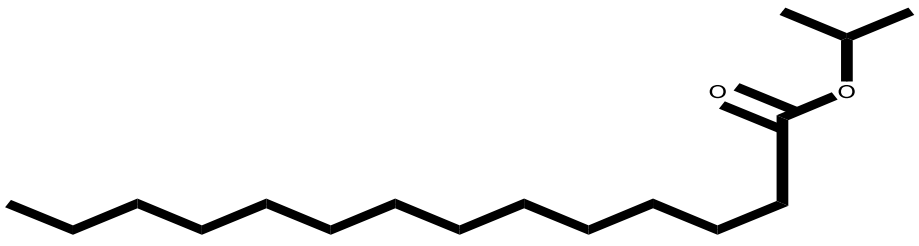



**Figure 3.1: Chemical structure of Isopropyl Myristate**

**Table 3.1 Physical and Chemical Properties of Isopropyl Myristate:**

| Name | IUPAC Name | Chemical Formula | Molar Mass | Density | Boiling Point | Source |
|---|---|---|---|---|---|---|
| Isopropyl Myristate | Propan-2-yl tetradecanoate | $C_{17}H_{34}O_2$ | 270.451 g/mol | 0.85 g/cm³, liquid | 167 °C | Spectrum Chemicals CAS No-110-27-0 |

### 3.1.2. Ethyl Oleate:

Another oil used as a continuous phase in the lecithin microemulsions was Ethyl Oleate (EO). Ethyl oleate is the oil formed by the condensation of the fatty acid oleic acid and ethanol. It is a colorless to light yellow liquid. Ethyl oleate is used as a solvent for pharmaceutical drug preparations involving lipophilic substances such as steroids. It also finds use as a lubricant and a plasticizer [54].

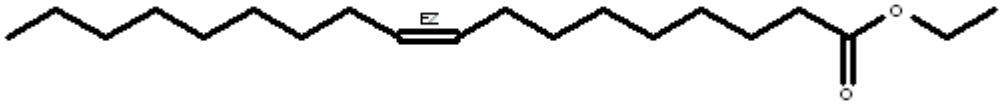


**Figure 3.2- Chemical structure of Ethyl Oleate**

**Table 3.2-Physical and Chemical Properties of Ethyl Oleate**

| Name | IUPAC Name | Chemical Formulae | Molar Mass | Density | Boiling Point | Source |
|---|---|---|---|---|---|---|
| Ethyl Oleate | Ethyl (*Z*)-octadec-9-enoate | $C_{20}H_{38}O_2$ | 310.51 g $mol^{-1}$ | 0.87 g/cm³, liquid | 210 °C | Spectrum Chemicals |

### 3.1.3 Lecithin

The surfactant used in the microemulsions was granular lecithin. Lecithin is a mixture of a number of phospholipids and it is generally present in a number of plant and animal tissues including egg yolk. In the pharmaceutical industry, it is used as a dispersing agent, wetting agent and stabilizing agent[55].

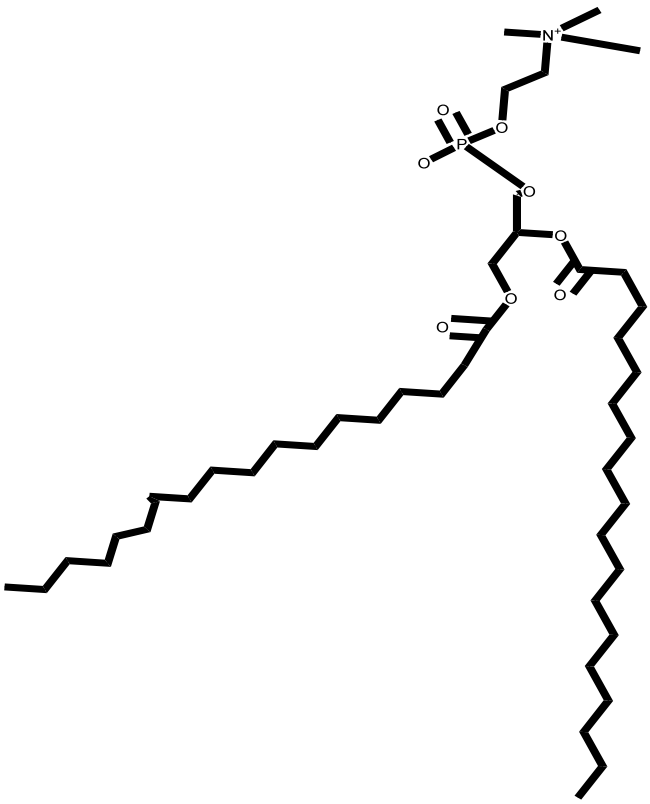


**Figure3.3- Chemical structure of Lecithin**

**Table 3.3 -Physical and Chemical Properties of Lecithin**

| **Name** | **IUPAC Name** | **Chemical Formulae** | **Color** | **pH** | **Source** |
|---|---|---|---|---|---|
| Soy Bean Lecithin | 2-[2, 3-bis [[(E)-nonadec-9-enoyl] oxy] propoxy-hydroxyphosphoryl] oxyethyl -trimethylazanium. | $C_{46}H_{89}NO_8P+$ | Golden to light tan. | 6.8 | Fischer Scientific AC41310-2500 Acros Organics No.:413102500 |

### 3.1.4. Isopropyl Alcohol:

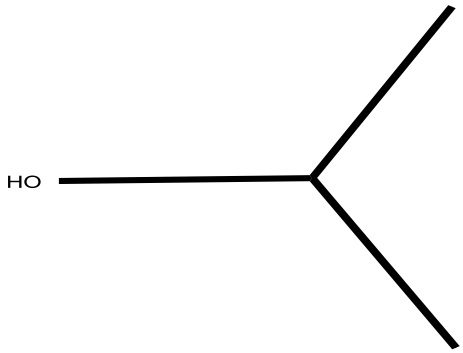



**Figure 3.4- Chemical Structure of Isopropyl Alcohol**

**Table 3.4 -Physical and Chemical properties of Isopropyl Alcohol:**

| Name | IUPAC Name | Chemical Formulae | Color | Density | Solubility in water | Source |
|---|---|---|---|---|---|---|
| Isopropyl Alcohol | 2-propanol | $C_3H_8O$ | Colorless liquid | 0.786 g/cm³ (20 °C) | Miscible | Chemical Stock Room |

### 3.1.5 Pluronic F-108.T

**Physical and Chemical Properties of Pluronic F-108**

**Name –** Pluronic F-108

**Molecular weight=** 14,600g/mol

**Color-** white to cream

**Synonyms-** Polyoxyethylene-Polyoxypropylene glycol.

**Form-** prills, cast solid, pastilles.

**Density-** 1.06 g/cm$^3$

**Solubility in water-** soluble

**pH-** 6-7.4

**Source-** 128-37-0 BHT 9003-11 BASF CORPORATION, Campus Drive, Florham Park, NJ 07932 USA.

### 3.1.6. Bovine Serum Albumin:

CAS No: 9048-46-8

**Chemical Structure:**

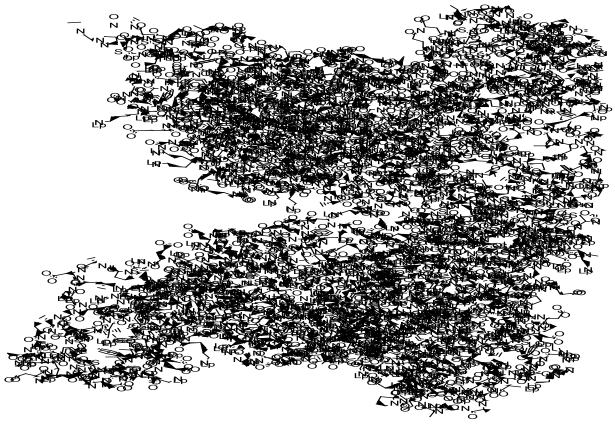


**Figure 3.5- Chemical Structure of Bovine Serum Albumin**

**Table 3.5 -Physical and Chemical Properties of Bovine Serum Albumin**

| Appearance | Odor | pH | Solubility | Molecular weight | Source |
|---|---|---|---|---|---|
| Off-white to tan powder | Slight | 6.7-7.3 | Soluble in water | 66382 Daltons | Fischer Scientific |

Bovine serum albumin is a serum albumin protein used widely in biochemical tests including ELISA, immunohistochemistry and immunoblots. At times, BSA is used as a nutrient in cell and microbial culture. In some of the restriction digests, BSA is used to stabilize enzymes during digestion of DNA, and also to prevent the adhesion of enzymes to reaction tubes and vessels [56].

BSA is also used to determine the quantity of other proteins, by comparing the unknown quantity of the protein to the known quantity of BSA. The advantages of using BSA as a standard is due to its stability, lack of effect in many biochemical reactions and low cost.

BSA has a number of uses as a carrier protein, and as a stabilizing agent in enzymatic reactions. BSA is also used as a blocking agent, in northern, southern and dot blot hybridizations. BSA is a common additive for polymerase chain reaction amplifications and gel shift assays. It is also used in buffers, for nick translation, polymerase reactions, and ligations [57].

## 3.2 Instruments and Softwares:

### 3.2.1 The DSC Unit [58] :

**Diamond DSC specifications:**

Power Compensation type of DSC.

Perkin Elmer Thermal Analysis Instruments

Cooling Apparatus: Liquid Nitrogen Cooling system.

**DSC Type:** Power- Compensation. Measures temperature and energy directly, rather than differential temperature (DT).

**DSC Cell:** Independent dual furnaces, constructed of platinum-iridium alloy, with independent platinum resistance heaters and temperature sensors with furnace mass less than 1g.

**Temperature Sensors:** Distributed, Platinum Resistance Thermometers for best linearity.

**Atmosphere:** Static or dynamic; including nitrogen, argon, helium, carbon dioxide, air, oxygen or other inert or active gases, over full temperature range. Oxygen can be used up to 730°C. A nitrogen atmosphere was used for the DSC experiments.

**Temperature Range:** -170°C TO 730°C.

**Software Used:**

The Diamond DSC was equipped with Pyris Series software used for recording and data analysis of the thermograms. The software can be used for changing the slope of the thermograms, shifting the curve, rescaling the axis and various other mathematical calculations such as peak area and enthalpy in joules per gram. The cooling apparatus used with the Diamond DSC, was a liquid nitrogen cooling system, with helium as the purge gas. The temperature range and thermal analysis rates are as mentioned below.

**Gas Control:**

Helium Gas was used as the purge gas with a flow rate of 50 ml per minute.

**Crucibles:**

Closed 20µL Aluminum crucibles were used for the DSC experiments. Approximately 3mg to 9mg of the samples and controls were weighed in the crucibles and then sealed with the help of a crimper used specially for liquid samples.

### 3.2.2. Polarized Light Microscope:

The polarized light microscope used was a Nikon model Tiu coupled with a photometric coolsnap EZ 20 monochrome camera, and was controlled by metamorph software. The samples were observed under a 40 X objective.

### 3.2.3. Conductivity Meter:

A Seven Multi, In Lab 741 conductivity meter from Mettler Toledo Instruments was used, with a conductivity probe cell constant of 0.102919$cm^{-1}$. The conductivity of microemulsions was measured in micro Siemens per centimeter.

### 3.2.4. Dynamic Light Scattering(DLS):

A Nicomp 380 ZLS [(Particle sizing Systems), Santa Barbara CA] was used to measure the particle size distribution of the microemulsions , with different $K_m$ (surfactant/co-surfactant) ratios, using both the oils Isopropyl Myristate and Ethyl Oleate. The channel width of the instrument was set to 200μsec, temperature set to $23^0$ C, and a scattering angle of $90^0$.

### 3.2.5. UV- Visible spectrophotometer:

A spectronic Max 190, UV instrument from Molecular Devices was used.

### 3.2.6. Other Softwares used:

Sigma Plot Software was used to construct the ternary and pseudo-ternary phase diagrams and other graphical representation of data.

## 3.3 Methods:

### 3.3.1 Microemulsion Formulation:

Microemulsions were formulated in various surfactant-oil mixtures. The surfactant concentration in these mixtures was modulated by varying the surfactant: oil ratio. Another parameter that was varied was the Km ratio or the surfactant/co-surfactant ratio.

The microemulsions were formulated at surfactant/oil ratios of 1:5, 1:10, 1:15, 1:2, 1:3 and 2:3, using the oils isopropyl myristate and ethyl oleate. The surfactant/co-surfactant or Km ratios used were 0.5, 1, 1.5, 1.77 and 1.94. The surfactant was accurately weighed and added to the oil. An appropriate amount of co-surfactant was then accurately added to prepare a mixture of the designated surfactant/co-surfactant ratio. This mixture of oil, surfactant, and co-surfactant was vortexed thoroughly with the aid of a vortex mixer for one minute at 3000 rpm. Water was titrated dropwise to this mixture and vortexed repeatedly, until a clear, transparent to translucent microemulsion was formed. Water was added until slight turbidity appeared in the microemulsions, this was noted as the maximum water that could be incorporated into the microemulsion [59].The samples were allowed to stand for 24 hours at room temperature, and then visually examined for any appearance of phase separation. This experiment was performed in triplicate, for each surfactant/oil ratio and each surfactant/cosurfactant ratio. The quantities, in grams for each of the surfactant, co-surfactant, oil and water were recorded [60].

### 3.3.2 Microemulsions formulated using poloxamer

Microemulsions with different surfactant to poloxamer ratio and different surfactant to oil ratios were chosen, in order to determine the amount of water incorporated increased or decreased, compared to when isopropyl alcohol was used as a co-surfactant. In this method, 5 grams of the poloxamer F-108, was weighed accurately and dissolved in 50ml of R.O water, by heating and stirring continuously in a beaker, until lumps of the poloxamer disappeared and a clear and transparent solution of the poloxamer in water was obtained. The oil-surfactant mixture was then titrated dropwise with the solution of poloxamer and vortexed repeatedly, until a clear-translucent to transparent viscous

solution was obtained [28, 61]. The amount of water incorporated using poloxamer was compared with the amount of water incorporated using the co-surfactant. The amount of water incorporated using the poloxamer was less, as compared to the amount of water incorporated using alcohol as the co-surfactant. The consistency of the microemulsion prepared using the poloxamer was thicker compared to the microemulsion prepared using alcohol as the co-surfactant.

### 3.3.3 Construction of Ternary Phase Diagrams:

Phase diagrams were constructed to identify the water-in-oil microemulsion forming compositions. The concentrations of the surfactant, co-surfactant, oil and water were normalized and converted into percentages using Sigma Plot® software. The pseudo-ternary phase diagram was constructed by representing (surfactant- co-surfactant binary mixture in one corner of the phase diagram, the concentration of the oil on the second corner of the phase diagram, and the concentration of water on the last corner of the phase diagram [62]. The region marked by the boundary of these plots was concluded to be the water in oil microemulsion region.

### 3.3.4 Polarized Light Microscopy Experiments:

The transparent microemulsions and milky emulsions were analyzed using a polarized light microscope, using both polarized and non-polarized light. The samples were prepared using the method described above and the milky emulsions were prepared by adding an excess of water to the transparent microemulsion. Approximately 10µL of each of these samples was pipetted onto a petri plate, with the help of a microsyringe and covered with a coverslip. The petri plate containing the sample was placed under the

eyepiece of the polarized light microscope. The image was analyzed in both polarized and non polarized light.

The images were analyzed when the maximum amount of light fell on the lens. The images were better resolved with the aid of the fine adjustment knob on the polarized light microscope. When the best resolution of the image was achieved, a picture of the image was taken with the help of the builtin camera. All images were analyzed on the computer attached to the microscope.

### 3.3.5. Conductivity Experiments:

The electrical conductivity of the microemulsions was measured using a conductivity meter. A ten milliliter sample of the oil-surfactant-cosurfactant mixture was placed in a vial. Water was then titrated dropwise to prepare a microemulsion containing a particular quantity of water. The conductivity probe was dipped into the microemulsion, until it gave a constant reading. The conductivity probe was rinsed and washed with R.O water, wiped clean, and the cleaning procedure repeated twice before taking another measurement. The conductivity of each of the other microemulsions, with different oil/surfactant and surfactant/co-surfactant ratios was measured following the same procedure described in triplicate.

### 3.3.6 Differential Scanning Calorimetry:

The sub ambient thermal analyses of microemulsions and controls were performed at a constant heat/cool/heat cycle. The first microemulsion sample chosen had a water concentration of 3.22%, another had a water concentration of 7.74%, and the third had a

water concentration of 9.063%.The controls chosen were isopropyl myristate, oil-surfactant mixture, oil-surfactant-co-surfactant mixture, isopropyl alcohol and water. The sample size ranged from 3mg to 10mg and the thermal analyses was performed in triplicate. The samples were weighed in 20μL aluminum pans, and sealed using a crimper. The samples were then made to undergo a heat/cool/heat cycle. The samples were cooled down to $-100^0$ C, at a constant rate of $5^0$ C /minute. These samples were then heated back to room temperature at a constant rate of $5^0$ C /min [63, 58].

### 3.3.7. Dynamic Light Scattering Experiments:

The microemulsions formulated using IPM and EO; were subjected to Dynamic Light Scattering experiments.

**Table 3.6- List of formulations chosen for DLS studies:**

| IPM | EO |
|---|---|
| Km =1, 2:3 | Km=1,2:3 |
| Km=1.94,2:3 | Km=1.94,2;3 |
| Km=1.94,1:5 | Km=1.77,2:3 |

To each of the IPM microemulsions, phosphate buffer of pH 7.4 was added, instead of water, for particle size analysis. The blank oil-surfactant-co-surfactant mixture was also tested for particle size analysis.

Before performing particle size analysis, the samples were centrifuged by transferring them to a 6x50mm Durex Borosilicate Glass Culture Tubes (VWR Scientific Products). The samples were then centrifuged at $23^0$C for 5 minutes at 5000 rpm, using an Eppendorf 5430 R centrifuge. Parameters including viscosity, channel width, and

refractive index were input into software in the Nicomp 380 ZLS (Particle sizing systems, Santa Barbara, CA) DLS instrument. The channel width was set at 200μSec; viscosity of IPM was 5.6cP, and refractive index as 1.434. The scattered light was collected at a scattering angle of $90^0$. The culture tubes were then placed in the DLS instrument and three cycles of 7 minutes each were performed in each sample.

### 3.3.8 Bovine Serum Albumin Formulations:

A total of 3 grams of bovine serum albumin (BSA) was dissolved in 100 ml of water, and 1.5 grams of bovine serum albumin was dissolved in 200 ml of phosphate buffer of pH 7.4. Selected oil- surfactant mixtures were prepared in IPM and EO. BSA solution was added dropwise to the surfactant-oil mixtures. All the experiments were done in triplicate and the maximum percentage of bovine serum albumin solution incorporated was compared to the maximum percentage of R.O water incorporated.

### 3.3.9 *In vitro* Release Studies:

The Hartree-Lowry albumin assay was used to evaluate *in vitro* release of BSA from these microemulsions [64]. A series of dilutions of 0.3mg/ml of bovine serum albumin was prepared in phosphate buffer of pH 7.4. Five albumin dilutions were prepared in test tubes containing 0.03, 0.06, 0.09, 0.12 and 0.15mg/ml of albumin in each of the test tubes. To 1 ml of each dilution of the standard and the buffer, 0.9 ml of reagent A was added which consisted of 2g of sodium potassium tartarate tetrahydrate, 100g of sodium carbonate, 500ml of 1N sodium hydroxide and water enough to make one liter. These test tubes were then incubated for 10 minutes in a $50^0$ C water bath and cooled to room temperature. To this 0.1ml of reagent B was added, which consisted of 2 gms of sodium potassium tartrate tetrahydrate, 1 gm of copper sulfate pentahydrate, 90 ml $H_2O$, and 10

ml 1N sodium hydroxide. The test tubes were again incubated for 10 minutes at room temperature, and 3 ml of reagent C, which consisted of 1 volume of Folin-Ciocalteau reagent diluted with 15 volumes of water, was added to each of the test tubes. The test tubes were again incubated for 10 minutes in the $50^0$ C bath, and then allowed to cool at room temperature. The final assay volume was 5 ml, and the absorbance was measured in each of these standards in triplicate by pipetting out 120 µL of the standard solution in a 96 well plate, and reading the absorbance in a spectronic max 190 Spectrophotometer (Molecular Devices) with phosphate buffer as a blank. The absorbance was measured at 650 nanometer and was plotted as a function of albumin concentration (mg/ml) to obtain a calibration curve.

The same assay procedure was followed for the microemulsion. Approximately 5 ml of the IPM/lecithin mixture, with a KM=1.94, 2:3 was placed in a test tube titrated with albumin containing solution and vortexed thoroughly. The sample was then transferred into a Spectra/Por Biotech Cellulose Ester Dialysis membrane with a molecular weight cutoff of 100,000 Daltons ( Spectrum Laboratories, Rancho Dominguez, CA), and clamped on both the ends to prevent spillage of the microemulsion. The filled dialysis membrane was then submerged in 1 liter of pH 7.4 phosphate buffer of placed in a beaker containing a magnetic stirrer. The medium temperature was maintained at $37^0$C by placing the *in vitro* release set-up on a magnetic stirrer/hotplate. Samples of 4 ml was withdrawn from the phosphate buffer and replaced with fresh buffer at various time intervals. The absorbance of the sample was measured at 650 nm in a 96 well plate, using a Spetronic max 190 spectrophotometer. The concentration of albumin was obtained from

the previously prepared calibration curve. Albumin concentration was plotted versus time to create a release profile. All the experiments were done in triplicate.

# Chapter 4

## 4.0 Results and Discussion:

### 4.1 Formulation of Microemulsions:

Lecithin Microemulsions were formulated in various surfactant-oil mixtures containing varying concentrations of surfactant and co-surfactant. The different surfactant/oil ratios used were 1:5, 1:10, 1:15, 1:2, 1:3 and 2:3, using two oils namely Isopropyl myristate and Ethyl oleate. The Km (surfactant/co-surfactant) ratios used were 0.5, 1, 1.5, 1.77 and 1.94 respectively [60]. The oil- surfactant-co-surfactant mixture was prepared and water was titrated dropwise into the mixture, and assessed visually, to determine the maximum amount of water which could be incorporated into the formulation. Phase diagrams were then constructed to visualize the microemulsion forming compositions [59]. This can be seen in Figures 9 to 13.

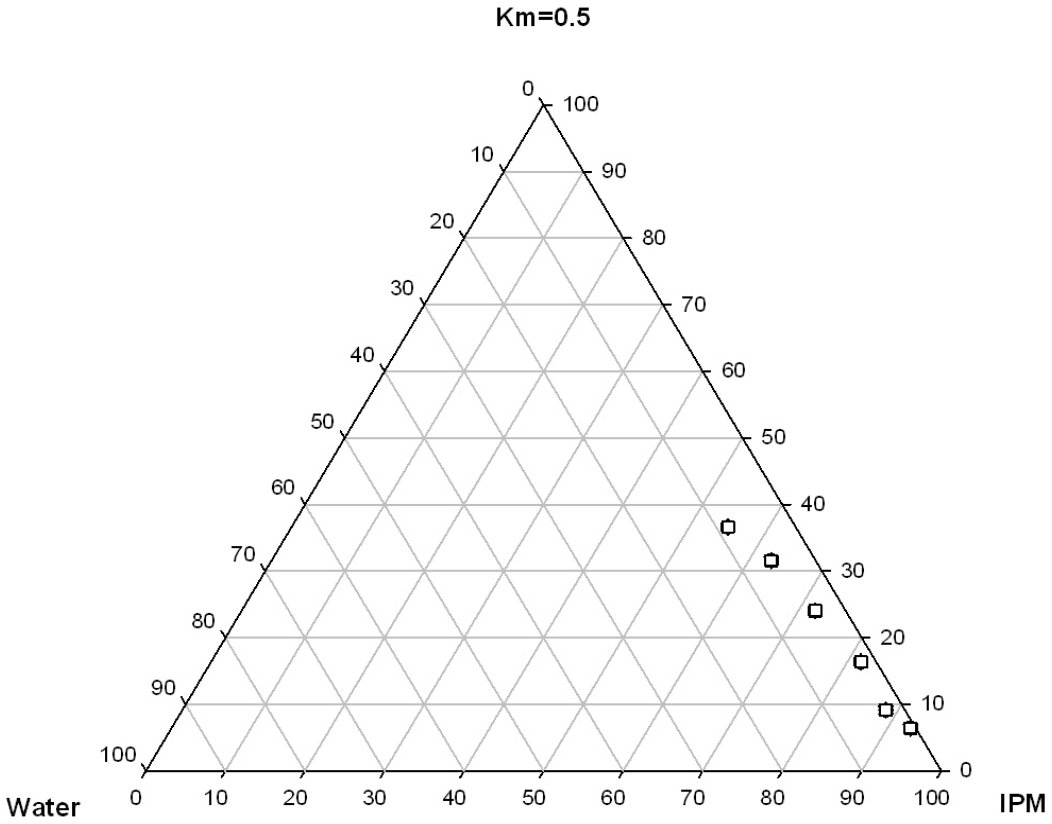



**Figure 4.1- Pseudo-ternary phase diagram representing microemulsion forming compositions in Km=0.5, for lecithin/IPM/IPA/Water systems.**

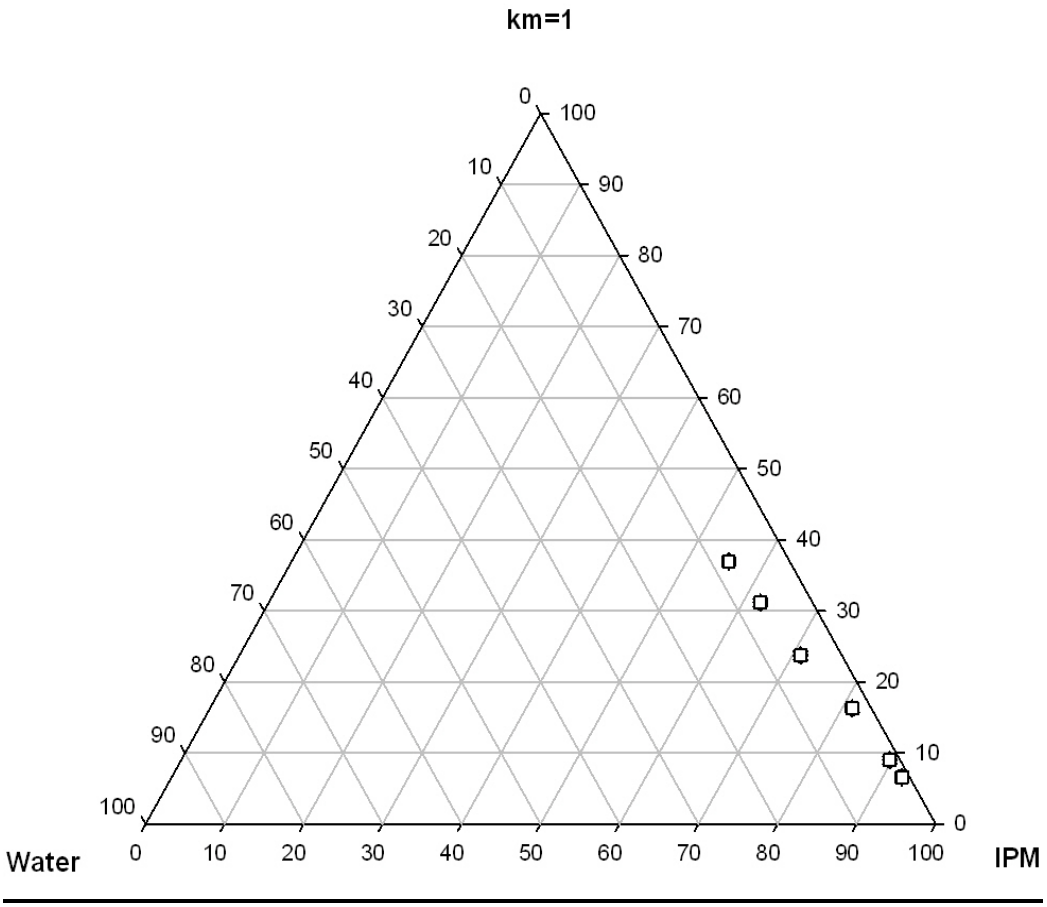



**Figure 4.2- Pseudo ternary phase diagram representing microemulsion forming compositions in Km=1, for lecithin/IPM/IPA/Water systems.**

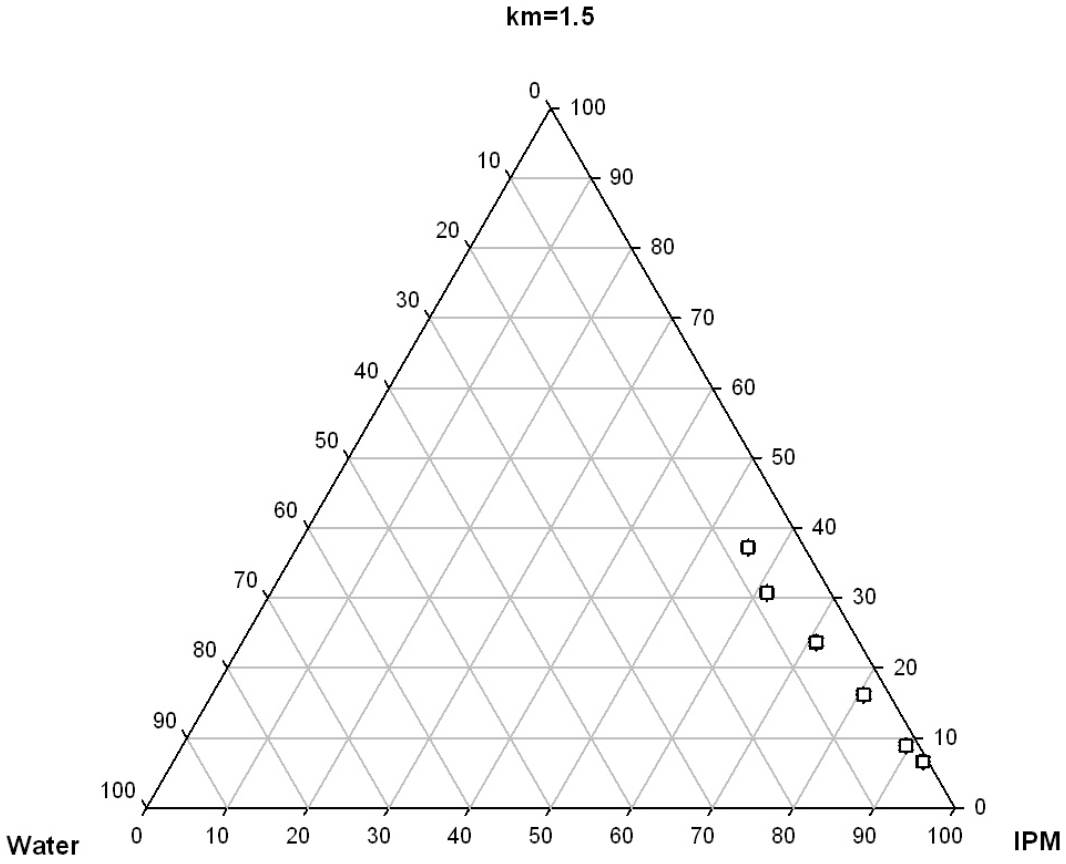



**Figure 4.3-Pseudo ternary phase diagram representing microemulsions forming composition in Km=1.5 for lecithin/IPM/IPA/Water systems.**

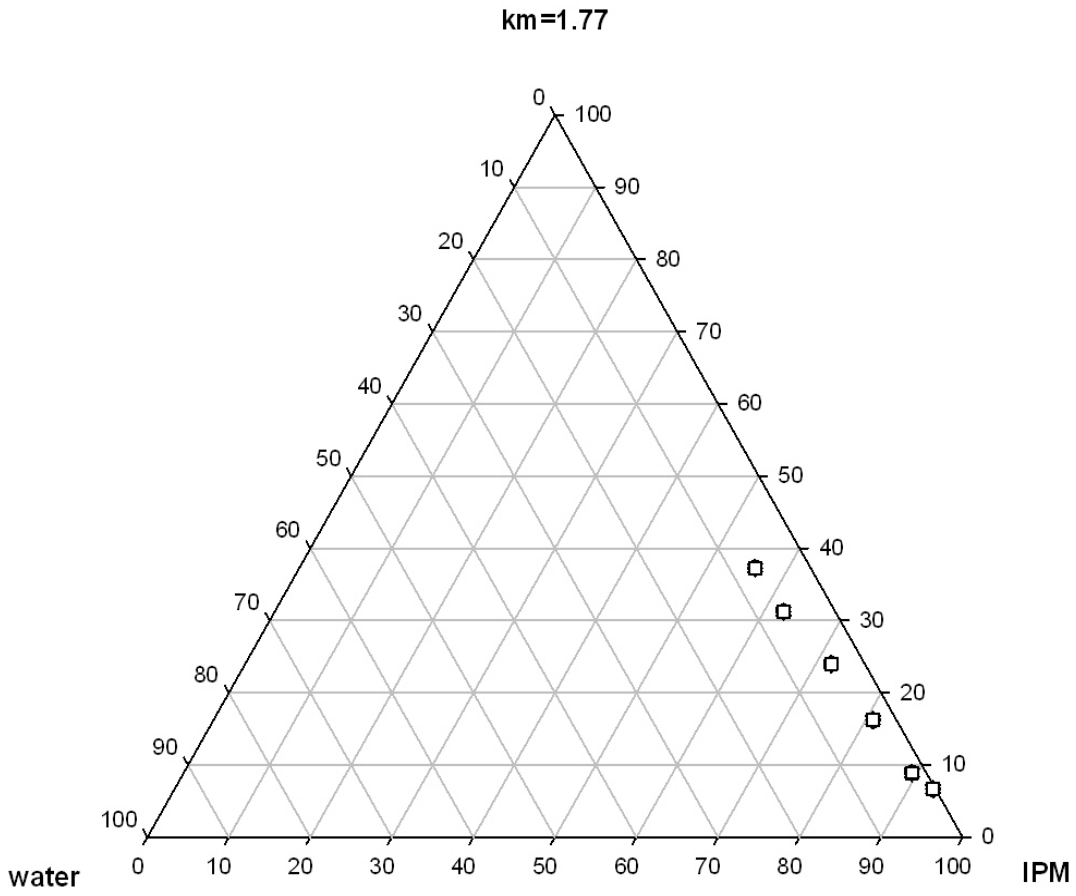



**Figure 4.4- Pseudo ternary phase diagram representing microemulsions forming composition in Km=1.77 for lecithin/IPM/IPA/Water systems.**

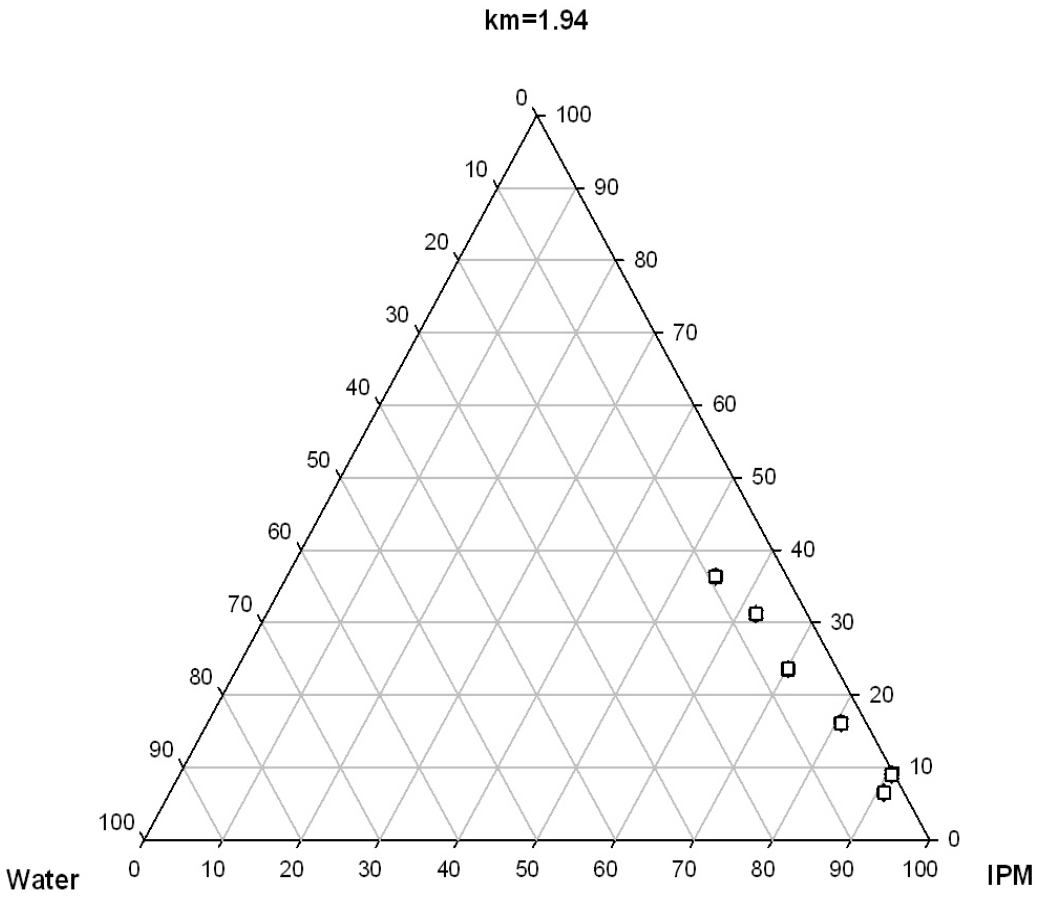



**Figure 4.5- Pseudo ternary phase diagram representing microemulsion forming composition in Km=1.94, for lecithin/IPM/IPA/water systems.**

**Ethyl Oleate Microemulsions:**

Microemulsions were formulated using ethyl oleate as the oil using a method similar to that used in IPM formulations. The surfactant was dissolved in the oil according to the surfactant to oil ratio used. Then IPA was added to the surfactant-oil mixture and vortexed thoroughly. Water was then titrated dropwise into the mixture. The surfactant/co-surfactant ratios used with ethyl oleate were Km= 0.5,1, 1.5, 1.77 and 1.94. Phase diagrams were constructed and these can be seen in the Figures 14 to 18 [65].

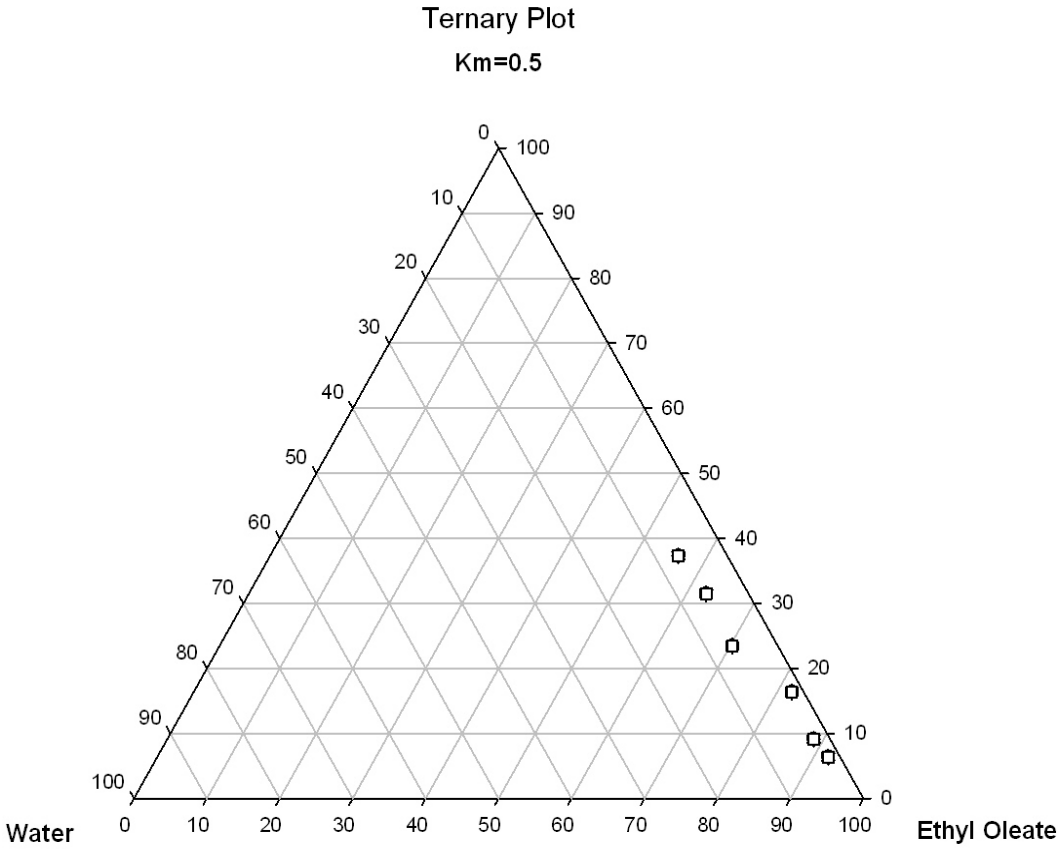



**Figure 4.6- Pseudo ternary phase diagram, representing microemulsion forming composition in Km=0.5, for lecithin/IPA/EO/water systems.**

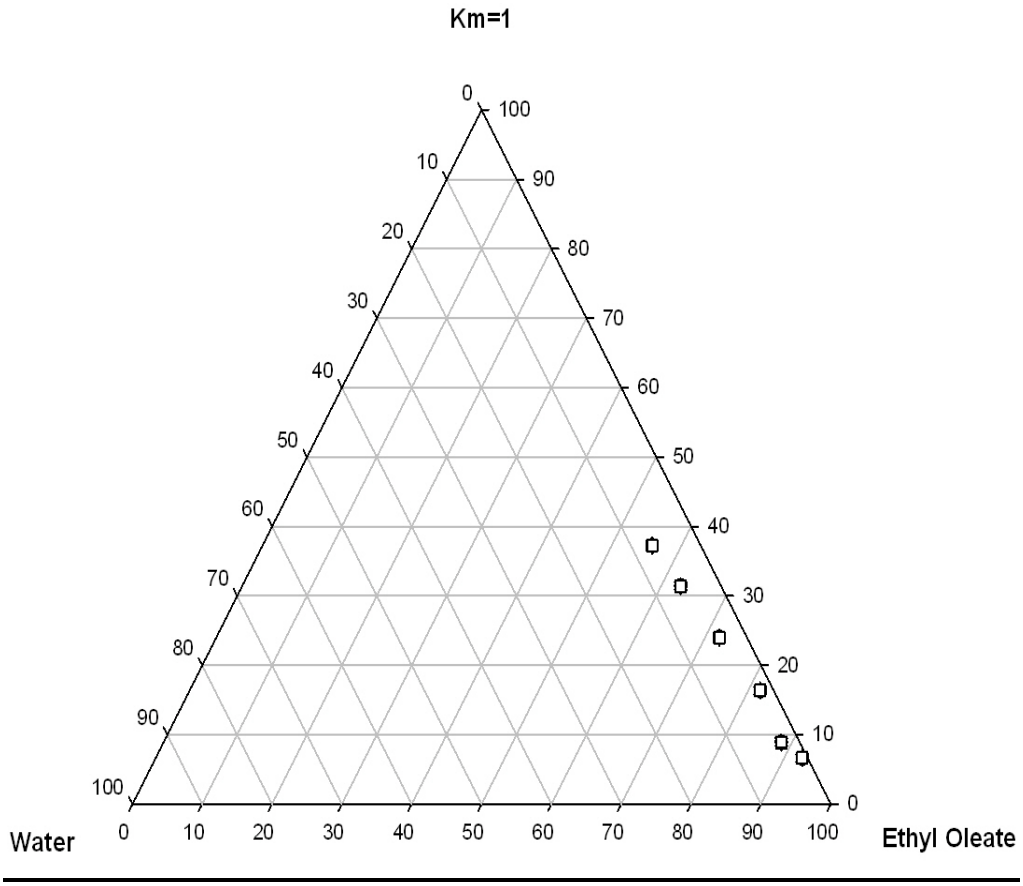



**Figure 4.7- Pseudo ternary phase diagram, representing microemulsion forming composition in Km=1, for lecithin/IPA/EO/water systems.**

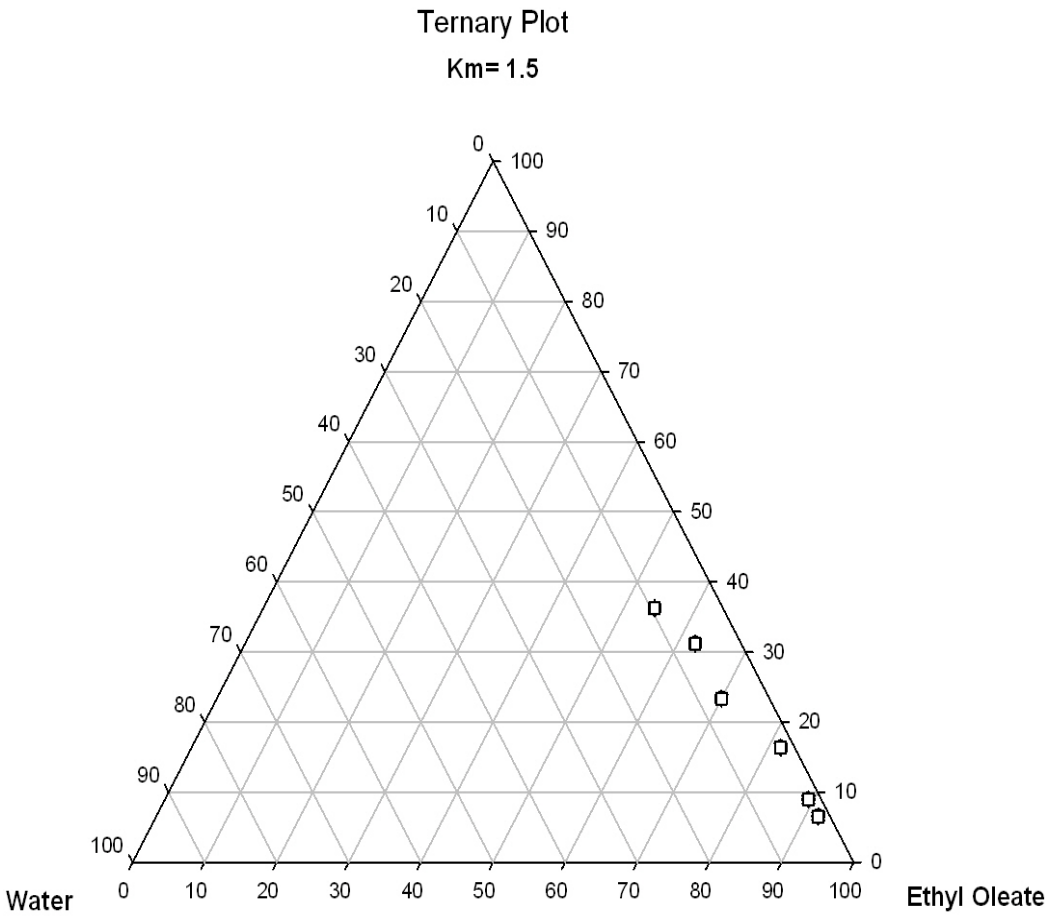



**Figure 4.8- Pseudo ternary phase diagram, representing microemulsion forming composition in Km=1.5, for lecithin/IPA/EO/water systems.**

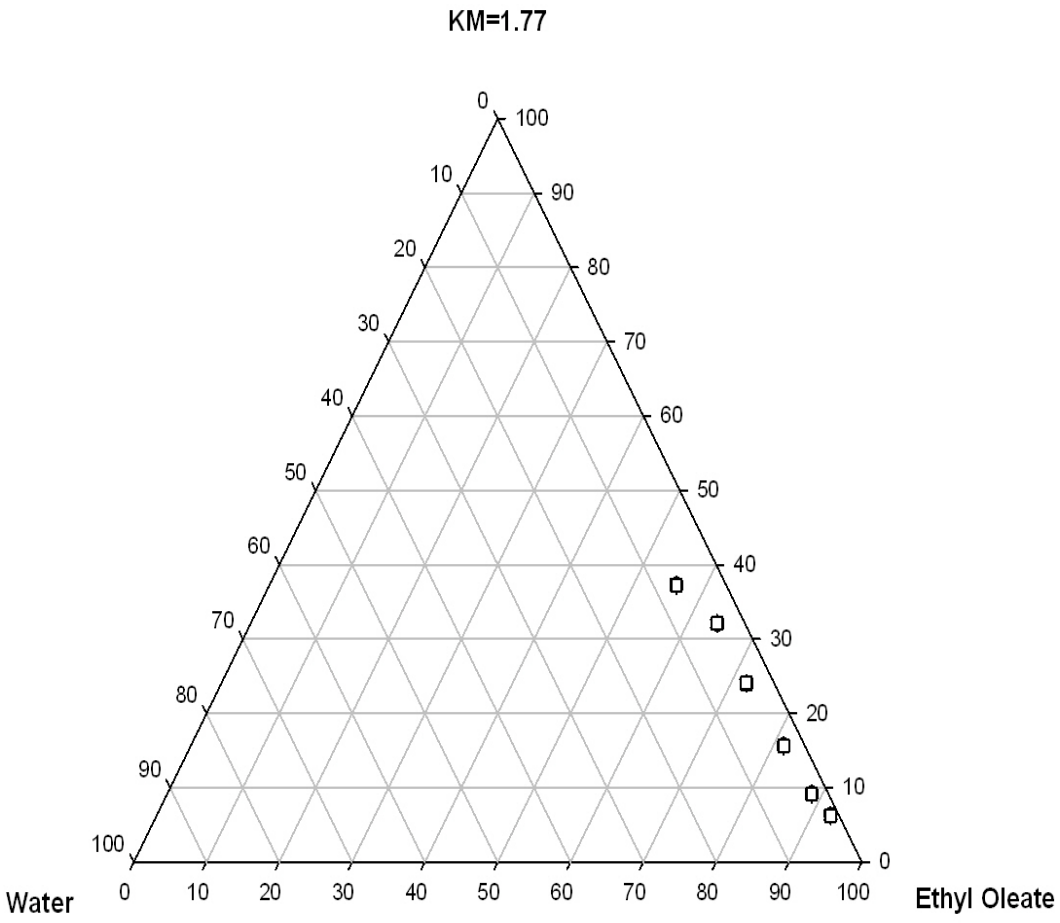



**Figure 4.9- Pseudo ternary phase diagram, representing microemulsion forming composition in Km=1.77, for lecithin/IPA/EO/water systems.**

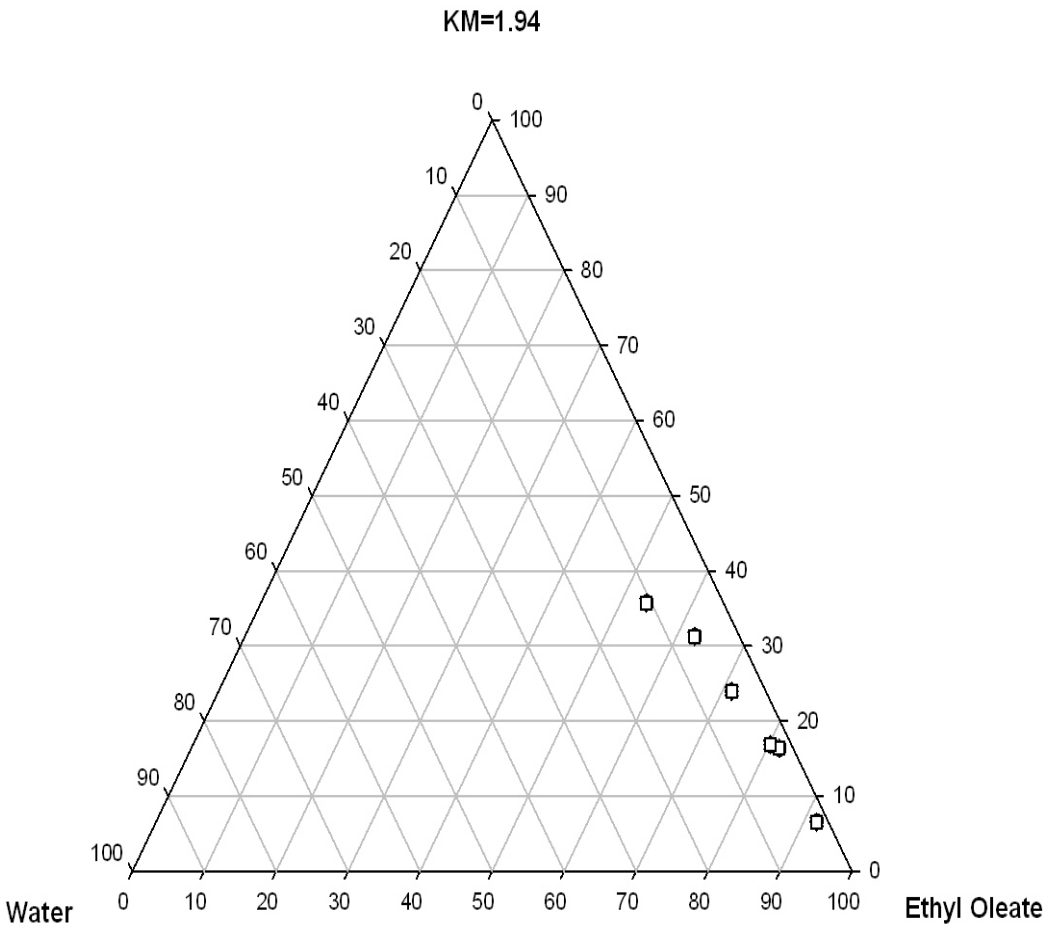



**Figure 4.10- Pseudo ternary phase diagram, representing microemulsion forming composition in Km=1.94, for lecithin/IPA/EO/water systems.**

Isopropyl myristate and ethyl oleate showed equal efficiency in forming microemulsions in the presence of lecithin and isopropyl alcohol. The surfactant to co-surfactant (Km) ratio did not influence the total area of the microemulsion forming region significantly. However, the surfactant-oil ratio affected the microemulsion forming region significantly. The surfactant/oil ratios of 1:5, 1:10 and 1:15, did not incorporate as much water as the 1:2, 1:3 and 2:3 ratios, which incorporated around 6%, 7% and 9% water, respectively. As the concentration of lecithin was increased in the microemulsions a greater amount of water was incorporated into the microemulsion [66]. Microemulsions which incorporated the maximum amount of water were those systems possessing a Km of 1.94, surfactant/oil 2:3 in IPM and EO. Since IPM has good skin permeability and emollient properties microemulsions formulated for further characterization in this project were prepared using IPM. Moreover, lecithin has good solubility in IPM. Lecithin organogels are formed when lecithin mixed with isopropyl myristate without the addition of alcohol. These organogels have been used for the topical delivery of a variety of medicaments. Lecithin IPM gels of broxaterol and scopolamine are used for the transdermal delivery of these compounds [67]. Propranolol hydrochloride, in 200 ml Lecithin/iso-octane organogel is used for the percutaneous delivery of compounds with poor skin permeability [67]. Ethyl oleate contains lipophilic surface active moieties. Lecithin is readily soluble in EO. However since soybean lecithin is a lipophilic surfactant of HLB 4 to 9, the mixture of ethyl-oleate and lecithin becomes too lipophilic. This necessitates the addition of a co-surfactant to reduce the interfacial tension and enables microemulsification to occur. The chemical nature of drugs and oils are factors that influence intermolecular interactions with the surfactant monolayer. A drug

BIBP3226; interacts with lecithin monolayer and partitions between the water phase and the surfactant film [68]. Due to the solubilization of this drug in the surfactant film; more water can be incorporated in the microemulsion.

### 4.1.1 Effect of the Surfactant/Oil ratio on Microemulsion Formation

Different surfactant-co-surfactant/oil systems were prepared using two oils, Isopropyl Myristate and Ethyl Oleate. These systems were titrated with water dropwise in order to determine the microemulsion forming regions [59]. Tables 7.1-7.5 represent the maximum amount of water which could be incorporated into these surfactant-oil systems which formed a transparent and homogenous microemulsion.

**Table 4-Tables showing percentage of water incorporated into the microemulsion in triplicate.**

**Km=0.5**

| **Oil** | **1:5** | **1:10** | **1:15** | **1:2** | **1:3** | **2:3** |
|---|---|---|---|---|---|---|
| IPM | 2.0±0.4 | 2.43±0.7 | 0.663±0.08 | 6±0.05 | 4±0.1 | 8.536±0.01 |
| EO | 1.6±0.03 | 2.27±0.2 | 1.64±0.05 | 6.2±0.07 | 6.7±0.04 | 7.2±0.03 |

**Km=1**

| **Oil** | **1:5** | **1:10** | **1:15** | **1:2** | **1:3** | **2:3** |
|---|---|---|---|---|---|---|
| IPM | 2.44±0.03 | 1.32±0.05 | 0.1±0.7 | 6.63±0.07 | 5.273±0.6 | 7.74±0.3 |
| EO | 3.22±0.05 | 2.62±0.08 | 0.8±0.2 | 6.0±0.12 | 5.8±0.2 | 6.97±0.05 |

**Km=1.5**

| Oil | 1:5 | 1:10 | 1:15 | 1:2 | 1:3 | 2:3 |
|---|---|---|---|---|---|---|
| IPM | 3.22±0.1 | 1.64±0.6 | 0.662±0.03 | 7.69±0.06 | 5.36±0.2 | 7.0±0.3 |
| EO | 2.0±0.2 | 1.817±0.13 | 1.7±0.2 | 6.4±0.3 | 6.68±0.1 | 9.5±0.2 |

**Km=1.77**

| Oil | 1:5 | 1:10 | 1:15 | 1:2 | 1:3 | 2:3 |
|---|---|---|---|---|---|---|
| IPM | 3.0±0.4 | 1.76±0.2 | 0.3±0.4 | 7.69±0.06 | 5.66±0.04 | 6.82±0.8 |
| EO | 2.34±0.2 | 2.0±0.3 | 1.153±0.3 | 5.39±0.7 | 3.8±0.5 | 6.8±0.4 |

**Km=1.94**

| Oil | 1:5 | 1:10 | 1:15 | 1:2 | 1:3 | 2:3 |
|---|---|---|---|---|---|---|
| IPM | 3.22±0.05 | 3.16±0.1 | 2.6±0.05 | 6.54±0.2 | 6.24±0.4 | 9.1±0.05 |
| EO | 1.96±0.06 | 3.0±0.3 | 1.6±0.3 | 6.25±0.3 | 4.75±0.5 | 11.0±0.03 |

In all the microemulsions prepared it was observed that if less than a threshold amount of water was added to the system the system was unstable. Only after the exact threshold amount of water was added did the mixture appear to be completely transparent and homogenous. The microemulsion formulated in IPM; at a Km ratio of 1.94, and surfactant/oil ratio of 2:3 incorporated 9.1% w/w of water. Initially when 1% w/w of water was added into the formulation it was hazy. When the amount of water in the microemulsion was increased gradually it cleared and became transparent in nature. After

the microemulsion was allowed to equilibrate at ambient temperature for 48 hours turbidity began to appear which may be due to the hydrolysis of the phospholipids in the microemulsion. This can be prevented by adjusting the pH of the microemulsion to between 7 and 8 [69]. Sometimes microemulsions are lyophilized to prevent hydrolysis of lecithin phosphatide groups and decomposition of these disperse systems [70].

### 4.2 Comparison of the % of water incorporated into microemulsions, formulated using Pluronic F-108 and Isopropyl Alcohol as Co-surfactants.

Pluronic F-108 was used as a co-surfactant to evaluate its potential to form microemulsions. The amount of water incorporated into poloxamer formulations were compared to the amount of water incorporated into isopropyl alcohol formulations. The microemulsions selected for the poloxamer efficiency study possessed the following Km, surfactant: oil ratios; Km=1, 1:10, Km=1.94, 2:3, Km=1.5, 1:5, Km=1.77, 1:5, and Km=1.77, 1:10.

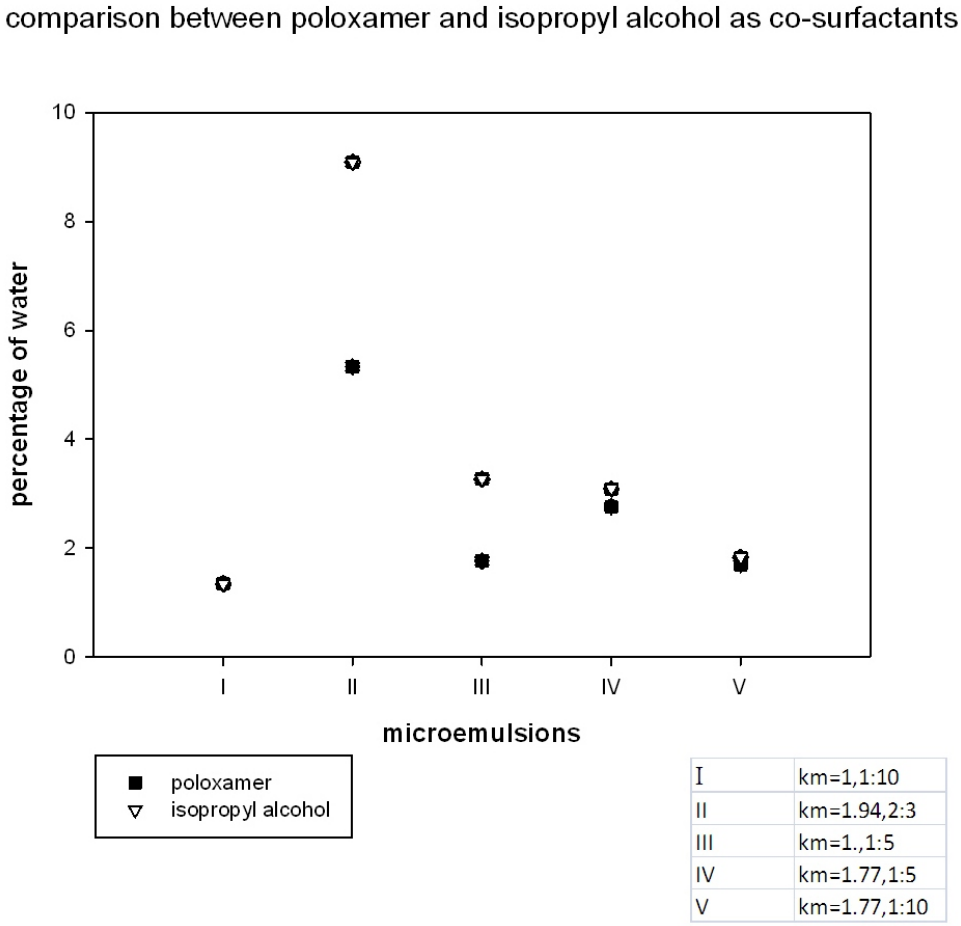



**Figure 19-Comparison of the amount of water incorporated into a microemulsion in IPA and poloxamer formulations.**

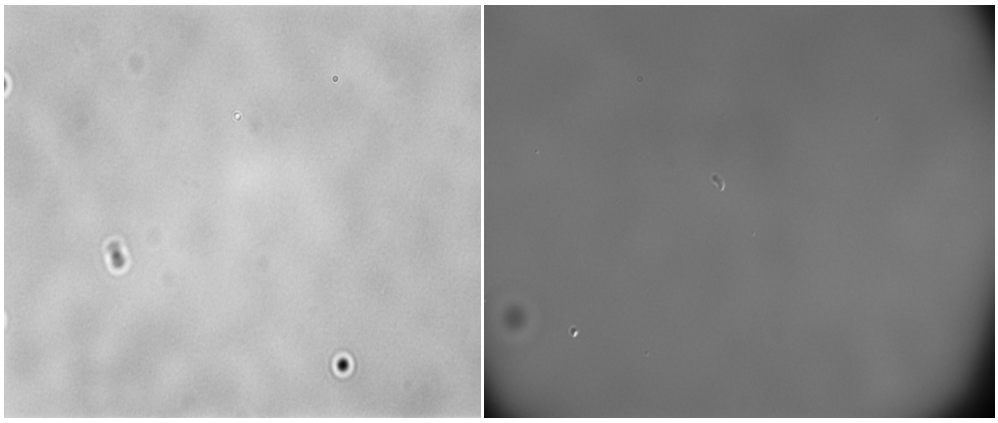
From the Figure 19, it can be concluded that isopropyl alcohol is a better co-surfactant than Pluronic F-108. IPA is able to incorporate a larger percentage of water into the microemulsion in all but two compositions compared to Pluronic F-108. This is evident from the Figure 19, in the Km 1.94, 2:3, Km 1.5, 1:5 and Km 1.77, 1:10, IPA incorporated larger amounts of water than poloxamer. In Km 1, 1:10 and Km 1.77, 1:10 systems IPA incorporated similar or slightly more amount of water than the poloxamer. It appears that alcohol is able to increase the flexibility of the surfactant film and reduce the

interfacial tension to a greater extent than poloxamer in the trial formulations. The advantage of using poloxamers as co-surfactants is that the microemulsion can be used for parenteral drug delivery, whereas IPA containing formulations cannot be used for parenteral drug delivery [71]. Microemulsions prepared using poloxamer were thicker and more viscous when compared to microemulsions prepared using IPA. This may extend their potential for dermal or transdermal delivery of drugs. The reduction in the amount of water incorporated in the presence of Pluronic F-108 may be partially due to its inability to lower the HLB of lecithin. IPA mixes with the aqueous phase making it less hydrophilic enabling spontaneous microemulsification to occur [72]. It was also observed that when the microemulsion containing poloxamer was mixed with the microemulsion containing alcohol the hydrolysis of phospholipids was decreased significantly. After 48 hours of equilibration the microemulsion did not show any visible precipitation or haziness that may be attributed to the hydrolysis of phospholipids. This may be due to an enhanced intermolecular interaction of these co-surfactants with the lecithin monolayer.

### 4.3 Polarized Light Microscopy:

When plane polarized light is passed through isotropic samples it transmits through the matrix undisturbed because isotropic samples are homogenous and do not show well defined structures. Since microemulsions are isotropic in nature they do not show optical birefringence [73]. The microemulsion with Km=1.94, and surfactant to oil ratio of 2:3 in lecithin/IPM and lecithin/EO; mixtures were selected for polarized light microscopy studies. Compositions that formed transparent microemulsions as demonstrated by the ternary phase diagram, and those beyond the microemulsion forming region, were

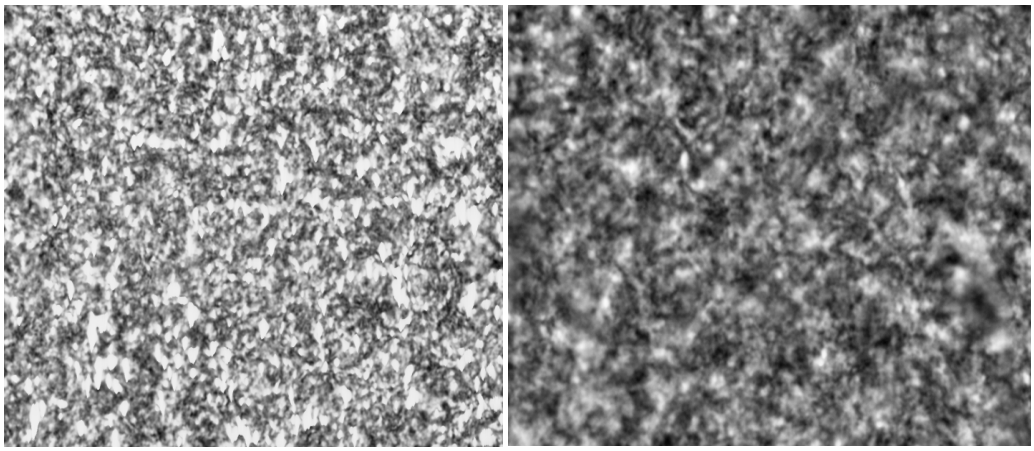
observed under the microscope. Microemulsions of Lecithin/IPM mixture containing 9.063% R.O water and 15% R.O water were prepared. The Lecithin/IPM microemulsion sample containing 9.063% did not show any birefringence in the presence of polarized light suggesting that it was a microemulsion. The Lecithin/IPM emulsion containing, 15% water, showed birefringence in polarized light. (See Figure 20)



**a)** **b)**

**Figure 4.11- Optical micrographs of Lecithin/IPM microemulsions (9.063%w/w water), a) without polarizing filter, b) with polarizing filter.**

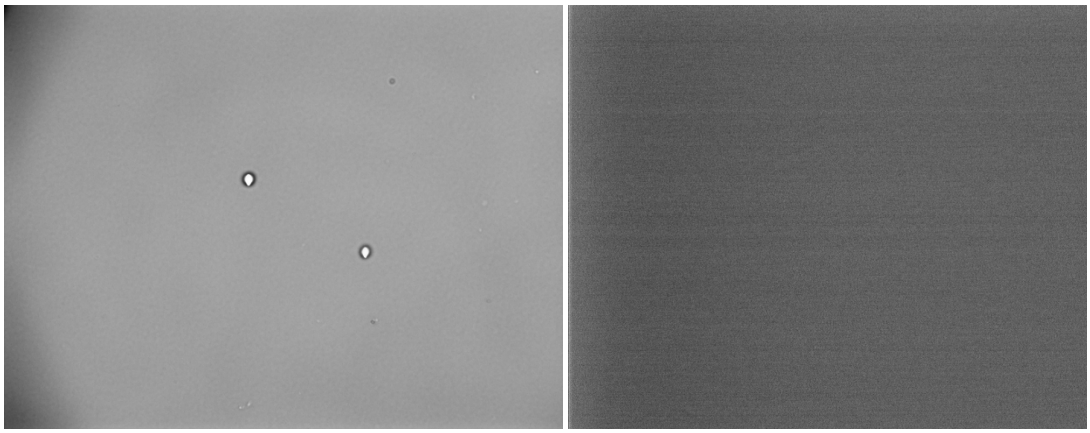
The microemulsion samples appeared to be clear and transparent and hence no birefringence is observed in these samples. When, these microemulsions were further diluted with 15% water, (i.e. beyond the microemulsion forming region of the ternary phase diagram) structural transition to a lamellar of vesicular nature appears to have occurred [74]. (See Figure 21)



**a)** **b)**

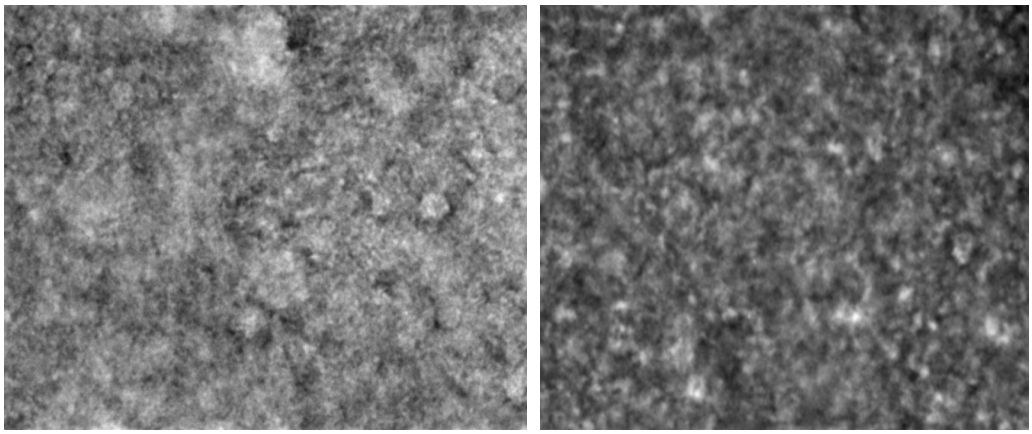
**Figure 4.12 - Optical micrographs of Lecithin/IPM emulsions (15% w/w water) a) without polarizing filter, and b) with polarizing filter.**

Microemulsion possessing Km value of 1.94 and surfactant: oil ratio of 2:3 was formulated in Lecithin/EO mixture. Two formulations were prepared. One microemulsion was formulated with 11% water and another emulsion contained 20% water. The sample containing 11% water did not show any optical birefringence when observed through a polarizing filter. The sample containing 20% water, showed optical birefringence [74]. (See Figures 22 and 23)



**a)** **b)**

**Figure 4.13-Optical micrographs of Lecithin/EO microemulsions (11% w/w water), a) without polarizing filter, b) with polarizing filter.**



**a)** **b)**

**Figure 4.14-Optical micrographs of Lecithin/EO microemulsions (20% w/w water), a) without polarizing filter, b) with polarizing filter.**

## 4.4 Conductivity:

Electrical conductivity of Lecithin/IPM and Lecithin/EO microemulsions were characterized. The microemulsion formulations chosen to measure the electrical conductivity for the Lecithin/IPM mixtures were Km=1.5, s/oil=1:5, Km=1, s/oil=2:3, Km=1.94,s/oil=1:5, Km=1.77,s/oil=1:5 and Km=1.94,s/oil=2:3. For the Lecithin/EO microemulsions the corresponding values were; Km=1.94, s/oil=2:3, Km=1.5, s/oil=2:3 and Km=1.5, s/oil=1:2. The conductivity in these formulations was evaluated by varying the percentage of water added to these microemulsions. The conductivity values obtained were then plotted as a function of the percentage of water added to the microemulsion. Very low conductivity values of 0.050μSiemens/cm, 0.055μSiemens/cm, 0.060 μSiemens/cm, 0.16 μSiemens/cm and 0.20 μSiemens/cm, were seen at 1%, 2%, and 3% percentages of water in the formulations. This demonstartes that oil is the continuous phase in these microemulsions. The water droplets are covered by the surfactant layer and are isolated in the non-conducting oil phase. But when the concentration of water is increased in these microemulsions, there is a gradual increase in the conductivity of the water in oil microemulsions, due to the presence of a large number of water droplets, up to the point it exists as a microemulsion known as the Percolation Threshold [75]. There is a gradual decrease in the conductivity after the percolation threshold since it leaves the boundary of the microemulsion forming region. The large sized droplets cannot migrate easily in the emulsion which forms eventually decreasing conductivity.

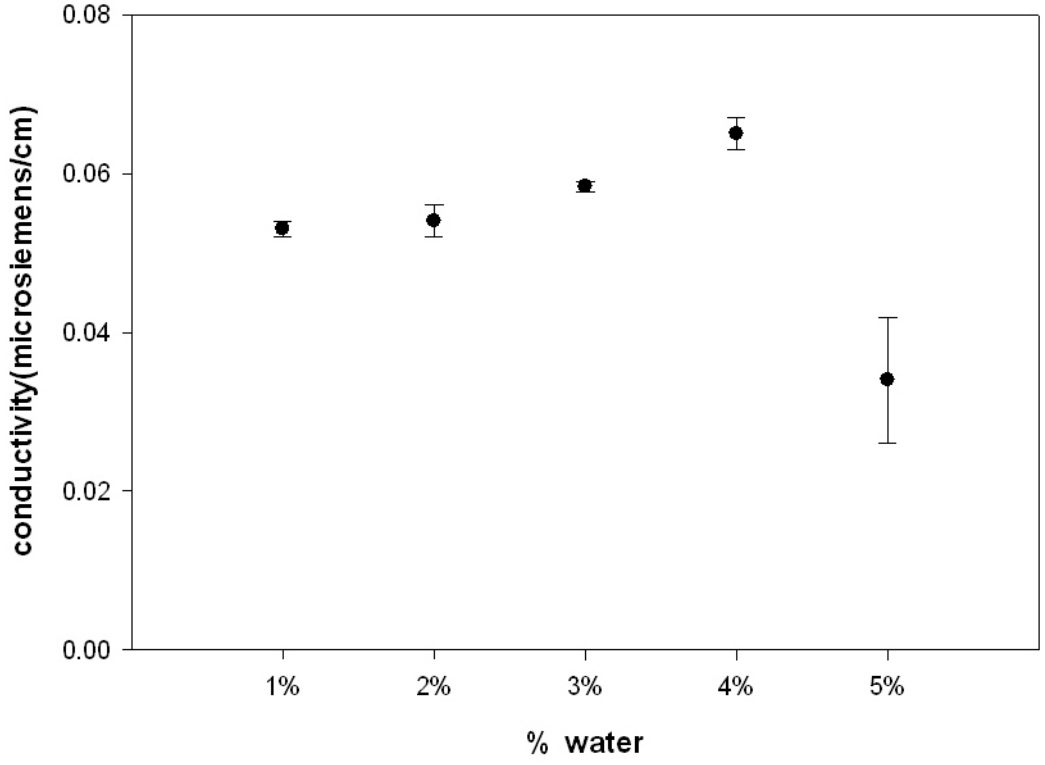



**Figure 4.15- Conductivity of microemulsion with Km=1.5, s/oil 1:5 (3.22% water), as a function of water concentration.**

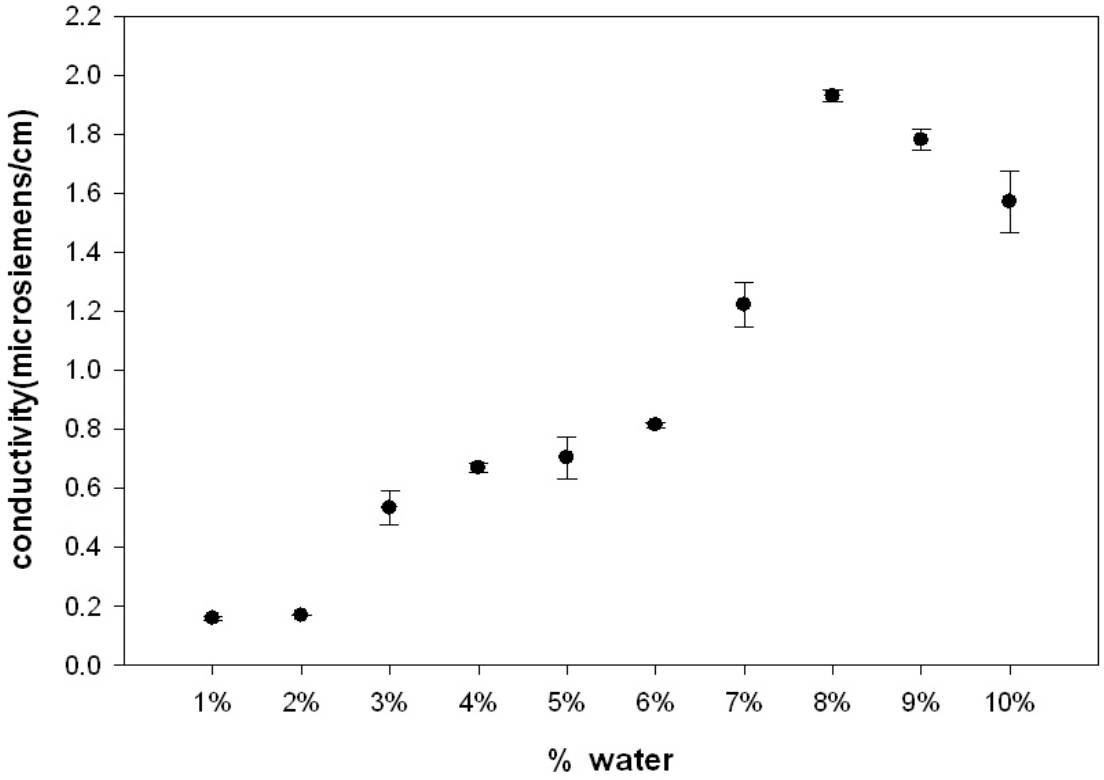



**Figure 4.16- Conductivity of microemulsion with Km=1, s/oil ratio 2:3 (7.74%), as a function of water concentration.**

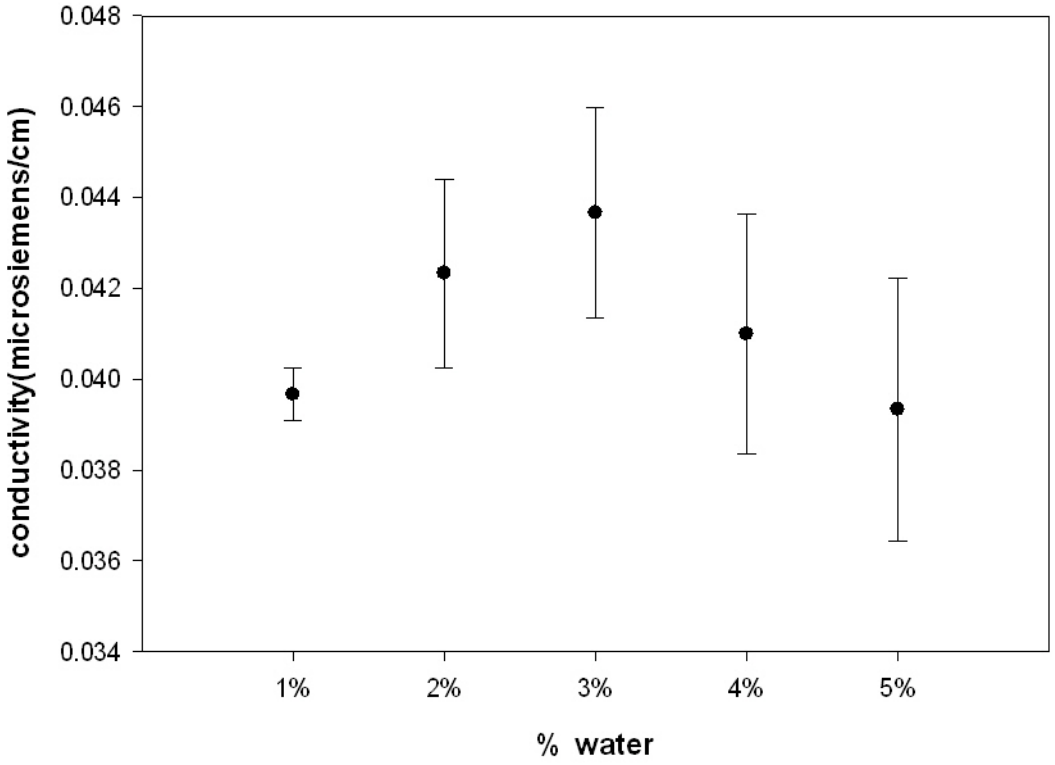



**Figure 4.17- Conductivity of microemulsion with Km=1.94, s/oil ratio of 1:5 (3.22% water) as a function of water concentration.**

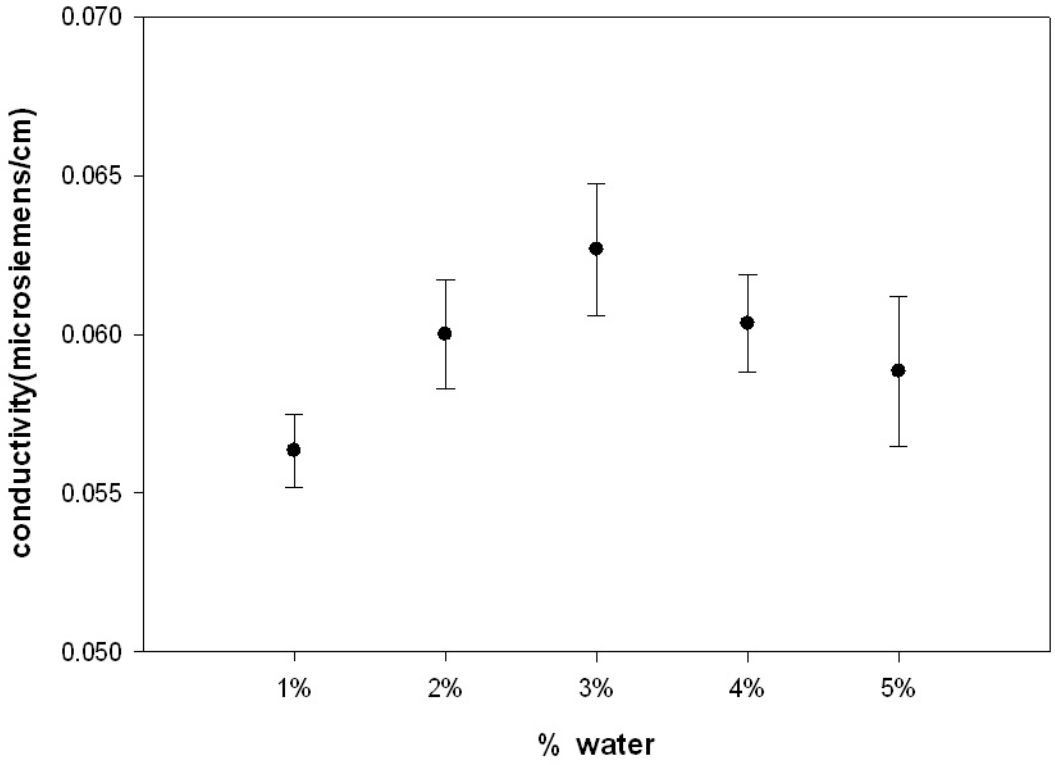



**Figure 4.18- Conductivity of microemulsion with Km=1.77, s/oil ratio of 1:5 (3%) as a function of water concentration.**

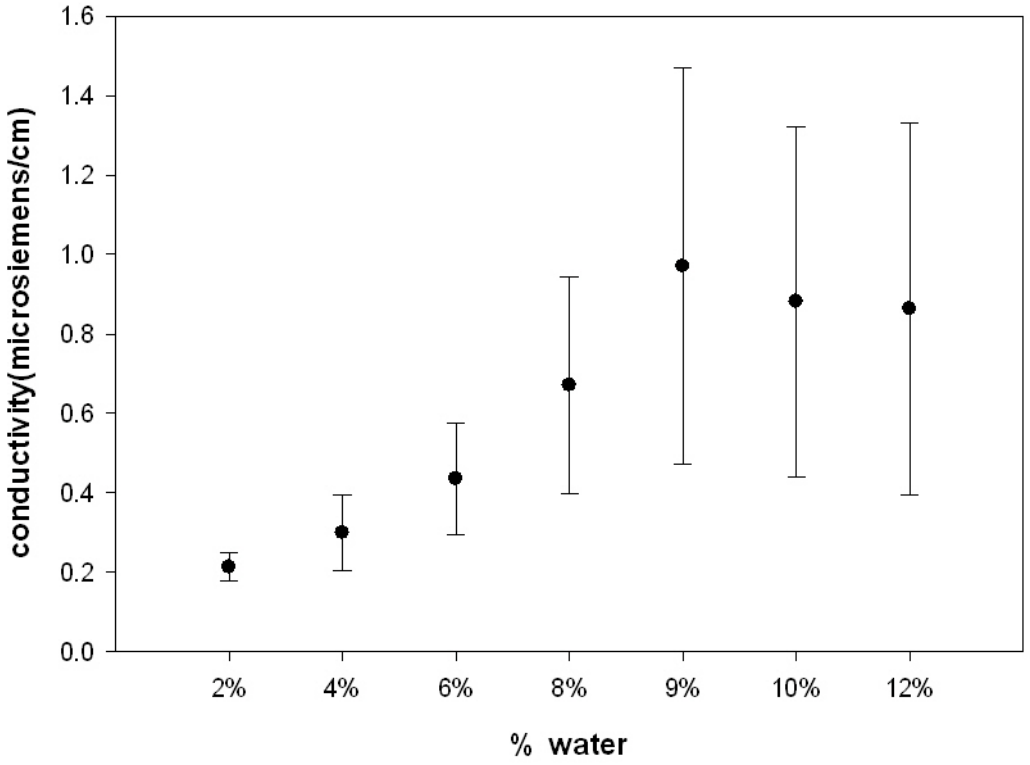



**Figure 4.19 -Conductivity of microemulsion with Km1.94, s/oil=2:3 (9.063% water) as a function of water concentration.**

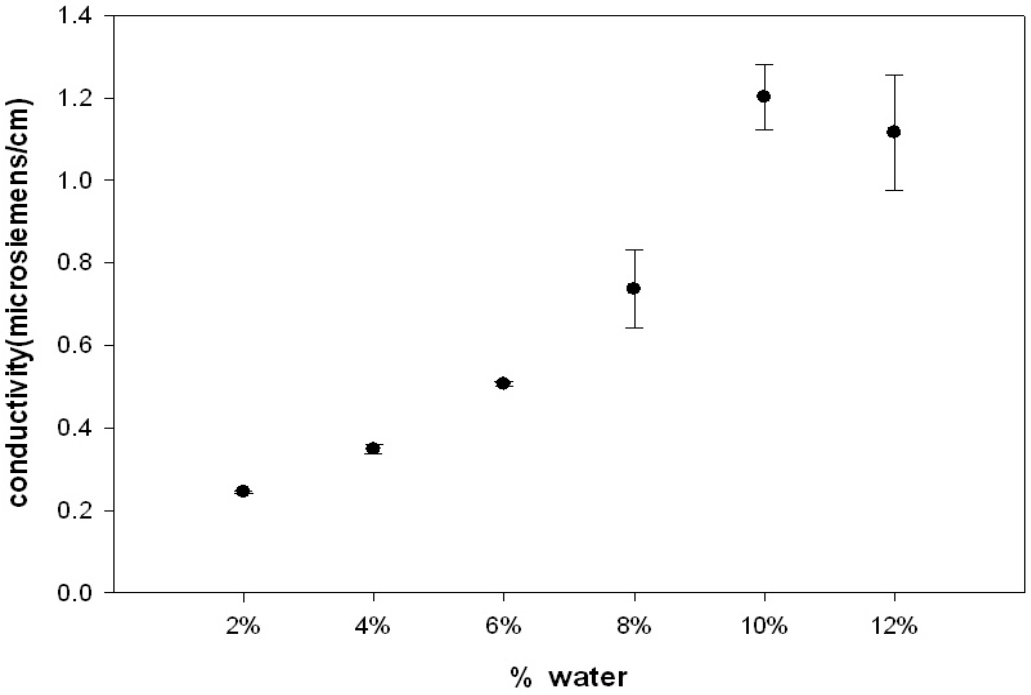



**Figure 4.20- Conductivity of lecithin/EO microemulsion with Km=1.94, s/oil 2:3(11% water), as a function of water concentration.**

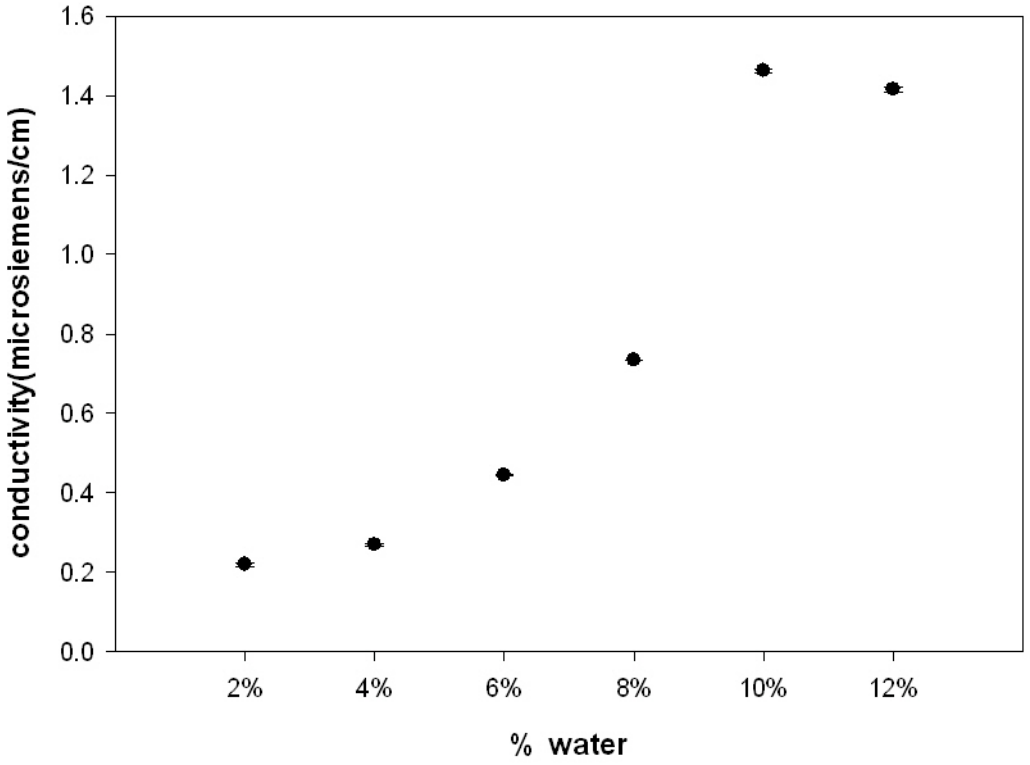



**Figure 4.21-Conductivity of Lecithin/EO microemulsion with Km=1.5, s/oil=2:3(9.5% water), as a function of water concentration.**

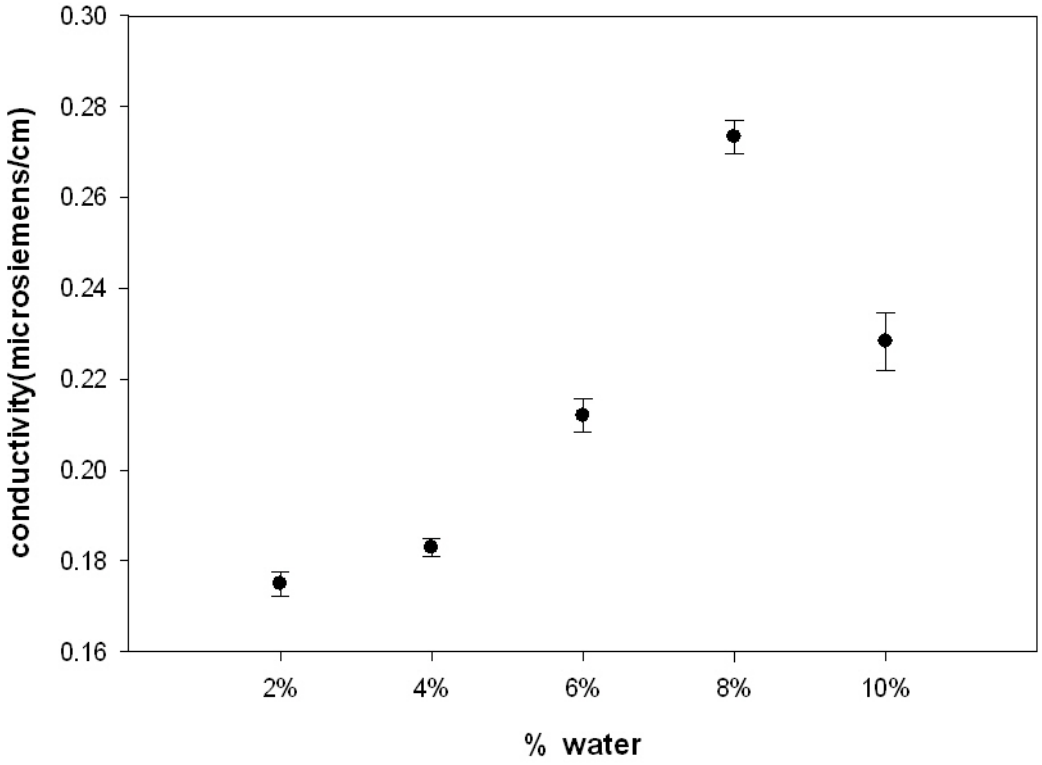



**Figure 4.22 - Conductivity of Lecithin/EO microemulsion with Km=1.5, s/oil=1:2(7.69% water), as a function of water concentration.**

In all of the conductivity measurements a percolation phenomenon was observed. The conductivity of the microemulsion increased with an increase in the percentage of added water until the microemulsion turned milky. In the lecithin/IPM microemulsion with Km=1.5,s/oil=1:5 (3.22% water), as is seen in Figure 24 the conductivity increased up to 4% water, because of gradual increase in the number of water droplets, in the continuous oil phase. A decrease in conductivity after 4% water could be due to the presence of large number of aggregates which cannot migrate easily in the non-conducting oil phase. In this system with Km=1.5, and surfactant/oil ratio of 1:5, any formulation which contained more than 4% water, appeared to be turbid, and had passed the w/o microemulsion

region, as is evident from the phase diagrams. The same kind of trend was observed in figure 25.

In the Figure 26, conductivity measured in the microemulsion with Km=1.94, and surfactant/oil ratio of 2:3, shows a rapid increase in the conductivity value from 0.2 μS/cm to 1.0 μS/cm, upto 9% of water, after which the conductivity values start decreasing at 10% and 12% water. The microemulsion starts turning cloudy at these compositions indicating that these formulations with 10% and 12% water are not microemulsions.Also when more than 12% water was added phase separation was observed immediately in this formulation. In all samples that were true microemulsion formulations the conductivity values were low indicating that the microemulsion formed is of water in oil type. The same kind of trend in conductivity was observed for figures 27, 28 and 29.

In the lecithin/EO microemulsion, with a Km of 1.5 and surfactant/oil ratio of 2:3, there is a gradual increase in conductivity from 0.2μS/cm to 0.7μS/cm, from 2% to 8% water, as is seen in the figure 30, after which there is a rise in conductivity to 1.5μS/cm. The conductivity decreases slightly in the presence of 12% water and the formulation turns cloudy. This sudden rise in conductivity may be due to clusters of water droplets migrating rapidly conducting electricity in the continuous phase [76]. Hence, this formulation remains a microemulsion, until it contains 10% water, but when 12% water is added to the formulation, it starts leaving the microemulsion region.

All the microemulsions demonstrated a percolation phenomenon, in which, a sharp increase in the conductivity is observed. As a greater amount of water is added to the alcohol based microemulsion, there is gradual drop in the conductivity. This is possibly due to a change in the shape of the aggregates which affects the movement of change within the microemulsion [77].

### 4.5 Differential Scanning Calorimetric Experiments:

Differential scanning calorimetry experiments, were performed with lecithin/IPM microemulsions. The microemulsions chosen varied according to the percentage of water incorporated into each of them. Microemulsion 1 had a Km of1.94, s/oil 1:5 with 3.22% water, microemulsion 2 had a Km of 1, s/oil 2:3 with 7.74% water and microemulsion 3 had Km of 1.94, s/oil 2:3 with 9.063% water. The DSC of the controls was also performed. The DSC for IPM, water, oil- surfactant-co-surfactant mixture and IPA was also performed from room temperature down to $-100^0$C, and back to room temperature, at a heating/cooling rate of $5^0$C/minute.

The figures below show the enthalpy, onset, end, area, and peak heights, of the freezing and melting curves obtained for each of the controls, as well as the microemulsion samples.

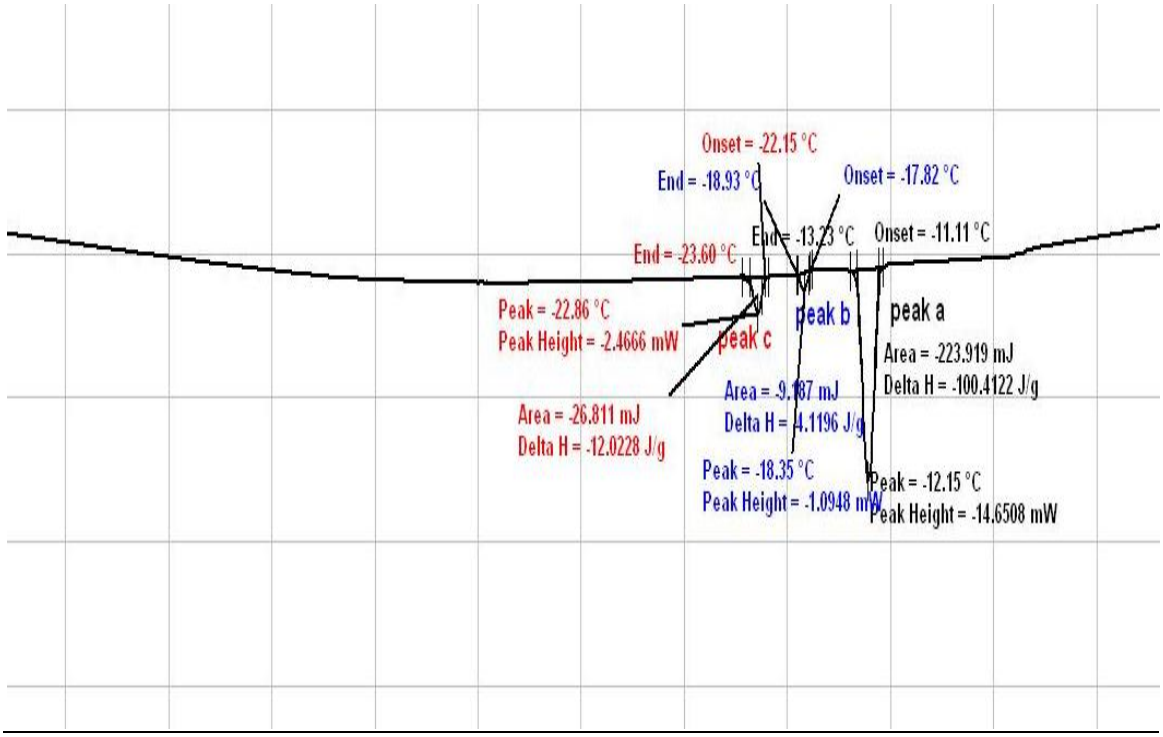



**Figure 4.23- Freezing curve of IPM.**

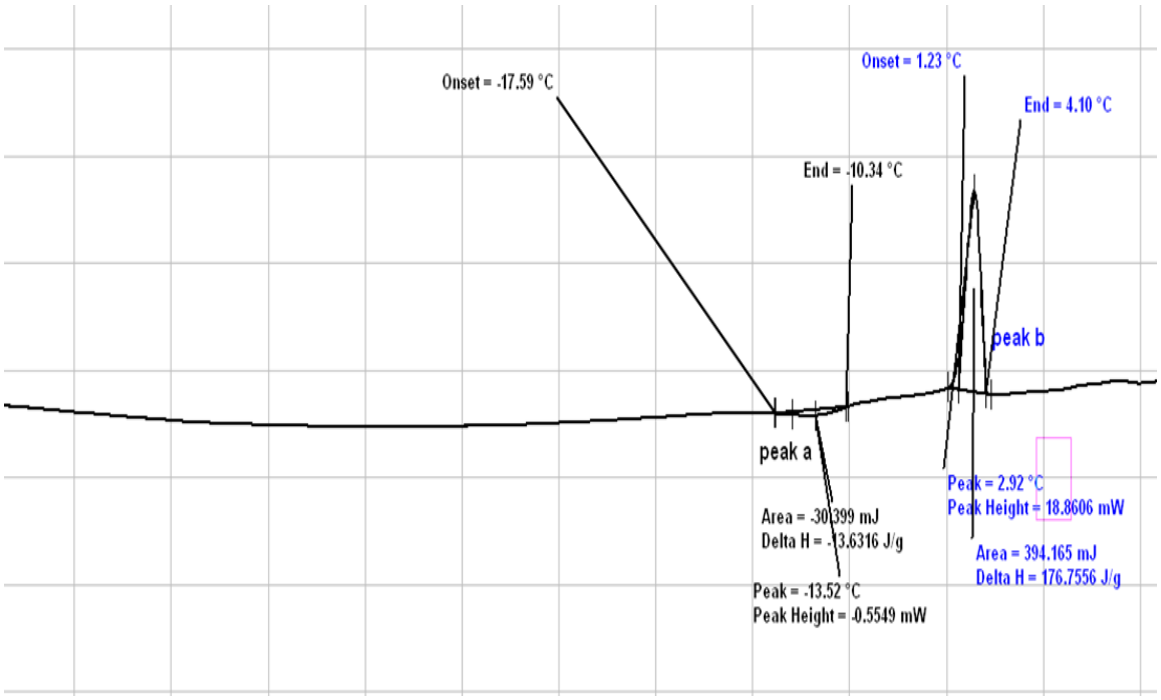



**Figure 4.24- Melting curve of IPM**

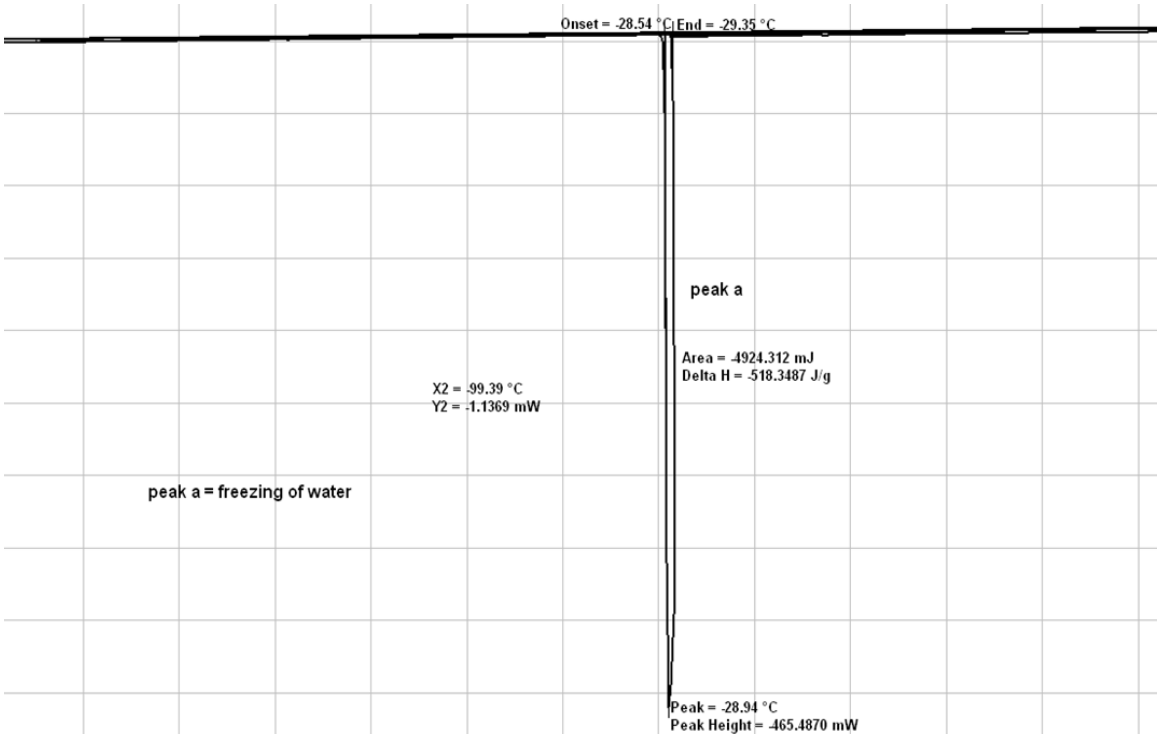



**Figure 4.25 -Freezing curve of water**

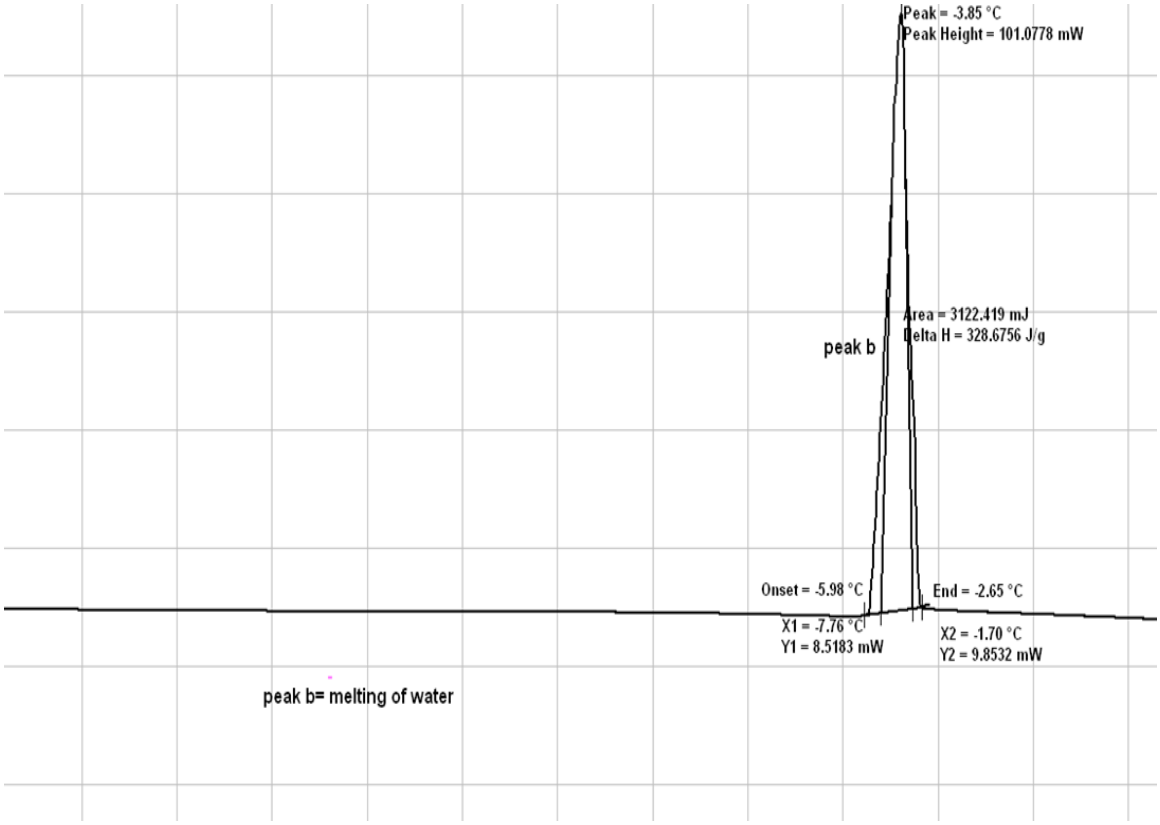



**Figure 4. 26 - Melting of water**

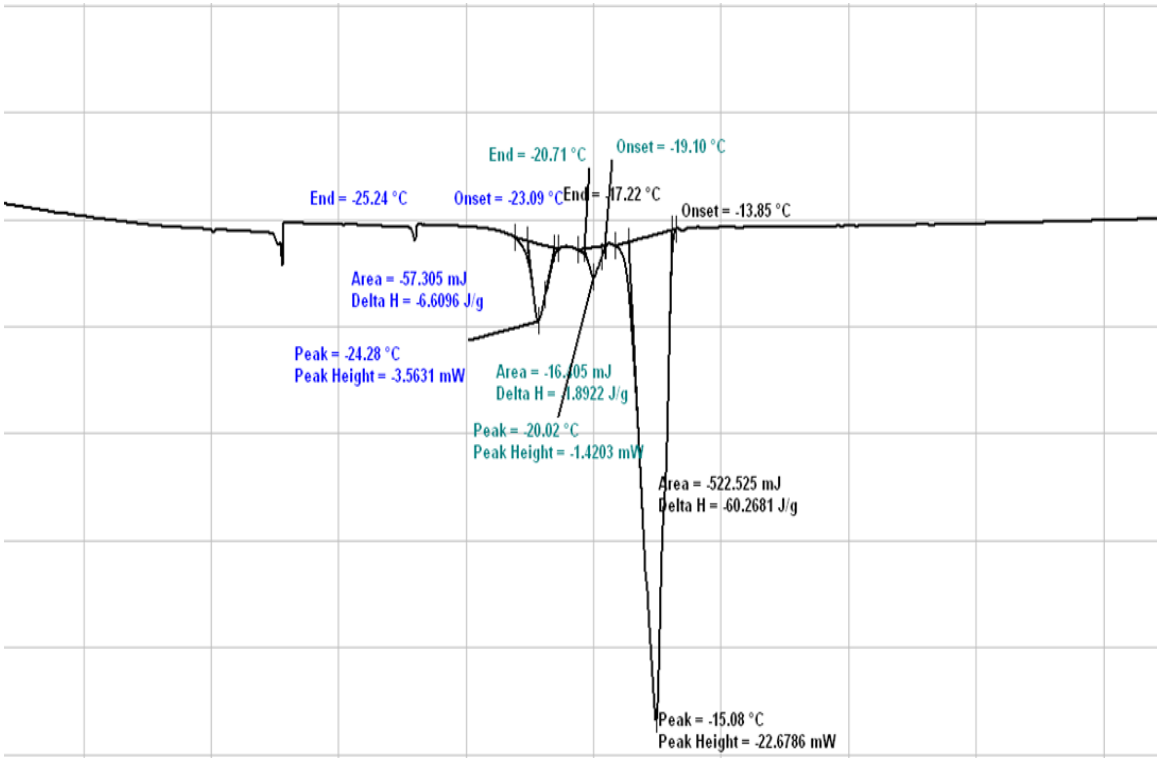



**Figure 4.27- Freezing curve of the oil+ surfactant**

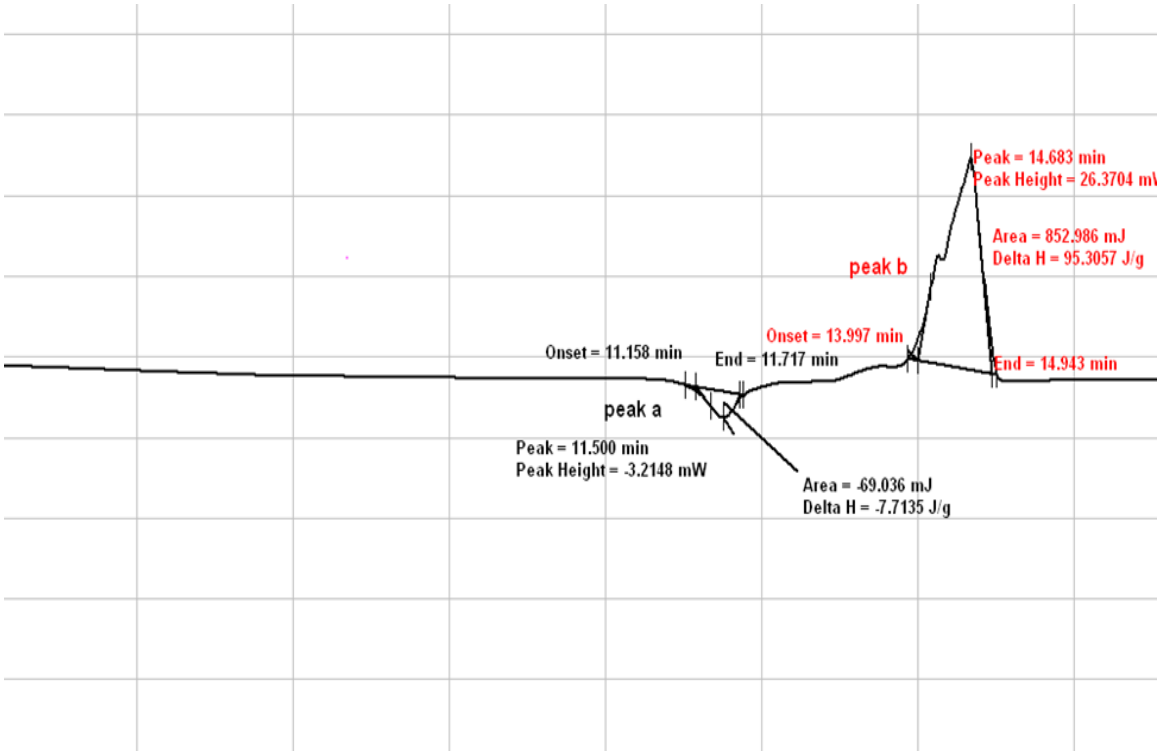



**Figure 4. 28-Melting curve of oil+ surfactant**

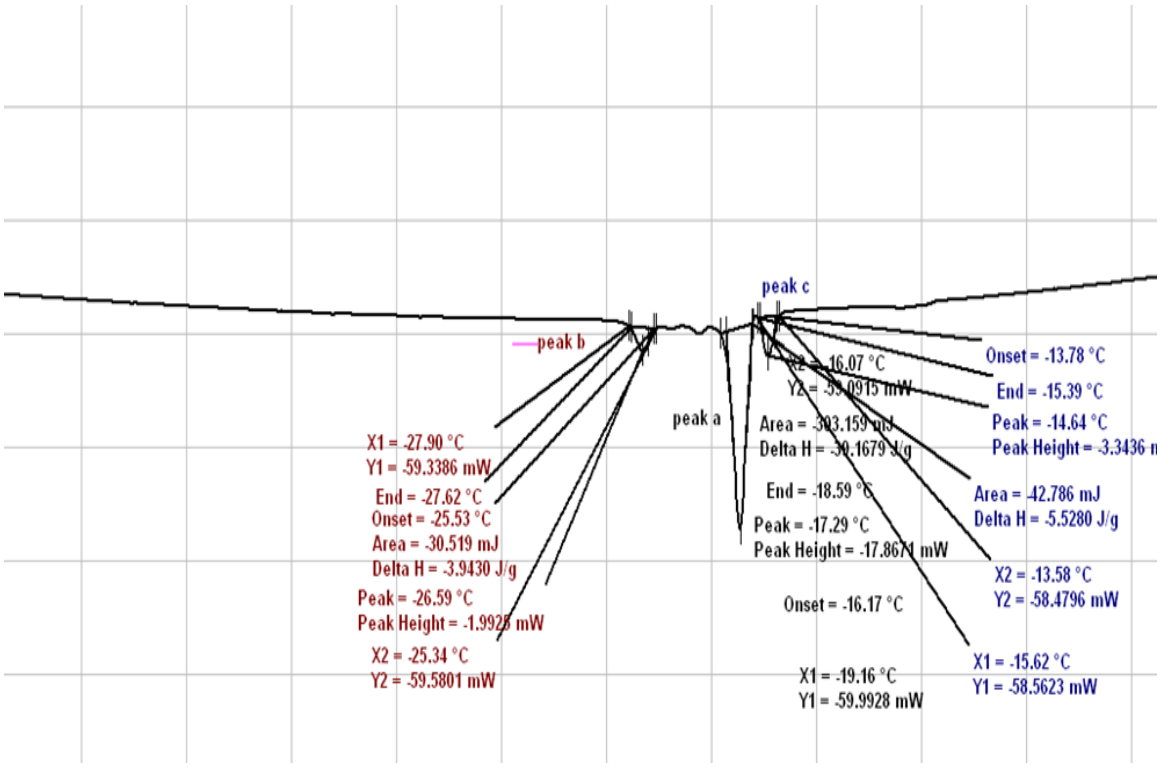



**Figure 4.29- Freezing curve of oil+ surfactant + co-surfactant**

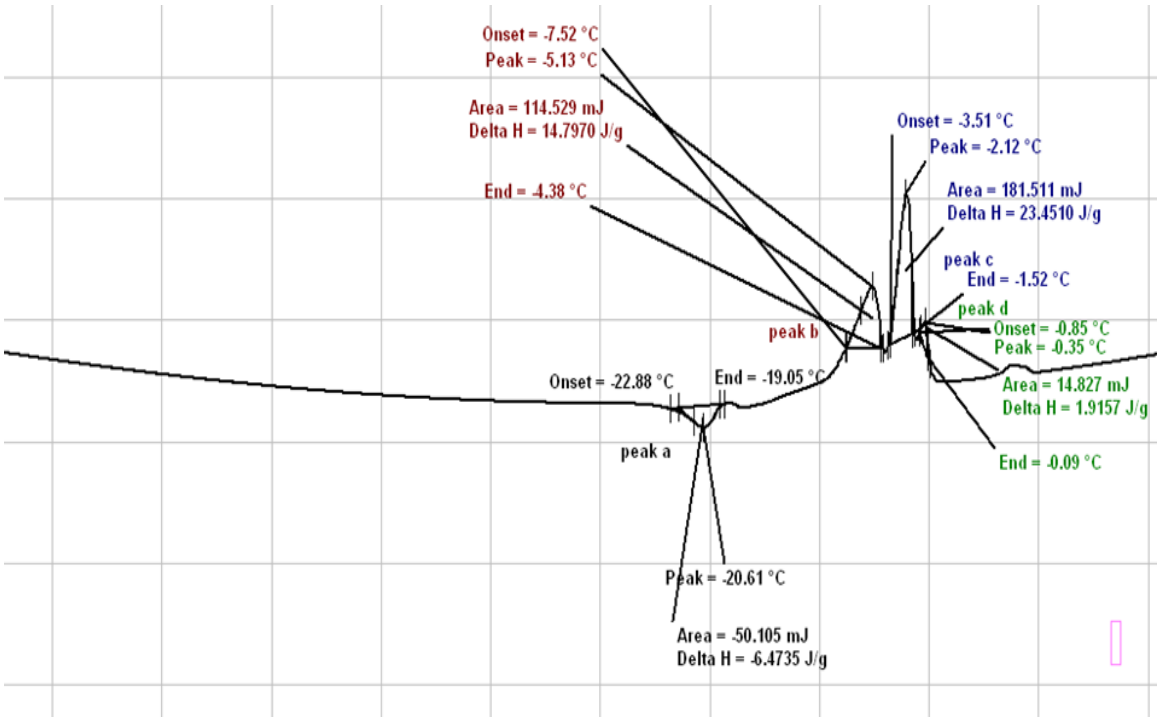



**Figure 4.30- Melting curve of oil+surfactant+co-surfactant**

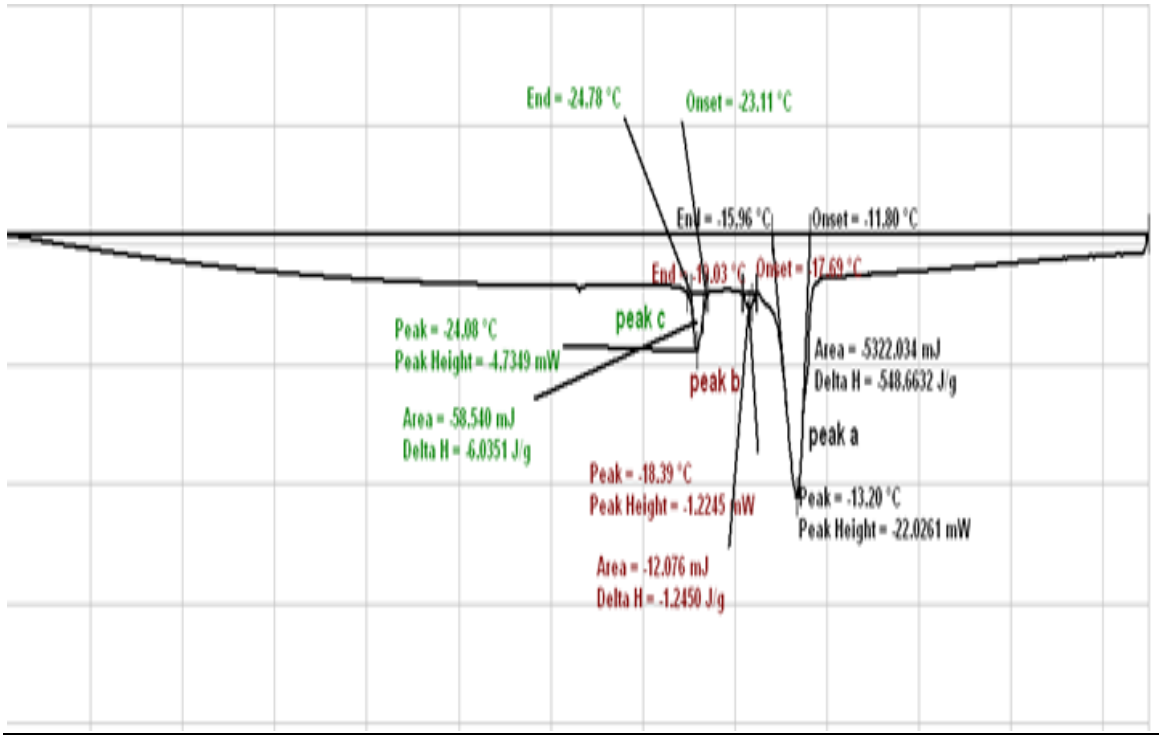



**Figure 4. 31-Freezing of microemulsion 1**

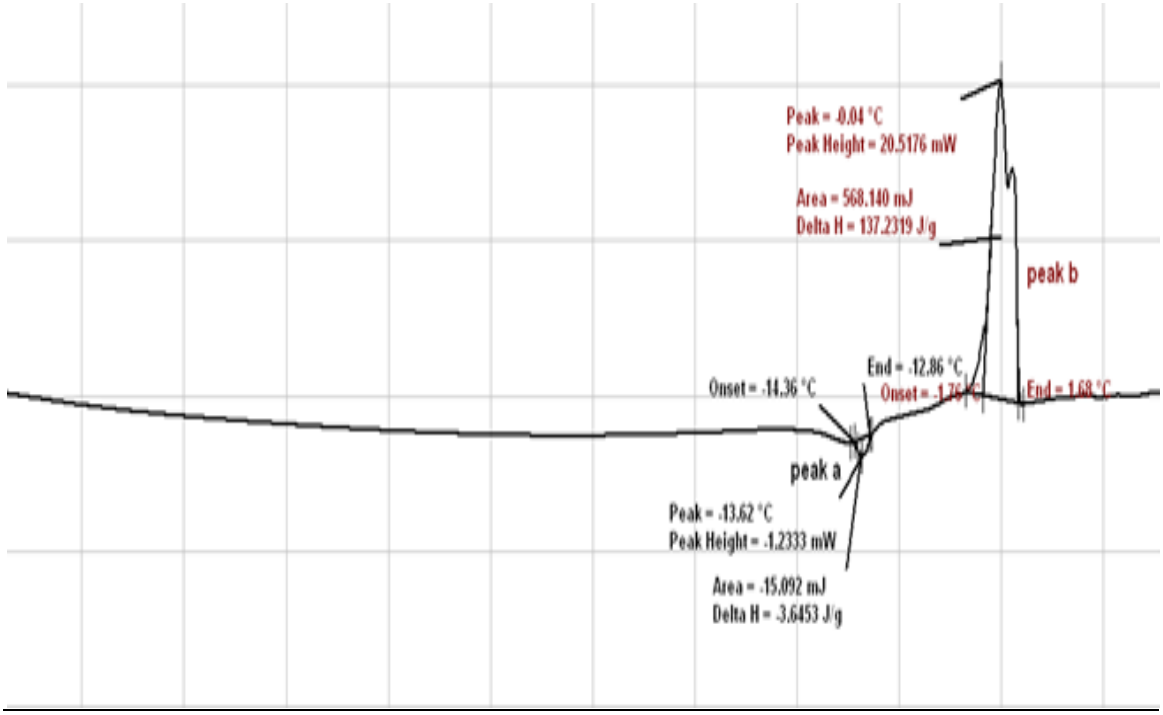



**Figure 4.32 - Melting of microemulsion1**

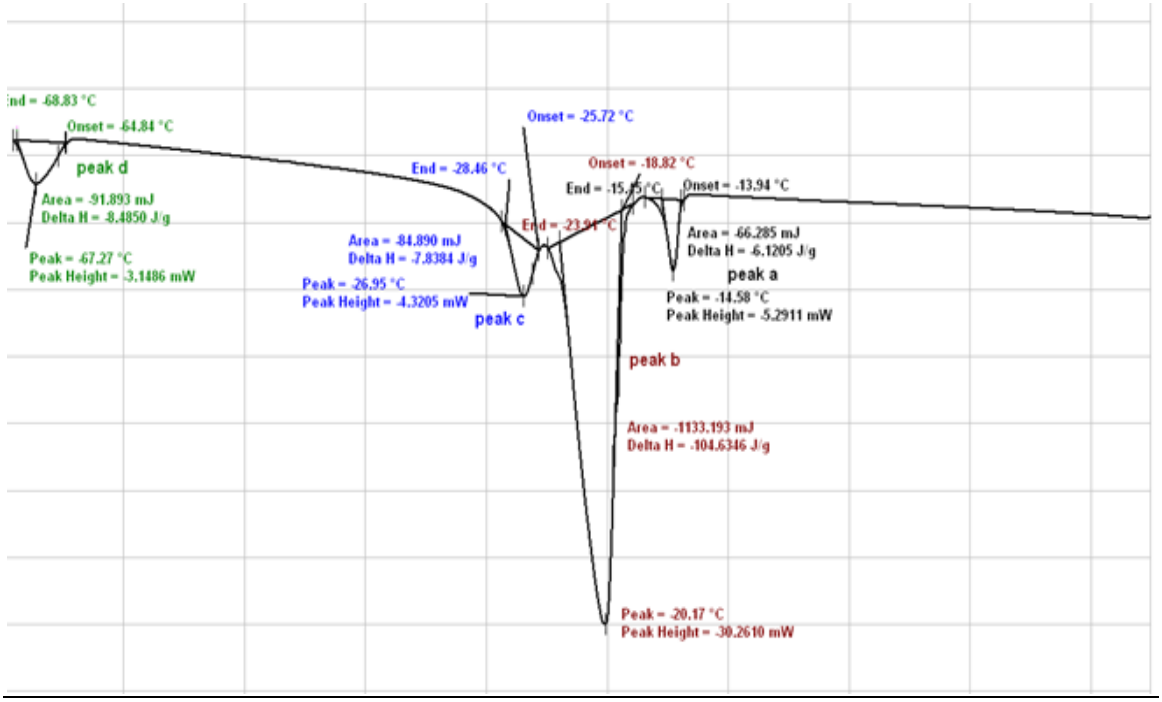



**Figure 4.33 -Freezing of microemulsion 2**

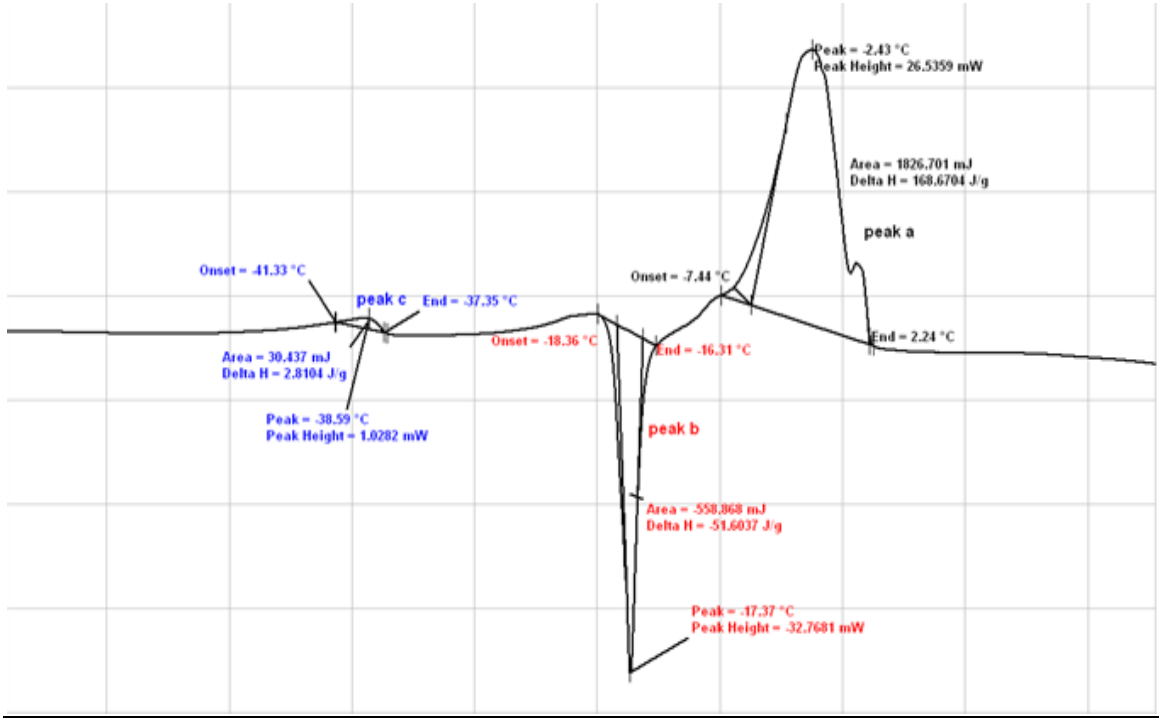



**Figure 4.34- Melting of microemulsion 2**

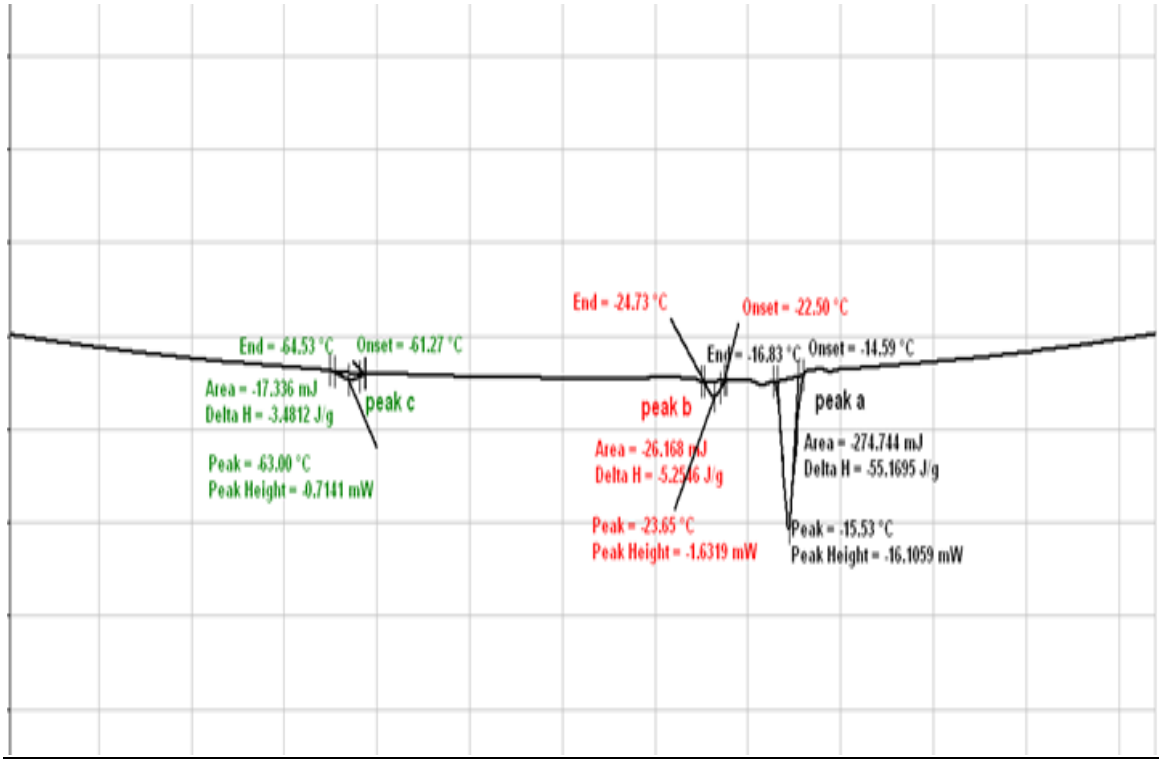



**Figure 4.35 – Freezing of microemulsion 3**

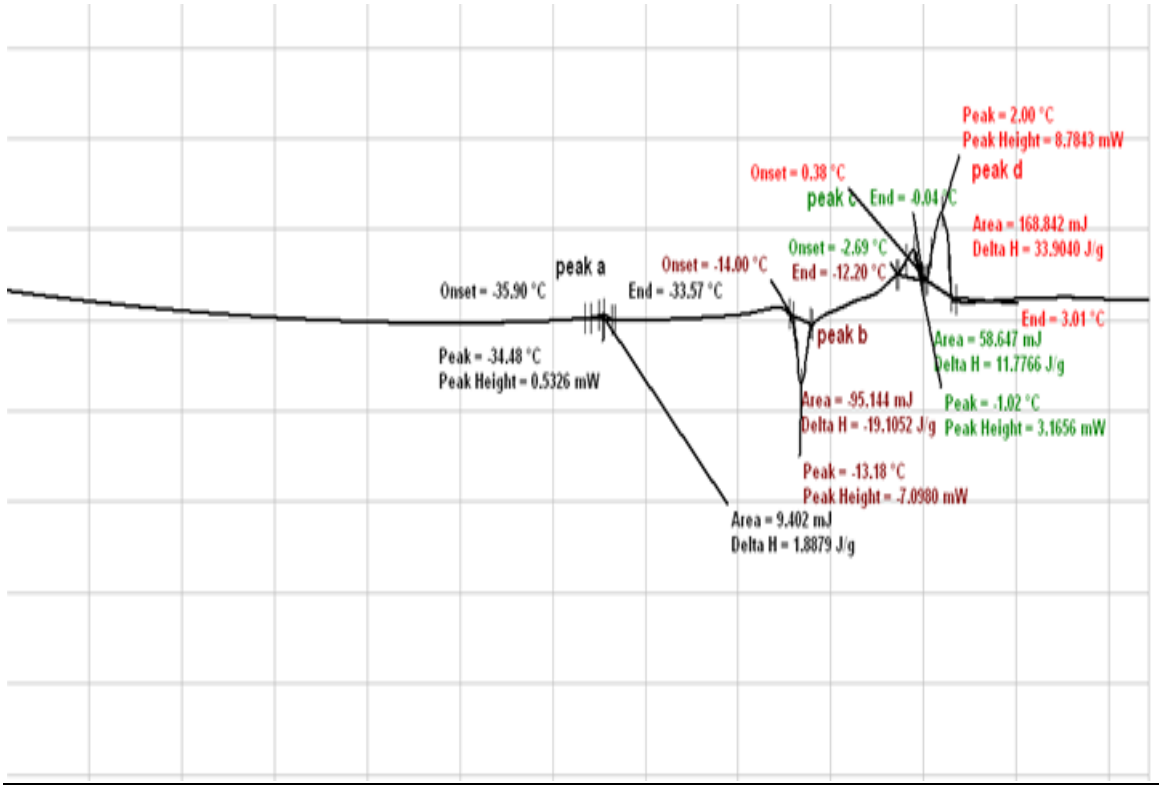



**Figure 4.36-Melting of microemulsion 3**

**Table 4.1- Summary of the peak areas, peak height, and enthalpy of the DSC curves above:**

**DSC of microemulsion 1: Run 1:**

| Freezing Curve | Onset ($^0$C) | End ($^0$C) | Area (mJ) | Enthalpy (J/g) | Peak ($^0$C) | Peak height (mW) |
|---|---|---|---|---|---|---|
| peak a | -11.8 | -15.96 | -5322.034 | -548.6632 | -13.20 | -22.0261 |
| peak b | -17.69 | -19.03 | -12.076 | -1.2450 | -18.39 | -1.2245 |
| peak c | -23.11 | -24.78 | -58.540 | -6.0351 | -24.08 | -4.7349 |
| Melting curve | | | | | | |
| peak d | 1.38 | 4.87 | 604.756 | 62.3460 | 3.28 | 22.5144 |
| peak e | -2.84 | -0.07 | 55.104 | 5.6808 | -0.83 | 2.5026 |

**DSC of microemulsion2: Run 1:**

| Freezing Curve | Onset (0C) | End (0C) | Area (mJ) | Enthalpy (J/g) | Peak (0C) | Peak Height (mW) |
|---|---|---|---|---|---|---|
| peak a | -17.13 | -19.48 | -375.229 | -50.0973 | -17.87 | -22.2477 |
| peak b | -23.43 | -25.61 | -33.281 | -4.4434 | -24.48 | -2.1384 |
| peak c | -63.94 | -67.43 | -29.025 | -3.8752 | -65.97 | -1.1216 |
| Melting curve | | | | | | |
| peak d | -17.17 | -15.70 | -214.212 | -28.5998 | -16.51 | -17.1521 |
| peak f | -38.02 | -33.89 | -13.069 | -1.7448 | -36.64 | -0.4155 |
| peak e | -5.72 | -0.69 | 535.702 | 71.5222 | -2.27 | 13.4378 |

**DSC of microemulsion 3-Run1:**

| Freezing Curve | Onset (0C) | End (0C) | Area (mJ) | Enthalpy (J/g) | Peak (0C) | Peak Height (mW) |
|---|---|---|---|---|---|---|
| Peak a | -14.59 | -16.83 | -274.744 | -55.1695 | -15.53 | 16.1059 |
| Peak b | -22.50 | -24.73 | -26.168 | -5.2546 | -23.65 | -1.6319 |
| Peak c | -61.27 | -64.53 | -17.336 | -3.4812 | -63.00 | -0.7141 |
| Melting Curve | | | | | | |
| Peak a | | | | | | |
| Peak b | -14.00 | -12.20 | -95.144 | -19.1052 | -13.18 | -7.0980 |
| Peak c | -2.69 | -0.04 | 58.647 | 11.7766 | -1.02 | 3.1656 |
| Peak d | 0.38 | 3.01 | 168.842 | 33.9040 | 2.00 | 8.7843 |

**DSC of water:- Run 1:**

| Freezing Curve | Onset (0C) | End (0C) | Area (mJ) | Enthalpy (J/g) | Peak (0C ) | Peak Height (mW) |
|---|---|---|---|---|---|---|
| Peak a | -28.54 | -29.35 | -4924.312 | -518.3487 | -28.94 | -465.4870 |
| Melting Curve | | | | | | |
| Peak b | -5.98 | -2.65 | 3122.419 | 328.6756 | -3.85 | 101.0778 |

**DSC of surfactant, co-surfactant and oil: Run 1:**

| Freezing Curve | Onset (0C) | End (0C) | Area (mJ) | Enthalpy (J/g) | Peak (0C) | Peak Height (mW) |
|---|---|---|---|---|---|---|
| Peak a | -16.17 | -18.59 | -303.159 | -39.1679 | -17.29 | -17.8671 |
| Peak b | -25.53 | -27.62 | -30.519 | -3.9430 | -26.59 | -1.9925 |
| Peak c | -13.78 | -15.39 | -42.786 | -5.5280 | -14.64 | -3.3436 |
| Melting Curve | | | | | | |
| Peak a | -22.88 | -19.05 | -50.105 | -6.4735 | -20.61 | |
| Peak b | -7.52 | -4.38 | 114.529 | 14.7970 | -5.13 | |
| Peak c | -3.51 | -1.52 | 181.511 | 23.4510 | -1.52 | |
| Peak d | -0.85 | -0.09 | 14.827 | 1.9157 | -0.35 | |

**DSC of surfactant + oil:**

| Freezing Curve | Onset (min) | End (min) | Area (mJ) | Enthalpy (J/g) | Peak (min) | Peak Height (mW) |
|---|---|---|---|---|---|---|
| Peak a | 8.145 | 8.852 | -519.112 | -58.0013 | 8.450 | -20.8802 |
| Peak b | 9.963 | 10.419 | -58.413 | -6.5266 | 10.200 | -3.3197 |
| Peak c | 9.178 | 9.505 | -13.596 | -1.5191 | 9.350 | -1.1110 |
| Melting Peak | | | | | | |
| Peak a | 11.158 | 11.717 | -69.036 | -7.7135 | 11.50 | -3.2148 |
| Peak b | 13.997 | 14.943 | 852.986 | 26.3704 | 14.683 | 26.3704 |

**DSC of IPM-Run 1:**

| Freezing curve | Onset ($^0$C) | End ($^0$C) | Area (mJ) | Enthalpy (J/g) | Peak ($^0$C) | Peak Height (mW) |
|---|---|---|---|---|---|---|
| peak a | -11.11 | -13.23 | -223.919 | -100.4122 | -12.15 | -14.6508 |
| peak b | -17.82 | -18.93 | -9.187 | 4.1196 | -18.35 | -1.0948 |
| peak c | -22.15 | -23.60 | -26.811 | -12.0228 | -22.86 | -2.4666 |
| Melting Curve | | | | | | |
| peak a | -17.59 | -10.34 | -30.399 | -13.6316 | -13.52 | -0.5549 |
| peak b | 1.23 | 4.10 | 394.165 | 176.7556 | 2.92 | 18.8606 |

In the freezing of Isopropyl myristate, three peaks were observed, with enthalpies and (onset temperatures) corresponding to -11.11°C (-100.4122 J/g), -17.82°C (4.12J/g) and -22.15°C (-12.023 J/g) respectively. In the melting curves for Isopropyl myristate enthalpies of approximately 176 J/g were observed and concurred with the other melting experiments for isopropyl myristate. The peak observed at approximately -22.15°C ( -12.023 J/g), may be due to structural rearrangement in the solidified IPM [47]. In the DSC experiment for the co-surfactant isopropyl alcohol, a peak at approximately-88$^0$ C corresponding to the freezing of IPA was observed.

In the DSC run for the surfactant, cosurfactant and oil, the peaks with enthalpies corresponding to onset points -16.17° C (-39.1679 J/g), -25.53° C (-3.9430J/g ) and -13.78° C (-5.5280J/g) were observed. In the melting curve for the surfactant, co-surfactant and oil mixture a recrystallization peak of lecithin was observed close to the freezing point of water [78]. This peak was observed at a peak with an onset point and enthalpy of (-22.88°C) -6.4735J/g. In the sub-ambient DSC for microemulsion 1, in

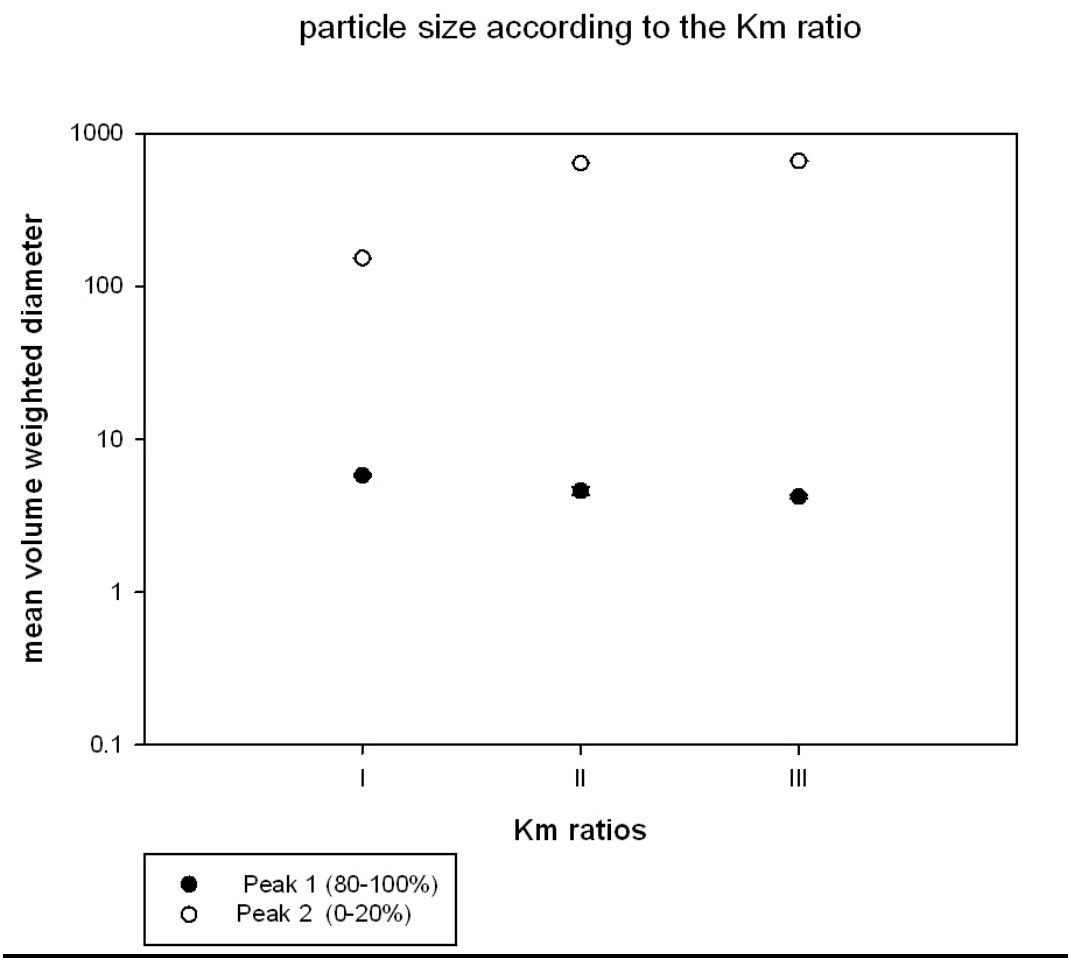
addition to the thermal events attributed to the controls no other freezing events were observed Since no events attributable to the freezing of water was observed in microemulsion 1 we conclude that most of the water present in the microemulsion containing 3% w/w water, is tightly bound to the surfactant and is non-freezable even at -$100^0$ C.

The DSC for microemulsion 2; which contains 7.74% w/w water, shows the freezing of water; at approximately -$70^0$ C (enthalpy of -3.8752J/g). This thermal event confirmed the presence of bound freezable water in this microemulsion. All the other thermal events corresponded to that observed in the controls. In the melting experiments for microemulsion 2 an endothermic event related to the melting of freezable bound water was observed at approximately – $41^0$C.

The DSC for microemulsion 3, which contains 9.063% water, in addition to the freezing of other formulation components an exothermic event observed at approximately -$70^0$ C is attributed to freezable bound water. Sub-ambient DSC for lecithin microemulsions delineated the presence of non-freezable and freezable bound water. The presence of bulk water could not be identified possibly due to other overlapping thermal events. The study of water behavior in closed domains such as microemulsions offers an interesting thermodynamic perspective regarding these systems. Water that remains unfrozen down to -$100^0$C and -$70^0$C is bound to hydrophilic moieties present in the surfactant. The non-freezable water is bound as the first hydration layer around the surfactant monolayer. Subsequent layers of bound water molecules have varying degrees of chemical potential and hence can be frozen at approximately -$70^0$C. This type of bound water has been used as modulators in the catalysis of various bioorganic reactions at low temperatures [79].

## 4.6 Dynamic Light Scattering Results:

**Figure 4.37-Variation of particle size in the IPM microemulsions according to the Km ratio.**



The microemulsions subjected to particle size analysis were Km=1.94, 1:5 containing 3.22% water; Km=1, 2:3 containing 7.74% water; and Km 1.94, 2:3 containing 9.063% water. All microemulsions showed bimodal particle size distributions that were analyzed via the Nicomp algorithm. Most of the particles are in the size range of 1-10 nm as is seen in Figure 47 and 48. A small group of particles in the size range of 100-1000 nm. Particle size for all the microemulsions increased with an increase in the percentage of water incorporated into the microemulsions. This is due to swelling of the surfactant layers in the presence of an increased amount of water.

**Figure 4.38-Particle size analysis of IPM microemulsions without phosphate buffer of Ph 7.4.**

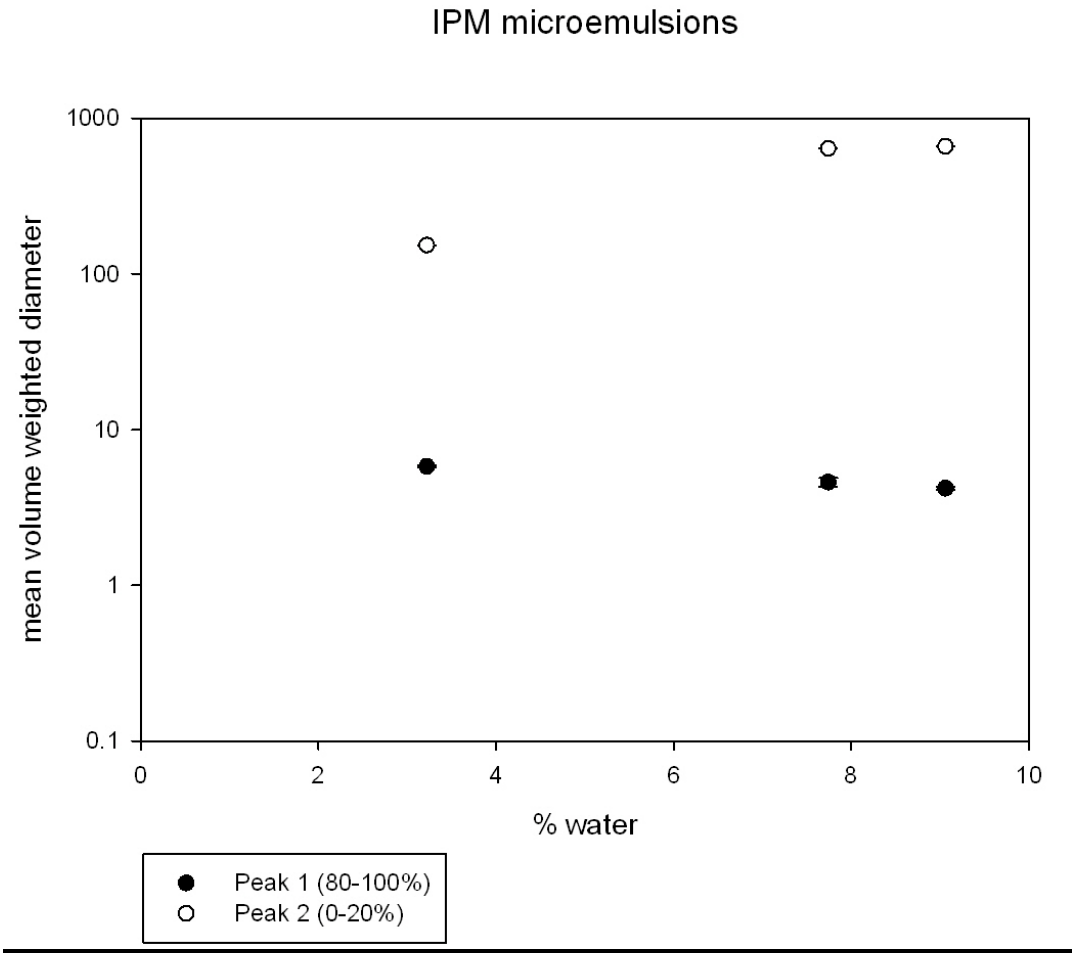
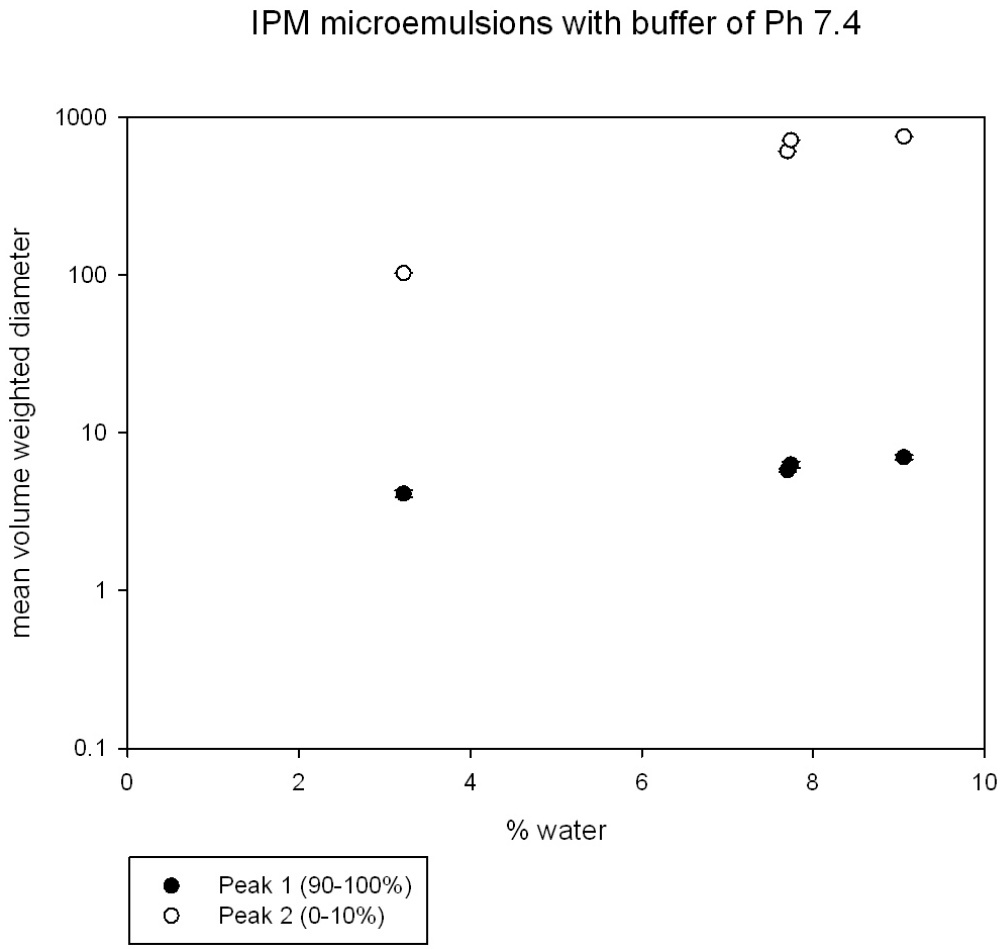


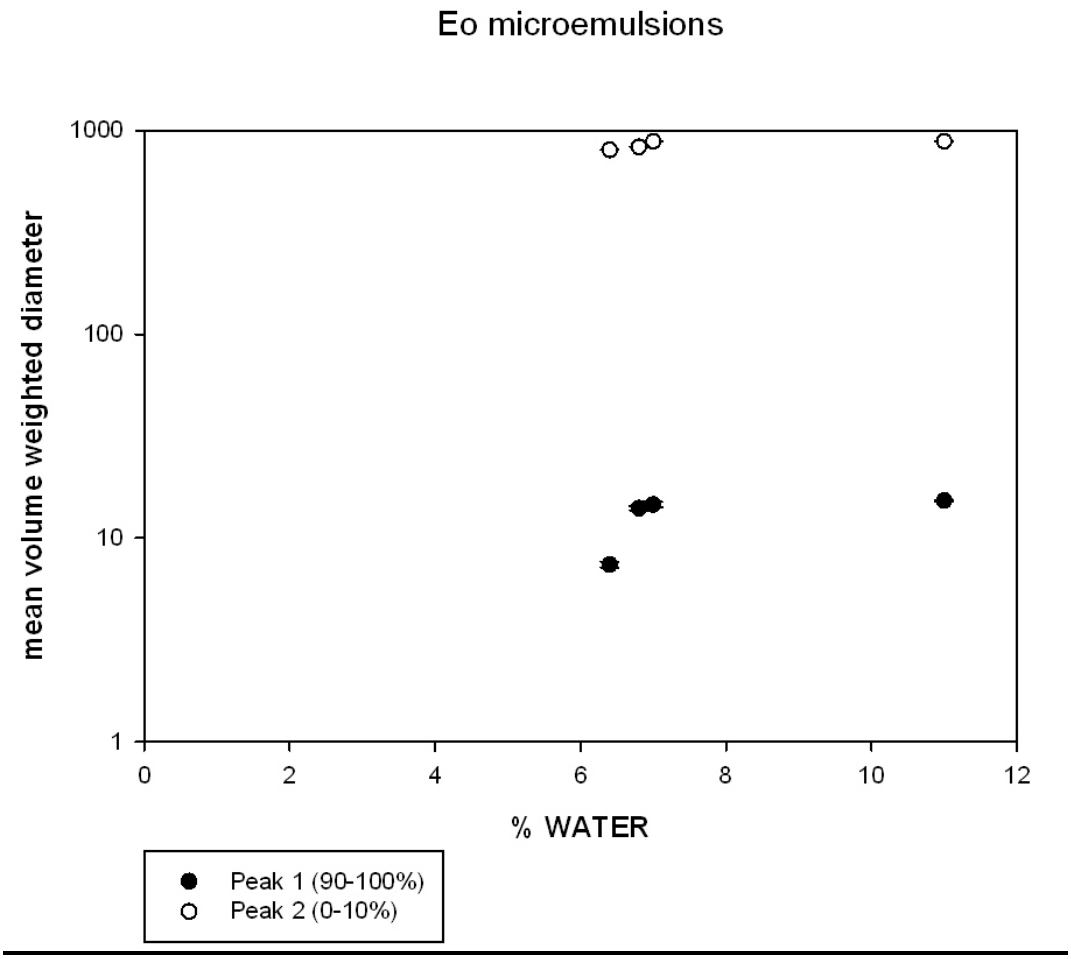
**Figure 4.39- Particle size analysis of IPM microemulsions, with the addition of phosphate buffer of Ph 7.4.**




It has been reported in the literature that when water is added to lecithin/cyclohexane or lecithin/isooctane solution, giant cylindrical and flexible microemulsion particles are formed. At higher lecithin concentrations, these polymer like aggregates entangle and form a transient and viscoelastic network which is similar to a semidilute polymer solutions leading to an increase in the viscosity. Hence, when light scattering studies are performed at a higher lecithin concentration, the data are dominated by intermicellar interactions, and hence will interpret the static and dynamic correlation in the viscous

network formed. When the lecithin concentration is very high, characterization of the microemulsions using the DLS is not possible [80]. In all microemulsions the primary peak in the particle size distribution (80% to 100%) is due to scattering by particles in the range of 1-10 nm indicating the presence of reverse micelles. The secondary peak (20% to 0%) may be attributed to the formation of large aggregates and/or presence of non-spherical elongated micelles.

**Figure 4.40-Particle size analysis of ethyl oleate microemulsions**



The microemulsions characterized for particle size analysis using ethyl oleate were Km=1.5, 1:2 containing 6.4% water; Km=1.77, 2:3 containing 6.8% water; Km=1, 2:3 containing 7% water; and Km=1.94, 2:3 containing 11% water. Most particles were in the size range of 1-12 nm. However, large particles in the size range between 800-1000 nm were also present. The particles observed between 1-12 nm may be reverse micelles. The particles in the range between 800-1000 nm may be due to the presence of communicating network of micelles in the microemulsion.

## 4.7 Albumin containing Formulations:

A solution of albumin was prepared in 100 ml water, and a solution of albumin was also prepared in 200 ml phosphate buffer of pH 8. The microemulsions selected for the incorporation of the albumin solution were those which incorporated a sufficient amount of water. In IPM formulations, they were Km=1.94, 2:3, Km=1, 2:3, and Km=0.5, 2:3. In ethyl oleate containing formulations the compositions were Km=1.5, 2:3, Km=1.94, 2:3, and Km=1.77, 2:3. No significant difference was observed in the amount of aqueous phase microemulsified in all trial formulations when water was used as the solvent.

**Table 4.2- Percent (%) of albumin containing solution emulsified in IPM/Lecithin and EO/Lecithin microemulsions in triplicate**

| **IPM** | **KM 1.94,2:3** | **KM 1,2:3** | **KM 0.5,2:3** |
|---|---|---|---|
| With buffer | 11.09±0.06 | 10±0.06 | 9.1±0.02 |
| With water | 11.4±0.01 | 9.4±0.01 | 8.53±0.01 |
| **Ethyl Oleate** | **KM =1.5,2:3** | **KM=1.94,2:3** | **KM=1.77,2:3** |
| With buffer | 9.4±0.01 | 11±0.01 | 9.36±0.01 |
| With water | 10.3±0.01 | 11.31±0.01 | 9.64±0.01 |

There was a slight increase in the amount of aqueous phase microemulsified when the protein was dissolved in a buffer. This may be due to positive interactions between components present in the buffer and the protein reducing the interactions between lecithin monolayer and albumin. This type of intermolecular interactions has been reported [56].

### 4.8 Release Studies:

The calibration curve obtained for the albumin standard solutions using the Hartree Lowry procedure can be seen in figure 50 below. The equation for the line was calculated to be $y= 0.9533x + 0.0948$ with an $R^2$ value of 0.9557.

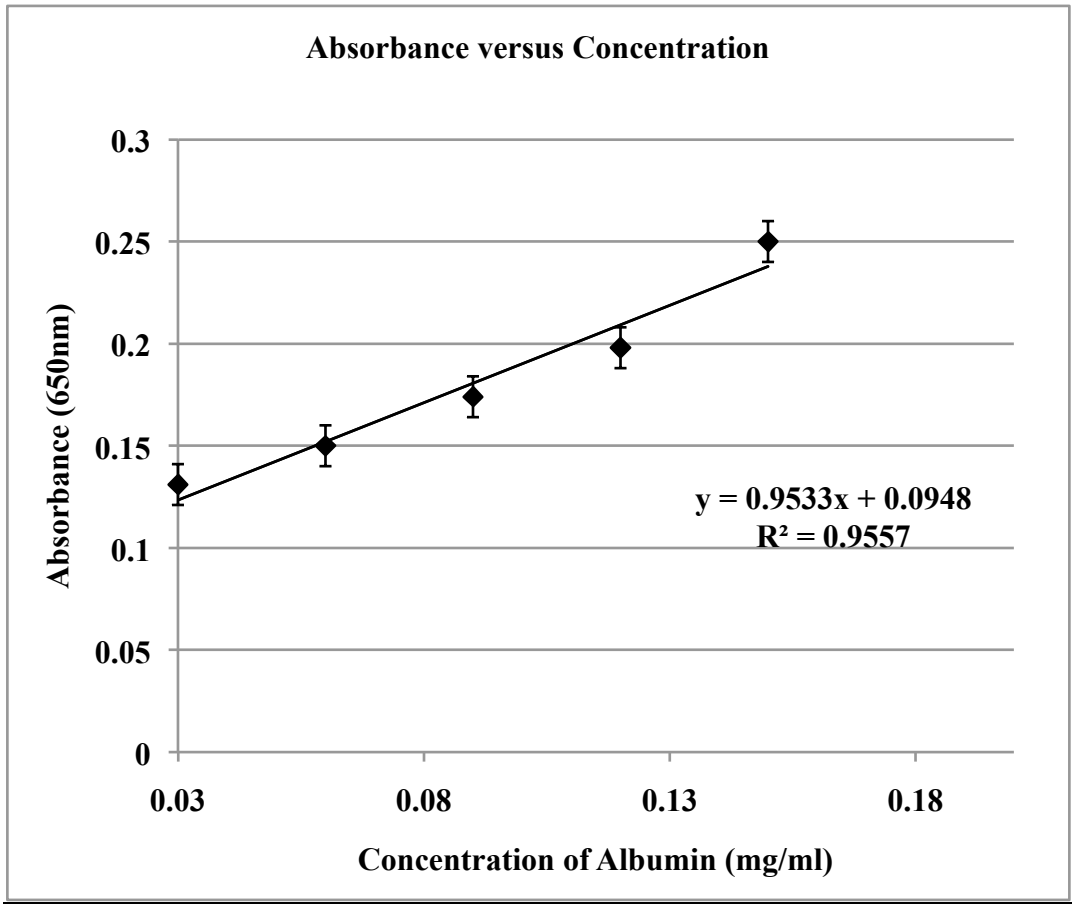



**Figure 4.41- Calibration curve for release studies, where y=0.9533x + 0.0948**

Absorbance obtained from the *in vitro* release studies were substituted into the equation obtained from the calibration curve to determine the concentration of the albumin released into the dissolution medium. A plot of albumin concentration as a function of time is shown in Figure 51.

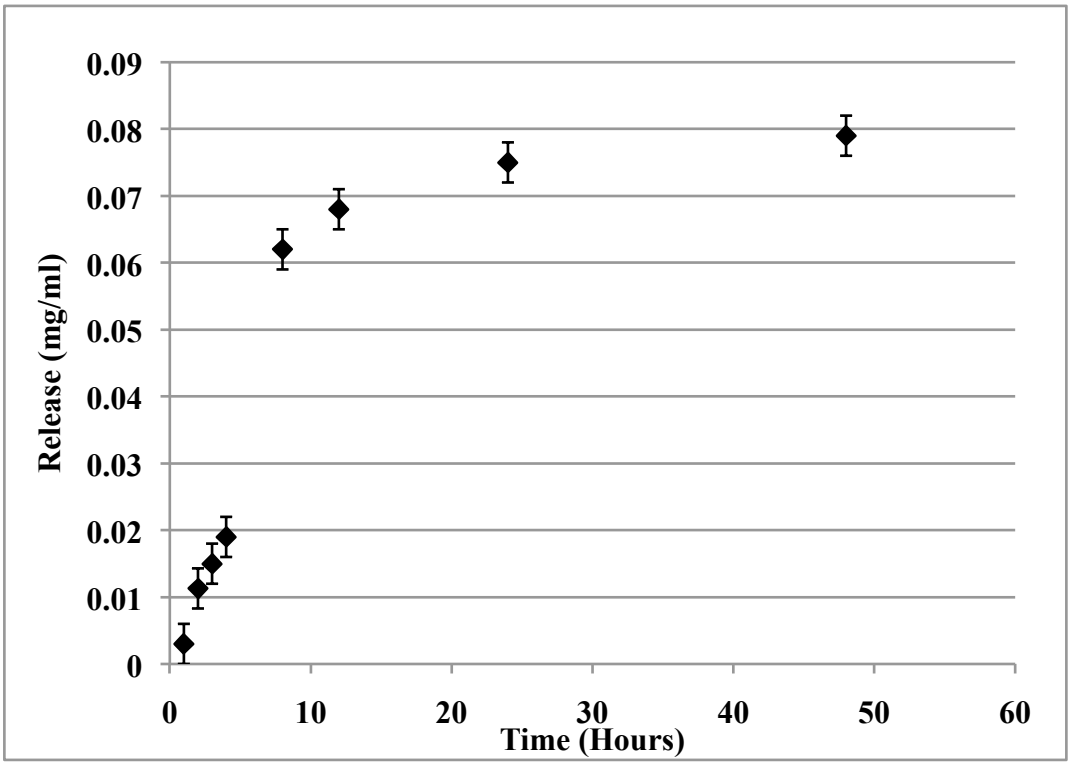



**Figure 4.42- Release profile of albumin from IPM/Lecithin microemulsion.**

The release of albumin was recorded over a 48 hour time period. There was a burst release at 8 hours, thereafter which the release seemed to plateau between 12 hours to 48 hour time period.

## 4.9 Statistical Analysis of the Data in Table 4.2:

T tests were performed using the SAS software inorder to compare between the two means, that is albumin incorporation with buffer and with water for each of the formulations shown in Table 4.2, and conclusions were made from the P value whether or not there was a significant difference between the two means.If the P value was greater than 0.05, it was concluded that there was no significant difference between the buffer and the water means and vice versa. The results of the statistical t-tests are as shown below.

### T TEST FOR IPM KM=1, surfactant/oil= 2:3 ( Performed using the SAS software)

### SAS code:

```
data one;
 infile 'G:/kush.csv' dlm=',' DSD;
 input  type $ nine five one enine efive eseven;
 options ls=100;
 proc sort; by type;
 proc print;
data two; set one;
 options ls=100;
 proc sort; by type;
 proc glm;
 class type;
 model =type;
 output out=one;
```

```
 means type/tukey;
data three; set one;
 proc ttest h0=0 alpha=0.05;
 class type;
 var one;

run;
```

## OUTPUT:

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The TTEST Procedure

Statistics

| Variable | type | N | Lower CL Mean | Mean | Upper CL Mean | Lower CL Std Dev | Std Dev | Upper CL Std Dev | Std Err |
|---|---|---|---|---|---|---|---|---|---|
| one | buffer | 3 | 0.3123 | 0.3267 | 0.341 | 0.003 | 0.0058 | 0.0363 | 0.0033 |
| one | water | 3 | 0.2454 | 0.2833 | 0.3213 | 0.008 | 0.0153 | 0.096 | 0.0088 |
| one | Diff (1-2) | | 0.0172 | 0.0433 | 0.0695 | 0.0069 | 0.0115 | 0.0332 | 0.009 |

T-Tests

| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| one | Pooled | Equal | 4 | 4.60 | **0.0101** |
| one | Satterthwaite | Unequal | 2.56 | 4.60 | 0.0271 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| one | Folded F | 2 | 2 | 7.00 | 0.2500 |

**Result: Since from the Pooled Method , it can be observed that the P value is < 0.05 (0.01). Hence there is a significant difference between the buffer and the water means.**

**T test for IPM with Km=1.94, 2:3 (Performed using the SAS software):**

**SAS code:**

```
data one;
 infile 'G:/kush.csv' dlm=',' DSD;
 input  type $ nine five one enine efive eseven;
 options ls=100;
 proc sort; by type;
 proc print;
data two; set one;
 options ls=100;
 proc sort; by type;
```

```
 proc glm;
 class type;
 model =type;
 output out=nine;
 means type/tukey;
data three; set one;
 proc ttest h0=0 alpha=0.05;
 class type;
 var nine;

run;
```

**OUTPUT:**

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The TTEST Procedure

Statistics

| | | | Lower CL | | Upper CL | Lower CL | | Upper CL | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | type | N | Mean | Mean | Mean | Std Dev | Std Dev | Std Dev | Std Err |
| nine | buffer | 3 | 0.3723 | 0.3867 | 0.401 | 0.003 | 0.0058 | 0.0363 | 0.0033 |
| nine | water | 3 | 0.4287 | 0.4667 | 0.5046 | 0.008 | 0.0153 | 0.096 | 0.0088 |
| nine | Diff (1-2) | | -0.106 | -0.08 | -0.054 | 0.0069 | 0.0115 | 0.0332 | 0.0094 |

T-Tests

| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| nine | Pooled | Equal | 4 | -8.49 | **0.0011** |
| nine | Satterthwaite | Unequal | 2.56 | -8.49 | 0.0061 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| nine | Folded F | 2 | 2 | 7.00 | 0.2500 |

**Result: There is a significant difference between the buffer and water means because the P value is< 0.05 (0.0011).**

**T TEST FOR IPM with KM=0.5, surfactant/oil=2:3 ( Performed using the SAS software.**

**SAS CODE:**

```
data one;
 infile 'G:/kush.csv' dlm=',' DSD;
 input  type $ nine five one enine efive eseven;
 options ls=100;
 proc sort; by type;
 proc print;
data two; set one;
```

```
options ls=100;
proc sort; by type;
proc glm;
class type;
model =type;
output out= five;
means type/tukey;
data three; set one;
proc ttest h0=0 alpha=0.05;
class type;
var five;

run;
```

**OUTPUT:**

The TTEST Procedure

Statistics

| Variable | type | N | Lower CL Mean | Mean | Upper CL Mean | Lower CL Std Dev | Std Dev | Upper CL Std Dev | Std Err |
|---|---|---|---|---|---|---|---|---|---|
| five | buffer | 3 | 0.2503 | 0.3 | 0.3497 | 0.0104 | 0.02 | 0.1257 | 0.0115 |
| five | water | 3 | 0.2852 | 0.31 | 0.3348 | 0.0052 | 0.01 | 0.0628 | 0.0058 |
| five | Diff (1-2) | | -0.046 | -0.01 | 0.0258 | 0.0095 | 0.0158 | 0.0454 | 0.0129 |

T-Tests

| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| five | Pooled | Equal | 4 | -0.77 | **0.4818** |
| five | Satterthwaite | Unequal | 2.94 | -0.77 | 0.4961 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| five | Folded F | 2 | 2 | 4.00 | 0.4000 |

**Result: There is no significant difference between the means of the buffer and the water, since the P value is >0.05 (0.48).**

**T test for EO, with KM=1.94, surfactant/oil =2:3 ( Performed using the SAS software)**

**SAS code:**

```
data one;
 infile 'G:/kush.csv' dlm=',' DSD;
 input  type $ nine five one enine efive eseven;
 options ls=100;
 proc sort; by type
```

```
 proc print;
data two; set one;
 options ls=100;
 proc sort; by type;
 proc glm;
 class type;
 model =type;
 output out=enine;
 means type/tukey;

data three; set one;
 proc ttest h0=0 alpha=0.05;
 class type;
 var enine;

run;
```

**OUTPUT:**

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The TTEST Procedure

Statistics

| | Lower CL | Upper CL | Lower CL | Upper CL |
|---|---|---|---|---|

| Variable | type | N | Mean | Mean | Mean | Std Dev | Std Dev | Std Dev | Std Err |
|---|---|---|---|---|---|---|---|---|---|
| enine | buffer | 3 | 0.3452 | 0.37 | 0.3948 | 0.0052 | 0.01 | 0.0628 | 0.0058 |
| enine | water | 3 | 0.3452 | 0.37 | 0.3948 | 0.0052 | 0.01 | 0.0628 | 0.0058 |
| enine | Diff (1-2) | | -0.023 | 0 | 0.0227 | 0.006 | 0.01 | 0.0287 | 0.0082 |

T-Tests

| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| enine | Pooled | Equal | 4 | 0.00 | **1.0000** |
| enine | Satterthwaite | Unequal | 4 | 0.00 | 1.0000 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| enine | Folded F | 2 | 2 | 1.00 | 1.0000 |

**Result: There is no significant difference between the buffer and the water means , since the P value is greater than 0.05 (1.0000). Hence both the buffer and the water incorporate same amount of albumin containing solution.**

**T test for EO with Km=1.5, surfactant/oil=2:3 ( Performed using the SAS software).**

**SAS code:**

```
data one;
 infile 'G:/kush.csv' dlm=',' DSD;
 input  type $ nine five one enine efive eseven;
 options ls=100;
 proc sort; by type;
 proc print;


data two; set one;
 options ls=100;
 proc sort; by type;
 proc glm;
 class type;
 model =type;
 output out=efive;
 means type/tukey;


data three; set one;
 proc ttest h0=0 alpha=0.05;
 class type;
 var efive;


run;
```

**OUTPUT:**

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The TTEST Procedure

Statistics

| Variable | type | N | Lower CL Mean | Mean | Upper CL Mean | Lower CL Std Dev | Std Dev | Upper CL Std Dev | Std Err |
|---|---|---|---|---|---|---|---|---|---|
| efive | buffer | 3 | 0.2952 | 0.32 | 0.3448 | 0.0052 | 0.01 | 0.0628 | 0.0058 |
| efive | water | 3 | 0.3952 | 0.42 | 0.4448 | 0.0052 | 0.01 | 0.0628 | 0.0058 |
| efive | Diff (1-2) | | -0.123 | -0.1 | -0.077 | 0.006 | 0.01 | 0.0287 | 0.0082 |

T-Tests

| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| efive | Pooled | Equal | 4 | -12.25 | **0.0003** |
| efive | Satterthwaite | Unequal | 4 | -12.25 | 0.0003 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| efive | Folded F | 2 | 2 | 1.00 | 1.0000 |

**Result: There is a significant difference between the buffer and the water means, since the P value is less than 0.05 ( 0.0003).**

**T test for EO, with Km=1.77 surfactant/oil=2:3 ( Perfoemd using the SAS software).**

**SAS code:**

```
data one;
 infile 'G:/kush.csv' dlm=',' DSD;
 input  type $ nine five one enine efive eseven;
 options ls=100;
 proc sort; by type;
 proc print;
data two; set one;
 options ls=100;
 proc sort; by type;
 proc glm;
 class type;
 model =type;
 output out=eseven;
```

```
 means type/tukey;
data three; set one;
 proc ttest h0=0 alpha=0.05;
 class type;
 var eseven;

run;
```

**OUTPUT:**

The SAS System 11:33 Tuesday, August 17, 2010 34

The TTEST Procedure

Statistics

| | | | Lower CL | | Upper CL | Lower CL | | Upper CL | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | type | N | Mean | Mean | Mean | Std Dev | Std Dev | Std Dev | Std Err |
| eseven | buffer | 3 | 0.2852 | 0.31 | 0.3348 | 0.0052 | 0.01 | 0.0628 | 0.0058 |
| eseven | water | 3 | 0.2854 | 0.3233 | 0.3613 | 0.008 | 0.0153 | 0.096 | 0.0088 |
| eseven | Diff (1-2) | | -0.043 | -0.013 | 0.0159 | 0.0077 | 0.0129 | 0.0371 | 0.0105 |

T-Tests

| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| eseven | Pooled | Equal | 4 | -1.26 | **0.2746** |
| eseven | Satterthwaite | Unequal | 3.45 | -1.26 | 0.2846 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| eseven | Folded F | 2 | 2 | 2.33 | 0.6000 |

**Result: There is no significant difference between the buffer anf the water means, since the P value is greater than 0.05 ( 0.2746). Hence both buffer and water are able to incorporate similar amount of albumin solution**

**T test for the two oils IPM and Eo comparing the best formulation with Km=1.94, 2:3.**

**SAS code:**

```
data one;
 infile 'G:/Thesis/oil.csv' dlm=',' DSD;
 input  type $ nine ;
 options ls=100;
 proc sort; by type;
 proc print;
data two; set one;
 proc ttest h0=0 alpha=0.05;
 class type;
```

```
var nine;

run;
```

**OUTPUT:**

The TTEST Procedure

Statistics

| Variable | type | N | Lower CL Mean | Mean | Upper CL Mean | Lower CL Std Dev | Std Dev | Upper CL Std Dev | Std Err |
|---|---|---|---|---|---|---|---|---|---|
| nine | eo | 6 | **0.3606** | 0.37 | 0.3794 | 0.0056 | 0.0089 | 0.0219 | 0.0037 |
| nine | ipm | 6 | **0.3794** | 0.4267 | 0.4739 | 0.0281 | 0.045 | 0.1104 | 0.0184 |
| nine | Diff (1-2) | | -0.098 | -0.057 | -0.015 | 0.0227 | 0.0325 | 0.057 | 0.0187 |

T-Tests

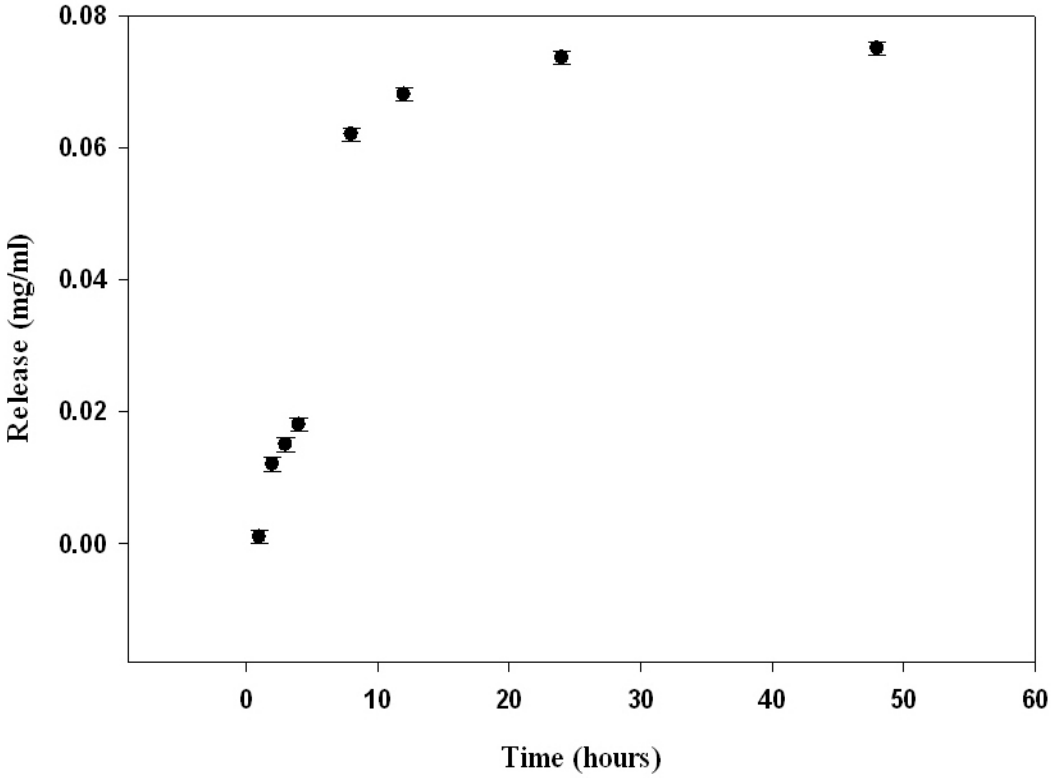
| Variable | Method | Variances | DF | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| nine | Pooled | Equal | 10 | -3.02 | **0.0128** |
| nine | Satterthwaite | Unequal | 5.39 | -3.02 | 0.0266 |

Equality of Variances

| Variable | Method | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|---|
| nine | Folded F | 5 | 5 | 25.33 | 0.002 |

**RESULT: There is a significant difference betwee the means of the two formulations, prepared with Ethyl Oleate and Isopropyl Myristate, since the P value is less than 0.05 ( 0.0128). Since the mean of the IPM is greater than the Ethyl Oleate mean, if forms a better formulation, and incoporates more amount of albumin solution than the formulation prepared with Ethyl Oleate.**

**5.The *invitro* Release Data for Albumin, Curve Fitting, and Nonlinear Regression , Dynamic Fitting ( Performed using Sigma Plot).**



## Curve Fitting

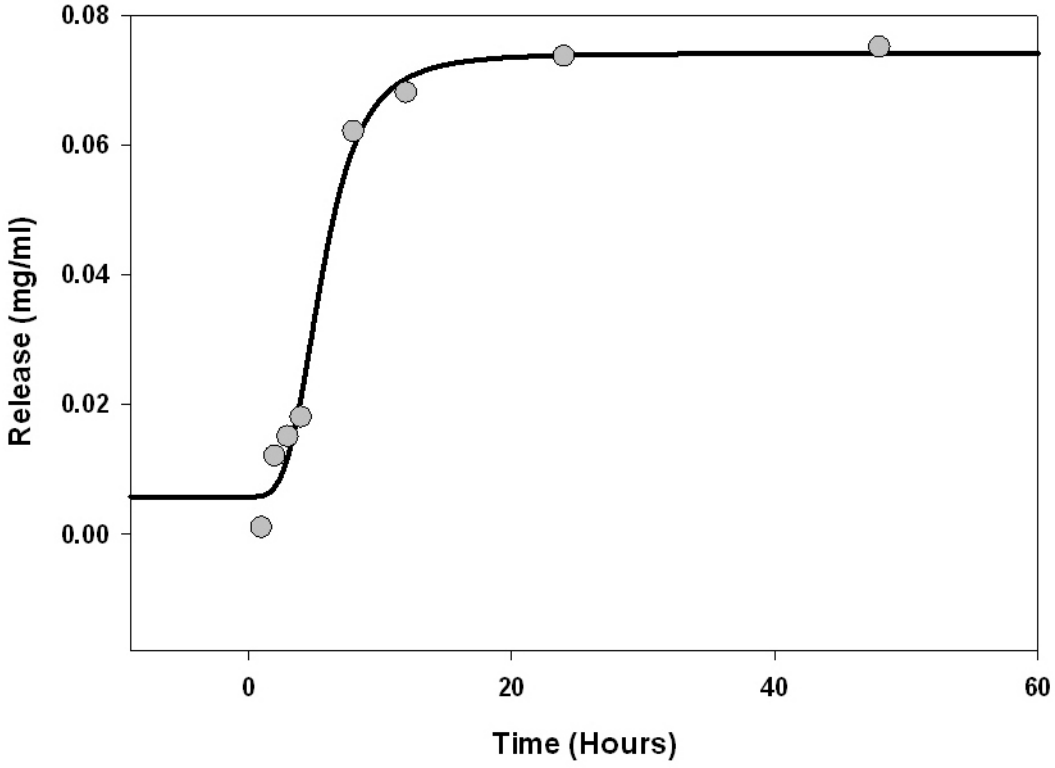



**Figure 4.43- Curve fitting data for the *invitro* release of albumin**

## Nonlinear Regression :

**Nonlinear Regression - Dynamic Fitting**

**Data Source: Data 1 in Notebook1**
**Equation: Standard Curves, Four Parameter Logistic Curve**
f1 = min + (max-min)/(1 + (x/EC50)^(-Hillslope))
f = if(x<=0, if(Hillslope>0,min,max), f1)

**Dynamic Fit Options:**

| | |
|---|---|
| Total Number of Fits | 200 |
| Maximum Number of Iterations | 1000 |

**Parameter Ranges for Initial Estimates:**

| | Minimum | Maximum |
|---|---|---|
| min | -0.0010 | 0.0030 |
| max | -0.0750 | 0.2250 |
| EC50 | 0.0000 | 17.4545 |
| Hillslope | -1.0000 | 3.0000 |

**Summary of Fit Results:**

| | |
|---|---|
| Converged | 100.0% |
| Singular Solutions | 38.5% |

**Results for the Overall Best-Fit Solution:**

| R | Rsqr | Adj Rsqr | Standard Error of Estimate |
|---|---|---|---|
| 0.9946 | 0.9892 | 0.9810 | 0.0044 |

| | Coefficient | Std. Error | t | P |
|---|---|---|---|---|
| min | 0.0058 | 0.0034 | 1.6801 | 0.1682 |
| max | 0.0741 | 0.0030 | 24.7136 | <0.0001 |
| EC50 | 5.5782 | 0.5100 | 10.9369 | 0.0004 |
| Hillslope | 3.6674 | 0.7878 | 4.6555 | 0.0096 |

**Analysis of Variance:**

Analysis of Variance:

| | DF | SS | MS |
|---|---|---|---|
| Regression | 4 | 0.0201 | 0.0050 |
| Residual | 4 | 7.6224E-005 | 1.9056E-005 |
| Total | 8 | 0.0202 | 0.0025 |

Corrected for the mean of the observations:

| | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 3 | 0.0070 | 0.0023 | 121.6954 | 0.0002 |
| Residual | 4 | 7.6224E-005 | 1.9056E-005 | | |
| Total | 7 | 0.0070 | 0.0010 | | |

**Statistical Tests:**

**Normality Test (Shapiro-Wilk)** Passed (P = 0.9094)

W Statistic= 0.9715 Significance Level = 0.0500

**Constant Variance Test** Failed (P = <0.0001)

**Fit Equation Description:**

```
[Variables]
x = col(1)
y = col(2)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2
'Automatic Initial Parameter Estimate Functions
sign(p,q) = if(xatymax(p,q)-xatymax(p,max(q)-q)>0,1,-1)
[Parameters]
min = min(y) "Auto {{previous: 0.00578136}}
max = max(y) "Auto {{previous: 0.0741142}}
EC50 = x50(x,y) "Auto {{previous: 5.57821}}
Hillslope = sign(x,y) "Auto {{previous: 3.66741}}
[Equation]
f1 = min + (max-min)/(1 + (x/EC50)^(-Hillslope))
f = if(x<=0, if(Hillslope>0,min,max), f1)
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
max>min
EC50>0
[Options]
tolerance = 0.0000000001
stepsize = 1
iterations=1000

Number of Iterations Performed = 200
```

# Chapter 5

## 5.1 Conclusion:

Lecithin microemulsions were formulated with soybean lecithin, water and two oils namely, IPM and EO. Characterization of these microemulsions, using polarizing light microscopy, conductivity, Differential Scanning Calorimetry and Dynamic Light Scattering, helped to understand the phase behavior and microstructure of these formulations. Microemulsions could be easily differentiated from macroemulsions, since they did not show any birefringence under polarized light. Low conductivity values, indicated the formation of water in oil microemulsions. There was a continuous increase in conductivity values, as the concentration of water in the microemulsion was increased. The conductivity values slightly decreased when the microemulsions turned milky. The DLS experiments indicated the presence of particles in the range of 5-10 nm that may be reverse micelles. Another small group of large particles in the range of 500-1000 nm formed by aggregation was also seen in the formulation.

When poloxamer and alcohol were compared as co-surfactants, it was observed that isopropyl alcohol was a better co-surfactant than the poloxamer. IPA was able to incorporate a larger quantity of water than the poloxamer in most of the Km

compositions. Albumin solution in water and buffer was successfully incorporated into the Lecithin/IPM and Lecithin/EO mixtures. The in vitro release profile and curve fitting data of albumin containing microemulsion formulation indicates a controlled release profile, and these microemulsions have a potential for use as protein/peptide delivery systems. From the ststistical tests performed on albumin incorporation in water and in buffer, it was found that IPM is a better oil than EO, since it incorporated more amount of albumin solution comparitively.

**Chapter 1:**

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