เรียนรู้เพื่อรับใช้สังคม

# การพัฒนาและการแสดงลักษณะเฉพาะของไมโครอิมัลชันน้ำมันไพลสำหรับการนำส่งพร้อมกันของส่วนประกอบน้ำมันไพลและยาอินโดเมทาซิน

# DEVELOPMENT AND CHARACTERIZATION OF PLAI OIL MICROEMULSIONS FOR SIMULTANEOUS DELIVERY OF PLAI OIL COMPONENT AND INDOMETHACIN

SUWIT KHAJITKHAJONWONG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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# การพัฒนาและการแสดงลักษณะเฉพาะของไมโครอิมัลชันน้ำมันไพลสำหรับการนำส่งพร้อมกันของส่วนประกอบน้ำมันไพลและยาอินโดเมทาซิน

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## บทคัดย่อ

การศึกษานี้เพื่อทดสอบการใช้น้ำมันไพลเป็นวัตภาคน้ำมันในการเตรียมตำรับไมโครอิมัลชันและเพื่อตรวจสอบผลของยาอินโดเมทาซินต่อรูปแบบลักษณะการซึมผ่านของส่วนประกอบน้ำมันไพล การดำเนินการทดลองเป็นลำดับขั้นตอน ขั้นตอนที่หนึ่ง การสร้างแผนภาพไตรภาคเทียมโดยใช้น้ำมันไพลเป็นวัตภาคน้ำมัน ขั้นตอนที่สอง การประเมินหาปริมาณความเข้มข้นที่เหมาะสมของน้ำมันไพล ขั้นตอนที่สาม การออกแบบตำรับไมโครอิมัลชันน้ำมันไพล โดยใช้ $2^3$แฟกทอเรียลเต็มรูป สามารถเตรียมตำรับไมโครอิมัลชันน้ำมันไพลได้แปดสูตรตำรับและทำการศึกษาคุณลักษณะเฉพาะ ขั้นตอนสุดท้ายเป็นการศึกษาความคงตัวและการซึมผ่านคราบงูในหลอดทดลอง ผลการทดลองพบว่าน้ำมันไพลสามารถใช้เป็นวัตภาคน้ำมันจากการสร้างแผนภาพไตรภาคเทียม และพบว่าระบบของน้ำมันไพล/ทวีน 80-เอทานอล บริสุทธิ์ (1:1 และ 2:1)/น้ำ มีพื้นที่บริเวณระบบวัตภาคเดี่ยวที่กว้าง น้ำมันไพลที่ระดับความเข้มข้น 14% โดยน้ำหนัก ถูกเลือกนำไปใช้ในการเตรียมไมโครอิมัลชันน้ำมันไพล ไมโครอิมัลชันน้ำมันไพลทั้งแปดตำรับที่เตรียมได้ มีลักษณะเป็นของเหลวใสสีเหลือง เป็นระบบไมโครอิมัลชันชนิดน้ำมันในน้ำ มีขนาดอนุภาคเล็ก การกระจายขนาดอนุภาคแคบ มีความหนืดต่ำและมีค่าพีเอชค่อนข้างเป็นกรด ภายหลังเก็บรักษาเป็นเวลา 3 เดือน ไม่พบการแยกชั้นหรือการตกตะกอนของผงยา จากการวิเคราะห์ปริมาณเทอร์พินีน-4-ออล ที่ใช้เป็นตัวบ่งชี้ของส่วนประกอบน้ำมันไพลและยาอินโดเมทาซิน ภายหลังการเก็บรักษาเป็นเวลา 3 เดือน พบว่า เทอร์พินีน-4-ออล มีความคงตัวค่อนข้างดี ส่วนยาอินโดเมทาซินมีความคงตัวในระดับหนึ่ง ผลการศึกษาการซึมผ่านคราบงูในหลอดทดลอง พบว่าการมียาอินโดเมทาซินร่วมในตำรับ มีผลลดอัตราการซึมผ่านคราบงูของเทอร์พินีน-4-ออล

**คำสำคัญ:** ไมโครอิมัลชัน น้ำมันไพล อินโดเมทาซิน เทอร์พินีน-4-ออล

# DEVELOPMENT AND CHARACTERIZATION OF PLAI OIL MICROEMULSIONS FOR SIMULTANEOUS DELIVERY OF PLAI OIL COMPONENT AND INDOMETHACIN

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## ABSTRACT

This study was to determine the use of Plai oil, the oily active substance, as oil phase in microemulsion preparation and to investigate the effect of indomethacin on the *in vitro* permeation characteristic of Plai oil component. The experiments were carried out as follows: firstly, the pseudoternary phase diagram of investigated systems using Plai oil as oil phase was constructed. Secondly, the optimization of Plai oil concentration was studied. Thirdly, eight Plai oil microemulsions were formulated with a $2^3$ full factorial design, prepared and characterized. Finally, the stability study and the *in vitro* permeation study through shed snake skin were investigated. Plai oil was successfully used as oil phase in the pseudoternary phase diagram study and in microemulsion preparation. In the pseudoternary phase diagram study, the system of Plai oil/Tween® 80-absolute ethanol (1:1 and 2:1)/water provided a larger single phase area. A 14% w/w Plai oil was chosen to subsequently prepare Plai oil microemulsions. All eight Plai oil microemulsions were isotropic transparent homogenous yellowish liquid mixtures. They were oil in water microemulsions. They had small particle size with a narrow distribution, low viscosity, and a slightly acidic pH value. No phase separation and drug precipitation were observed after three-month stroage. Terpinen-4-ol as a chemical marker of Plai oil component seemed to be stable while indomethacin was stable to some extent after three-month storage. In *in vitro* permeation study, the presence of indomethacin considerably decreased the permeation flux of terpinen-4-ol.

**Keywords**: Microemulsion, Plai oil, indomehacin, terpinen-4-ol

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Suwit Khajitkhajonwong

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| % w/w | Percent weight of a substance of the total weight |
| % v/v | Percent volume of a substance of the total volume |
| °C | Degree Celsius |
| µg | Microgram |
| µS | Micro-Siemens |
| AOAC | Association of Official Analytical Chemists |
| CPP | Critical packing parameter |
| cm | Centimeter |
| DMPBD | (E)-1(3,4 -dimethoxyphenyl) butadiene |
| g | Gram |
| GC | Gas chromatography |
| HLB | Hydrophilic-lipophilic balance |
| HPLC | High performance liquid chromatography |
| $J_S$ | Pseudo-steady state flux |
| min | Minute |
| ml | Milliliter |
| NA | Not assessment |
| NSAIDs | Non steroidal anti-inflammatory drugs |
| o/w | Oil in water |
| rpm | Revolutions per minute |
| SD | Standard deviation |
| SC | Stratum corneum |
| SR | Shear rate |
| SS | Shear stress |
| $T_L$ | Lag time |
| µl | Microliter |
| w/o | Water in oil |

# CHAPTER 1
# INTRODUCTION

Microemulsion is a transparent liquid mixture system consisting of appropriate amount of water, oil, and surfactant normally in combination with a co-surfactant. Microemulsion has optical isotropic property and is thermodynamically stable. (1-4) It forms spontaneously after mixing the proper ratio of components together. Therefore, the component selection is important to formulate receivable microemulsion system. As was known, microemulsion is recognized as a good vehicle for topical delivery of both hydrophilic and lipophilic drugs. (5-7) In microemulsion preparation, investigated water-insoluble compounds were incorporated into oil phase. Commonly used oils are either fatty acids or esters of fatty acids. However, the use of natural oils or vegetable oils with good biocompatibility are limited and remain interesting. (8)

Plai (*Zingiber cassumunar* Roxb.) is a herbal which has been used in Thailand and South-East Asia for topical treatment of various conditions such as muscular pain, sprains and skin diseases. An essential oil obtained by distillation from the rhizome of Plai was called Plai oil. Plai oil consists of five major components that are (E)-1(3,4 -dimethoxyphenyl) butadiene (DMPBD), terpinen-4-ol, sabinene, $\gamma$-terpinen, and $\alpha$-terpiene. (9-11) Due to the main active compounds, Plai oil is found to have anti-inflammatory effect and also show antimicrobial activity. Its therapeutic effect associated with muscle pain and inflammatory was well recognized. The commercial products of Plai oil for topical application are now available in the dosage forms of cream and ointment.

Indomethacin is a non steroidal anti-Inflammatory drug with analgesic and antipyretic effects. (11-12) Indomethacin for oral dosage form has been used for treatment the symptoms of rheumatoid arthritis, gout, and osteoarthritis. Indomethacin, like other non steroidal anti-Inflammatory drugs (NSAIDs), has potential side effects such as gastric ulcer, gastrointestinal irritation, gastrointestinal bleeding. (4, 13) Therefore, the topical route is an alternate choice of administration.

Indomethacin for topical application is commercially available such as SATOGESIC® GEL, Elmetacin®.

In this study, I would like to investigate the potential of the essential Plai oil itself as an oil phase in microemulsion preparation. If it is successful, it will be an example for pharmaceutical formulators to use an active oil ingredient itself without combination with other oils as oil phase in microemulsion preparation. In addition, in order to gain synergistic effect of the formulation, indomethacin was added.

**1.1 Objectives**

1. To investigate whether an essential Plai oil itself can be used as oil phase in microemulsion preparation
2. To characterize the physicochemical properties of the obtained formulations.
3. To investigate the effect of indomethacin on the *in vitro* permeation characteristic of the Plai oil component.

**1.2 Scope of the study**

1. To construct the pseudoternary phase diagram for surfactant screening.
2. Using a $2^3$ full factorial design to determine the effect of the three factors: weight ratios (Km) of surfactant to cosurfactant, the concentration of the mixture of surfactant and cosurfactant and the presence of indomethacin on the physicochemical characteristics of the obtained formulations**.**
3. To determine the acceptable formulation by investigating the physicochemical properties, the stability after three months and the *in vitro* permeation study of the obtained formulations.

**1.3 Expected outcome of the research**

An acceptable Pial oil microemulsion formulation for topical application.

# CHAPTER 2
# LITERATURE REVIEWS

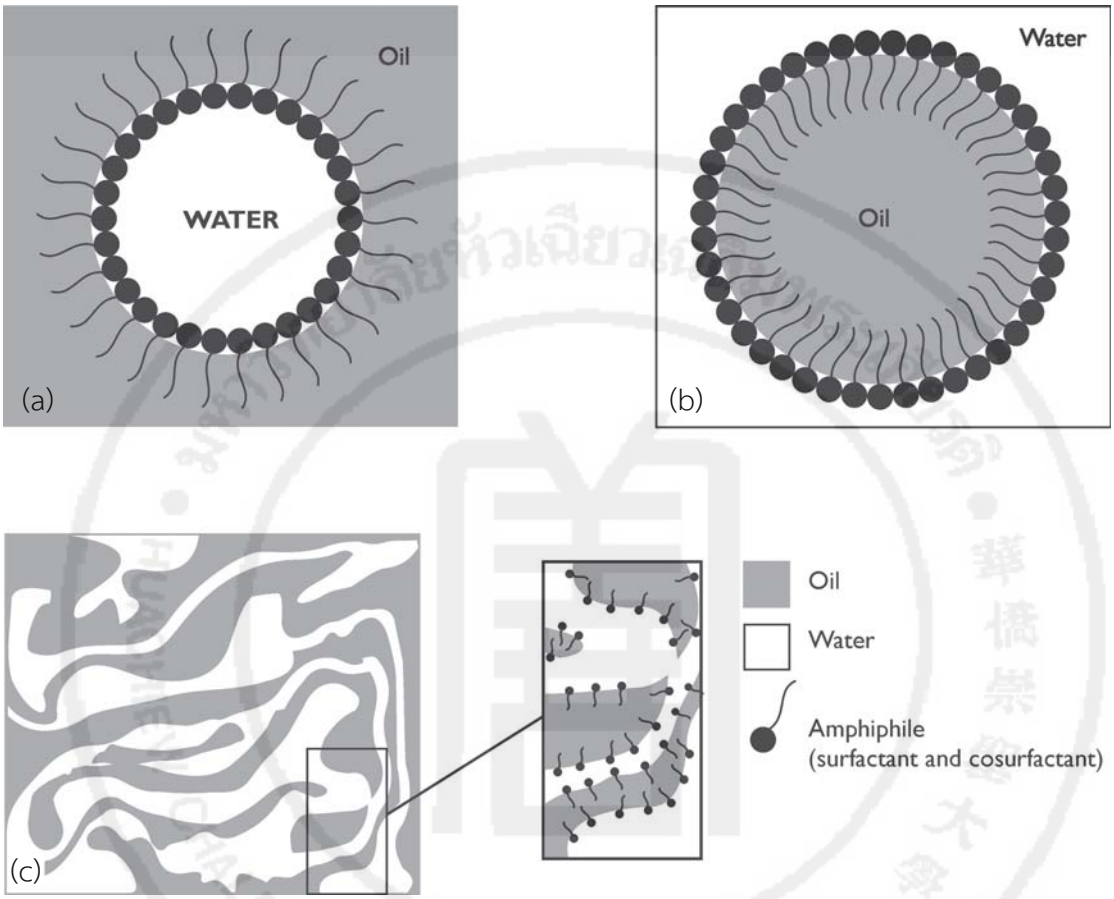
In the 1940s, the term microemulsion was introduced by Hoar and Schulman. Since then the term has been used to explain systems consisting of water, oil, and surfactant/co-surfactant. Microemulsions are the optically isotropic transparent and thermodynamically stable systems. Their droplet sizes are less than 200 nm and can be prepared without considerable input of high mechanical energy. (1, 14) By this definition the following systems are not microemulsions :(1)

- Aqueous solutions of surfactants (micellar and nonmicellar) without additives or with water soluble nonelectrolytes as additives
- Liquid crystalline phases (mesophases)
- Coarse emulsions including micronized coarse emulsions
- Systems that are surfactant free

## 2.1 Structure and formation of microemulsion systems

A microemulsion can be classified according to the compositions into three types as follows: oil in water (o/w), bicontinuous, and water in oil (w/o). (1-2, 15) Each type has an interfacial surfactant/co-surfactant monolayer separating oil phase and water phase. An interfacial surfactant monolayer surrounds the droplets (Figure 1a and 1b). The type of microemulsion droplets is dependent on the volume fraction of oil and water. The oil in water (o/w) droplets occur when the volume faction of water is high. In contrast, water in oil (w/o) microemulsion droplets form when the volume faction of oil is high. When the amounts of water and oil are similar, a bicontinuous microemulsion may exist (Figure 1c). In such system, both oil and water exist as continuous phase that are separated by surfactant-stabilized interface with a net curvature of zero. (1, 15)

**Figure 1** (a) w/o microemulsion, (b) o/w microemulsion, (c) water-and-oil bicontinuous microemulsion. (1)




Thermodynamic approach with reference to the equation has been used to explain microemulsion formation

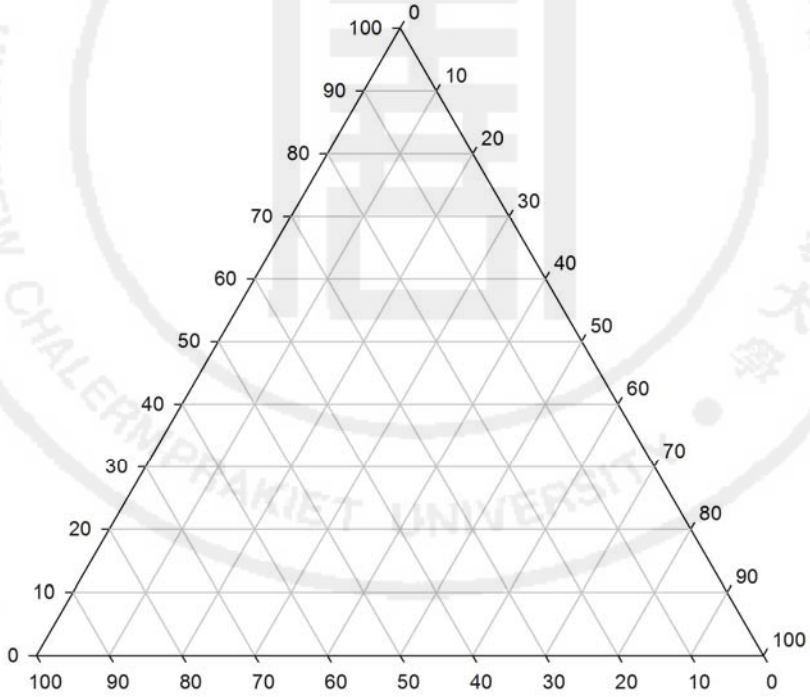
$$\Delta G = \gamma \Delta A - T\Delta S \qquad (1)$$

Where $\Delta G$ is the free energy of microemulsion formation, $\gamma$ is the interfacial tension of the oil-water interface, $\Delta A$ is the change in interfacial area, $\Delta S$ is the change in entropy, and T is the temperature. The process of microemulsion formation occurs without high energy input (spontaneous); therefore, $\Delta G$ is a negative value. (1, 15)

**2.1.1 Phase behavior studies**

The phase behavior of simple microemulsion systems comprising oil, water and surfactant can be studied with the aid of ternary phase diagram using a Gibbs triangle (Figure 2) in which each corner of the diagram represents 100% of the particular component. The phase diagrams graphically provide i) information on the appropriate ratio of ingredients to obtain a specific composition, and ii) opportunity to predict the composition of the formulation resulting from various ratios of the ingredients. (16) Generally, the microemulsion will contain additional components such as a cosurfactant and/or drug.

**Figure 2** Gibbs triangle.




When the systems are formed by four or more components, the pseudoternary phase diagrams are used where a corner will typically represent a binary mixture of two components such as surfactant/cosurfactant, water/drug or oil/drug. (15)

The ternary phase diagram can be constructed by two methods as follows:

1. Titrating a mixture of two components with the third components.

2. Preparing a large number of samples of different composition.

If all mixtures reach equilibrium rapidly, both methods give identical results. For mixtures that do not reach equilibrium quickly, the second method is recommended. (1)

When oil phase, water phase, and the surfactant system are mixed at different ratios of the components and the phase behavior of such system is constructed, the areas of single phase and multiple phase can be indicated where water-in-oil microemulsions and oil-in-water microemulsions were formed. Not every combination of components produces microemulsions over the whole range of possible compositions, in some instances the extent of microemulsion formation may be very limited. (15)

It should be noted that in the area outside the microemulsion region, there are four types of microemulsion phase equilibrium as follows: (17)

Type I (Winsor I): the surfactant is preferentially soluble in water and oil-in-water (o/w) microemulsions form. The surfactant-rich water phase coexists with the oil phase where surfactant is only present as monomers at small concentration.

Type II (Winsor II): the surfactant is mainly in the oil phase and water-in-oil (w/o) microemulsions form. The surfactant-rich oil phase coexists with the surfactant-poor aqueous phase.

Type III (Winsor III or middle-phase microemulsion): a three-phase system where a surfactant-rich middle-phase coexists with both excess water and oil surfactant-poor phases.

Type IV: a single-phase (isotropic) micellar solution that forms upon addition of a sufficient quantity of amphiphiles (surfactant plus alcohol).

## 2.2 Microemulsion composition

### 2.2.1 Oil or organic phase

Hydrocarbons pack well within the surfactant tails. Hydrocarbons with too long tail are not good oil phases because of lack of solubility but medium and short chain triglycerides are candidates to serve as the oil phase. Commonly used oils are either fatty acids such as oleic acid, myristic acid or esters of fatty acids such as methyl or ethyl esters of lauric acid, isopropyl palmitate and isopropyl myristate. (8, 18) Boonme et al. (2) prepared microemulsion system containg isopropyl palmitate (IPP) as oil phase, polyoxyethylene-10-oleyl ether (Brij-97) as surfactant, 1-butanol as cosurfactant at weight ratio of the surfactant to cosurfactant of 2:1, and water. Podlogar et al. (19) also succeeded formulating microemulsions containing isopropyl myristate (IPM) as oil phase, Tween®40 as surfactant, glyceryl caprylate (Imwitor®308) as cosurfactant, and water.

### 2.2.2 Surfactants

Surfactants so-called amphiphilics are organic compounds containing both hydrophobic groups and hydrophilic groups. Surfactants used in the formulations are the substances at least generally regarded as safe and preferably pharmaceutical-grade ingredients. (1)

Surfactants usually diffuse in water and absorb at the interface between air and water or at the border between oil and water. A short chain surfactant has less pronounced character so rather high concentrations of amphiphiles are needed. A long chain surfactant is a stronger amphiphile so lower amounts of surfactant are needed to from a microemulsion. High surfactant levels are essential because of the increase in interface between the aqueous and oil phase.

The HLB concept is not relevant but the critical packing parameter (CPP) can be a useful guide to surfactant selection. The CPP is a measure of the preferred geometry adopted by the surfactant and, as a consequence, is predictive of the type of aggregate that is likely to form. (15) The effect of changing CPP is illustrated as shown in Figure 3. CPP is defined by the equation

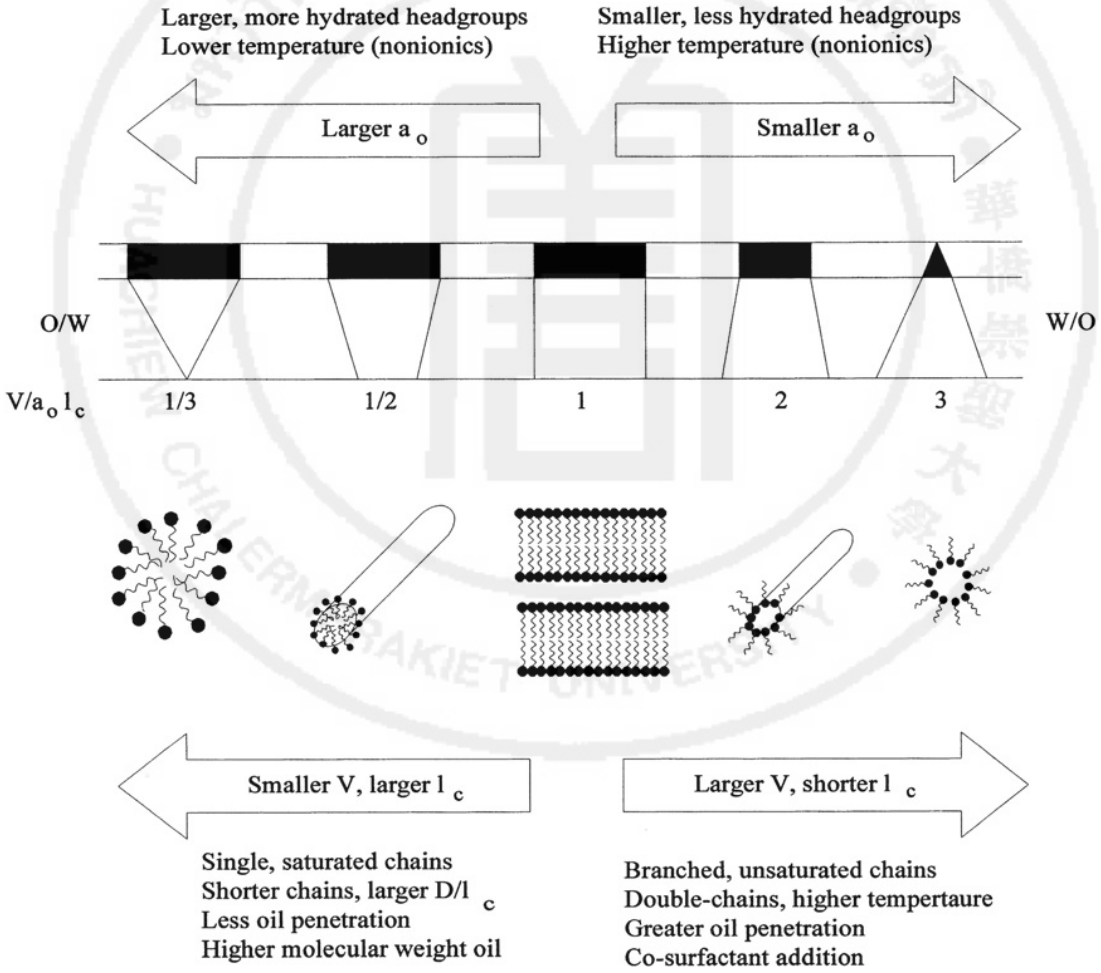
$$CPP = v/(l_c a_o) \quad (2)$$

V = hydrophobic group volume

$l_c$ = critical hydrophobic group length

$a_o$ = optimal head group area

**Figure 3** Effect of molecular moieties and solution conditions on the CPP of a surfactant and the resulting range of possible surfactant aggregates in water or aqueous solution. (15)



The surfactants used to stabilize microemulsion system may be: (i) non-ionic, (ii) zwitterionic, (iii) cationic or (iv) anionic surfactants. (15) Nonionic surfactants are commonly used in microemulsion preparation. Among the range of nonionic surfactants used are sucrose esters, polyoxyethylene alkyl ethers, polyglycerol fatty acid esters, polyoxyethylene hydrogenated castor oil, and sorbitan esters. (1)

Cho et al. (20) investigated the phase behavior of system based on a hydrophilic surfactant (Tween®80). They found that the combination of different hydrophobic surfactants with Tween®80 in microemulsion formation produced smaller droplets than Tween®80 alone.

**2.2.3 Cosurfactants**

Cosurfactant helps the surfactant reduce the interfacial tension of a system to very low values (near zero) to achieve thermodynamic stability. They both modify the curvature of the interface by incorporating additional apolar groups and provide more fluidity to the film in order to prevent crystallization of the tails of surfactant. Commonly, the cosurfactants are short (ethanol) and medium-chain (propanol to octanol) alcohols. (18)

El Maghraby (7) investigated the effects of cosurfactants (ethanol, isopropanol and propylene glycol) on the transdermal delivery of hydrocortisone from eucalyptus oil microemulsion which contained 20% oil, 20% water and 60% either Tween 80 or 1:1 surfactant/cosurfactant mixture. The presence of cosurfactant can affect both the phase behavior and the transdermal delivery potential of microemulsion. In this study, ethanol produced the greatest effect followed by propylene glycol and isopropanol.

Liu et al. (21) investigated the effect of cosurfactant on microemulsion stability and curcumin transdermal delivery. The constituents were oils (limonene), surfactants, and a cosurfactant (ethanol, isopropanol, and propylene glycol). They found that the flux of curcumin through the skin was ranked in the following order ethanol > propylene glycol> isopropanol > cosurfactant-free.

## 2.3 Microemulsion characterization

### 2.3.1 Polarized light microscopy

Polarized light microscopy is a simple technique to learn and use, readily available, and of great value to differentiate between various anisotropic liquid crystalline systems. When a mixture of oil, water, surfactant, and cosurfactant is examined under a polarized light microscope, the resulting aggregates tend to show strong birefringence if they are anisotropic. On the other hand, if the resulting aggregates are isotropic as with microemulsion systems, the view remains dark because the analyzer absorbs light passing through the polarizer. (1)

Many liquid crystalline systems may appear transparent and can be easily misinterpreted as isotropic microemulsion systems. Thus it becomes essential when investigating systems to confirm findings based on visual appearance with polarized light microscopic examination. (1)

### 2.3.2 Electrical conductivity measurements

Electrical conductivity measurements can provide valuable information phase behavior of microemulsion systems. (1) Microemulsion can exist as water in oil or oil in water, therefore conductivity measurements can be applied for their characterization. The underlying principle for phase determination by conductivity is the ability of water to conduct an electric current, which is measured in S $cm^{-1}$ or μS $cm^{-1}$. If water forms the continuous phase of a microemulsion, the system will show a high conductivity. On the other hand, the system will exhibit its low conductivity if oil becomes the continuous phase. (22)

Electrical conductivity plays an important role in the characterization of colloidal system due to its ease of use and data interpretation, and low cost of the measurement equipment.

### 2.3.3 Viscosity measurements

Viscosity measurements are dynamic experiments. They will give information on dynamic properties of the microemulsion. These will depend on the microstructure, type of aggregates, or interactions within the microemulsion. Newtonian flow is usually observed for the viscosity of microemulsion system, from which the shear stress (SS) is directly proportional to the shear rate (SR). (22)

Newtonian flow behavior of microemulsion is a decisive feature in differentiation from other colloidal systems. It is point to the characteristic of microemulsion. (22)

The low viscosity of microemulsion reflects the fluid character of the overall structure which is a favorable feature for most microemulsion applications. (1)

**2.3.4 Other characterization techniques**

The other techniques used to characterize microemulsion systems with emphasis on droplet size determination are optical techniques such as static and dynamic light scattering and nonoptical techniques such as small-angle X-ray scattering, small-angle neutron scattering, pulsed field gradient NMR and dielectric measurements. (1)

## 2.4 Microemulsions as a transdermal drug delivery system

Transdermal drug delivery is an effective method to maximize the flux through the skin into the systemic circulation whereas dermal drug delivery aims at targeting either the epidermis or the dermis of the skin. (1, 23) The key challenge in both cases is to provide sufficient increase in drug flux with minimal or no significant irreversible alteration to the skin barrier function. Transdermal route offers distinct advantages compared to traditional routes by avoidance of first-pass metabolism, potential of controlled release, ease of administration, and possibility of immediate withdrawal of treatment when necessary. (1)

Various mechanisms for skin permeation enhancement of permeants by microemulsions have been proposed. Firstly, microemulsions act as drug reservoirs where loaded drug is released from the inner pseudophase to the outer pseudophase and finally progresses into the skin. Secondly, microemulsion droplets might breakdown on the surface of stratum corneum and then release their content into skin. Thirdly, loaded drug directly permeate from the microemulsion droplets to the stratum corneum without fusion at the stratum corneum. The last mechanism has been frequently supported by findings of researches and indicates that the enhancement effect of microemulsion is caused by the nano-sized droplets

dispersed in continuous phase which can move easily into the stratum corneum and carry the drug through the skin barrier. (3, 24)

Several studies have reported on the enhanced bioavailability of cutaneous drugs using oil in water and water in oil microemulsions. (1) A diverse range of drug molecules such as ketoprofen (3), diclofenac (5), indomethacin (7-8, 12), meloxicam (14), and ibuprofen (25) were incorporated into different microemulsion systems.

El Maghraby (7) studied the transdermal delivery of indomethacin from the self-microemulsifying and microemulsion systems. They found these formulations increased the transdermal drug flux compared to saturated drug solution in phosphate buffered saline. In addition, Chen et al. (12) reported that microemulsion showed a novel transdermal delivery vehicle for increasing the solubility and permeability of indomethacin.

Zhao et al. (23) reported that a microemulsion system might be the promising vehicle for the transdermal delivery of theophylline. They prepared a microemulsion composed of oleic acid as the oily component and Cremophor RH40/Labrasol (1:2) as surfactants. Their pharmacokinetic study in rabbits concluded that area under the plasma concentration-time curve from zero hour to infinity ($AUC_{0\rightarrow\infty}$) of transdermal administration was 1.65-fold higher than that of oral solution administration.

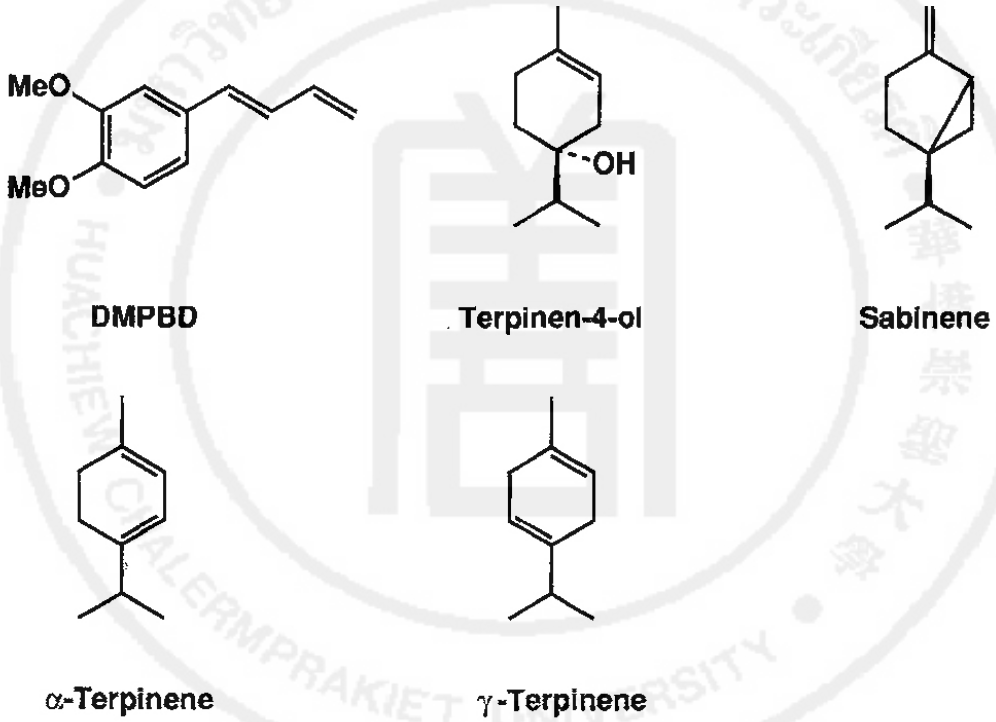
Ngawhirunpat et al. (3) studied a microemulsion for transdermal drug delivery of ketoprofen (KP). They found that the microemulsion system containing IPM, water, Cremophor RH40:PEG400 and terpenes resulted in significant enhancement in skin permeation of KP.

**2.5 Plai oil**

Plai has long been used in folklore remedies for the treatment of muscle pain, joint pain, sprains and skin diseases. (10, 26-27) An essential oil obtained by distillation from the rhizome of Plai (*Zingiber cassumunar* Roxb.) was called Plai oil. Plai oil consists of five major components that are (E)-1(3,4 -dimethoxyphenyl) butadiene (DMPBD), terpinen-4-ol, sabinene, γ-terpinen, and α-terpiene as shown in Figure 4. (10) Plai oil has been reported to have anti-inflammatory effect and analgesic action.

Pongprayoon et al. (10) studied the essential oil of the rhizome of *Zingiber cassumunar.* They reported the individual assessment of topical anti-inflammatory of the five major components of Plai oil. They found that (E)-1(3,4-dimethoxyphenyl) butadiene (DMPBD), terpinen-4-ol and α-terpiene had the topical anti-inflammatory effect. DMPBD was the most active compound.

**Figure 4** Chemical structure of DMPBD, terpinen-4-ol, sabinene, α-terpiene and γ-terpinen. (10)




Niempoog et al. (28) evaluated the efficacy of Plygersic® gel (combination of ginger and Plai oil, 14%) for the treatment of osteoarthrthritis of the knee. They used 1% Diclofenac® gel as a comparator. The study showed that both Plygersic gel and diclofenac gel could significantly improve knee joint pain symptoms and improve the quality of life in osteoarthritis knees during a six weeks treatment regimen with no difference to the 1% Diclofenac® gel group.

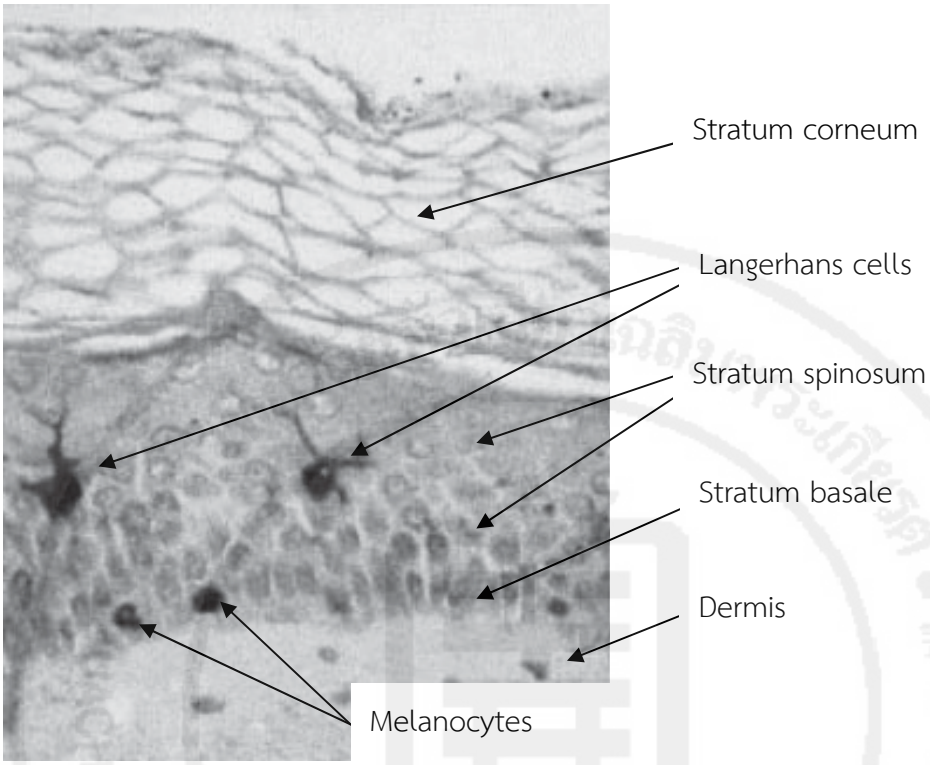
## 2.6 Skin

Skin is the largest organ of the body, which provides a barrier between the body and the external environment**.** This barrier protects against the permeation of ultraviolet (UV) radiation, chemicals, allergens and possible invasion of pathogens, and the loss of moisture and body nutrients. The thickness, pigmentation, and distribution of the appendages of the skin vary in different parts of the body. The skin provides an ideal site for administration of therapeutic compounds for local and systemic effects. Human skin is made up of three main regions: epidermis, dermis and subcutaneous tissues. (29-30)

### 2.6.1 Epidermis

The epidermis is a multilayered region that varies in thickness. The stratum corneum is the outermost layer of epidermis and is approximately 10-20 μm thick. The epidermis is in a constant state of renewal. Thus the structure of the epidermal cells changes from the stratum basale, through the stratum spinosum, stratum granulosum, and stratum lucidum to the outermost stratum corneum (Figure 5). The epidermis does not have any blood vessels; therefore epidermal cells must source nutrients and remove waste by diffusion across the epidermal – dermal layer to the cutaneous circulation in the dermis. (29)

**Figure 5** Structure of epidermal layer. (29)




The stratum corneum has been described as a brick wall-like structure of corneocytes as “bricks” in a matrix (or “mortar”) of intercellular lipids. The corneocytes lack a nucleus and are composed of about 70% – 80% keratin and 20% lipid within a cornified cell envelope. The cornified cell envelope is a protein/lipid polymer structure formed just below the cytoplasmic membrane that subsequently resides on the exterior of the corneocytes. The intercellular lipids to the corneocyte protein envelope is important in providing the structure and barrier function of the stratum corneum. (29)

The stratum corneum intercellular lipids and their structural arrangement in multiple lamellar layers within a continuous lipid domain are critical to the barrier function of the stratum corneum. The major components of the lipid domains are ceramides, cholesterol, free fatty acids, cholesterol esters, and cholesterol sulfate, with the notable absence of phospholipids. The stratum corneum contains about 15% – 20% water that is primarily associated with the keratin in the corneocytes. (29)

### 2.6.2 Dermis

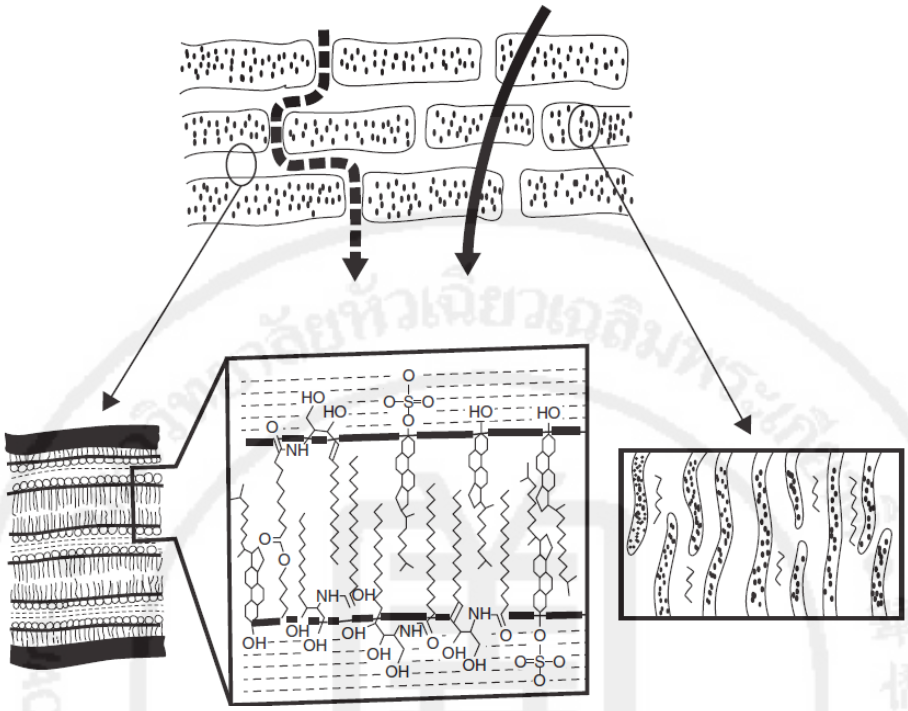
The dermis is about 2 to 5 mm thick. The dermis is composed of network of elastic connective tissue and collagen fibrils embedded in mucopolysaccharide matrix. Collagen fibrils provide support while elastic connective tissue provides elasticity and flexibility. Due to this structure, the dermis provides little barrier to the permeation of most drugs, but may reduce the permeation to deeper tissues of very lipophilic drugs. Within the dermis, an extensive vascular network, nerves and lymph vessels cross this matrix and skin appendages (the hair follicles and associated sebaceous glands, eccrine, and apocrine sweat glands). (29)

## 2.7 Skin permeation pathways (29)

### 2.7.1 Permeation via the stratum corneum: Transcellular route

Transport through the statum corneum is predominantly by the intercellular route. Transport by the transcellular route as shown in Figure 6 has been regarded by some as a polar route through the stratum corneum. While the corneocytes contain an intracellular keratin matrix that is relatively hydrated and thus polar in nature, permeation requires repeated partitioning between this polar environment and the lipophilic domains surrounding the corneocytes.

**Figure 6** Stratum corneum permeation pathways. (29)



### 2.7.2 Permeation via the stratum corneum: Intercellular route

The intercellular lipid route provides the only continuous route through the stratum corneum. Transport can take place via both lipid (diffusion via the lipid core) and polar (diffusion via the polar head groups) pathways. The diffusional rate-limiting region of very polar permeants is the polar pathway of the stratum corneum, which is fairly independent of their partition coefficient, while less polar permeants probably diffuse via the lipid pathway, and their permeation increases with increase in lipophilicity.

### 2.7.3 Permeation via appendages

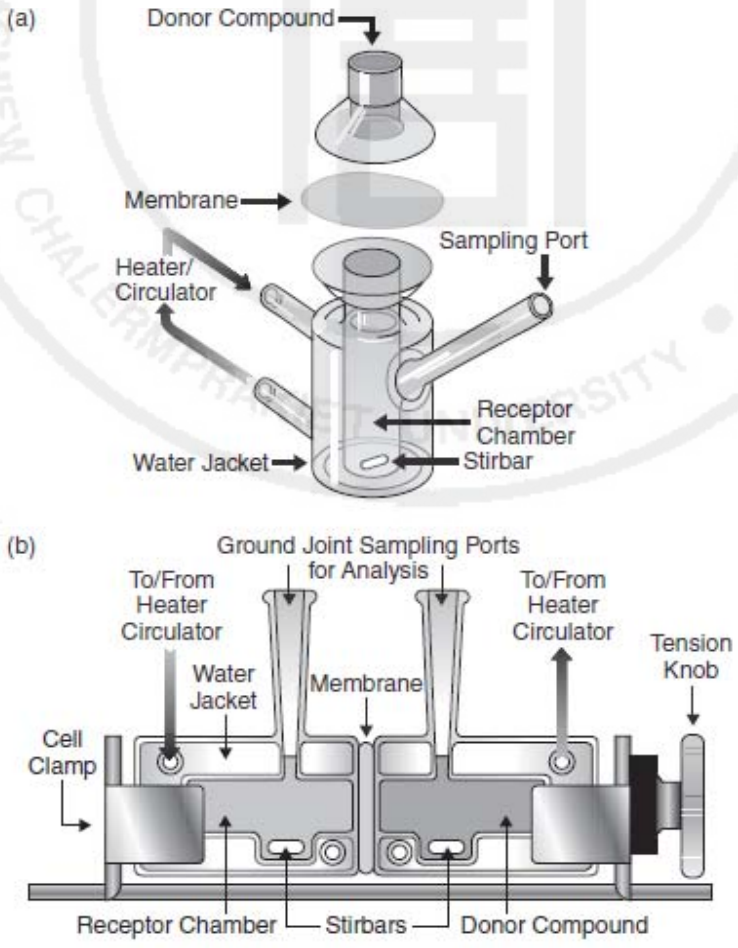
The appendages have been considered as low resistance shunts as the sweat glands are filled with aqueous sweat and the follicular glands with lipoidal sebum. The available diffusional area of the appendage route is approximately only 0.1% – 1.0% of the total skin surface area.

**2.8 *In vitro* skin diffusion cell**

*In vitro* systems range in complexity from a simple two-compartment ''static'' diffusion cell to multi-jacketed ''flow-through'' cells. Excised skin is always mounted as a barrier between a donor chamber and a receptor chamber as shown in Figure 7, and the amount of compound permeating from the donor to the receptor side is determined as a function of time.

The receptor chamber surface is bathed by some form of aqueous solution. The most commonly used receptor fluid is pH 7.4 phosphate-buffered saline (PBS) being the most frequent choice, and its temperature regulated by thermostatically controlled water circulating through a jacket surrounding the chamber in order to maintain the skin surface at 32°C. (31-32)

**Figure 7** Diffusion cell designs (a) Franz cell, (b) side - by - side cell. (32)



**2.9 Skin membranes**

A major potential variant in the design of *in vitro* skin permeation experiments is the nature of the skin membrane. Animal skin has been widely used as a substitute for human skin but some animal models are still occasionally promoted. (32) Shed snake skin was well studied and used as a model membrane for permeability study. When compared with human stratum corneum, it has similarities in terms of structure composition, lipid content and water permeability as well as activity. Shed snake skin was interesting to be used because of its ease of handling and storage and its low cost. (33-34) Many researchers concluded that shade snake skin was suitable to be used as a model membrane for in *vitro* percutaneous penetration study. (3, 33-35)

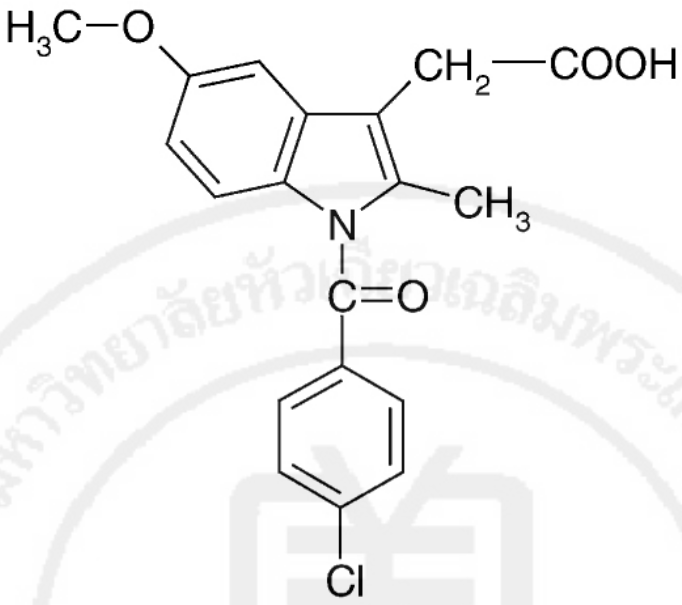
Itoh et al. (33) studied shed snake skin as a model membrane for *in vitro* percutaneous penetration studies in comparison with human skin. The permeability of various compounds and the contribution of several functional groups to the permeability were also found to be similar between shed snake skin and human skin.

Kumpugdee-Vollrath et al. (35) compared the difference among four types of shed snake skin as a model membrane for the pharmaceutical purpose instead of human stratum corneum. Penetration of ketoprofen in ethanol was studied. They found that the different kinds of shed snake skin showed only small variation from each other and could be used as an alternative membrane.

**2.10 Indomethacin**

Indomethacin (1-(4-chlorbenzoyl)-5-methoxy-2-methylindoleacetic acid) (Figure 8) is a non steroidal anti-inflammatory drug with analgesic and antipyretic properties. (11-12) Indomethacin is also a more potent antipyretic and analgesic than aspirin. Indomethacin is absorbed rapidly after oral administration. Side effects such as gastro-intestinal disturbances, headache, and dizziness are common with indomethacin. Gastro-intestinal ulceration and bleeding may also occur. (36-38)

**Figure 8** Molecular structure of indomethacin.




Indomethacin appears as pale yellow to yellow-tan, crystalline powder. It is odorless. Its molecular mass is 357.8 Daltons and pKa is 4.50. It is poorly water-soluble drug but soluble in ethanol. The solubility in water is about 0.40 mg/100 ml at 25°C but in phosphate buffer, the solubility increases when the pH of the buffer increases. Indomethacin undergoes alkaline hydrolysis to *p*-chlorobenzoate acid and 2-methyl-5-methoxy-indole-3-acetate. Aqueous solution of indomethacin is not stable because of the ease of hydrolysis. (36-38)

# CHAPTER 3

# EXPERIMENTAL

## 3.1 Materials

1. Essential Plai oil (Lot E064T, Make Scents Limited, Thailand)
2. Tween® 80 (Lot S6955987 446, Merck, Germany)
3. Kolliphor® RH40 (Lot 14275475L0, BASF, Germany)
4. Absolute ethanol (Lot K42133283, Merck, Germany)
5. Acetonitrile (Lot K45965891 446, I61469 147, Merck, Germany)
6. Methanol (Lot I746607 431, Merck Germany)
7. Ortho-Phosphoric acid 85% (Lot 13 04 0054, RCI Labscan, Thailand)
8. Terpinen-4-ol (4-Carvomenthenil) (Lot MKBQ5361V, Sigma-Aldrich, USA)
9. Sodium dihydrogen orthophosphate (Lot 0912460, UNIVAR® Ajax Finechem, New Zealand)
10. Di-Sodium hydrogen orthophosphate anhydrous (Lot F1J069, Asia Pacific Specialty Chemical limited, Australia)
11. Sodium chloride (Lot 0907321, UNIVAR® Ajax Finechem, New Zealand)
12. Sodium hydroxide pellets (Lot 1306251724, UNIVAR® Ajax Finechem, NewZealand)

## 3.2 Equipment

1. Stereomicroscope (Nikon Eclipse 50i, Japan)
2. pH meter (Lab 850, Schott® Instrument, Germany)
3. Photon correlation spectroscopy (Delsa™ Nano C Particle Analyzer, Beckman Coulter®, USA)
4. Brookfield® Digital Rheometer (Model DV-II+ Visometer, Brookfield Engineering Laboratory, USA)
5. Gas chromatography (GC-2014, Shimadzu®, Japan)
6. Flame ionization Detector (FID-2014, Shimadzu®, Japan)

7. Auto injector (Model AOC-20i, Shimadzu®, Japan)
8. Capillary column BP624 (SGE Analytical Science, Australia)
9. Liquid chromatography (Model LC-20AD, Shimadzu®, Japan)
10. Communication bus module (Model CBM-20A, Shimadzu®, Japan)
11. UV/VIS Detector (Model SPD-20A, Shimadzu®, Japan)
12. Reversed phases HPLC columns (model XDB1 C8, SiliaChrom®, Canada)
13. Magnetic stirrer (Heidolp Instruments, Germany)
14. Franz diffusion cell (PermeGear®, Germany)
15. Heated circulating baths (Grant®Instruments, England)
16. Centrifuge (Model D-78532, Hettich, Germany)

**3.3 Methods**

**3.3.1 Determination of phase diagram construction method**

The phase behavior of the particular combination of the investigated ingredients was established to determine the microemulsion formation region when such investigated ingredients were mixed in order to obtain the microemulsions. Two methods were used to construct the phase diagram of Plai oil/water/Tween®80-absolute ethanol in this study: i) titrating a mixture of two components with the third component and ii) preparing a large number of samples of different compositions.

**3.3.1.1 Construction of phase diagram by water titration method**

Tween®80 and absolute ethanol were mixed at a weight ratio of 1:1 to obtain a surfactant-cosurfactant mixture. Plai oil and the mixture of Tween®80 and absolute ethanol were mixed at the weight ratios of 1:9 to 9:1. Water was added drop by drop to the mixtures under moderate agitation until the clear mixture became turbid at a certain point. The concentrations of components were recorded to construct the phase diagram. The microemulsion region of the system was constructed on a triangular graph using SigmaPlot®12.5 software.

**3.3.1.2 Construction of phase diagram by preparing a large number of different compositions**

The amounts of Plai oil, water, and the mixture of 1:1 Tween® 80-absolute ethanol were calculated to obtain the total combinations of 100% by weight. The amount of each component varied in the range of 10% to 90% at 10% weight intervals. The three components were weighted and the mixed under moderate agitation. The mixtures were stored at ambient temperature for at least 24 hour to reach equilibrium before further investigation. The single phase samples were defined as microemulsions. The microemulsion region of the system was constructed on a triangular graph using SigmaPlot® 12.5 software.

**3.3.2 Screening of surfactants using Plai oil as oil phase**

In order to investigate the effect of different surfactants and the weight ratio (Km) of investigated surfactant to cosurfactant, absolute ethanol, to produce the single phase area, pseudoternary phase diagrams were constructed by water titration method to obtain the proper concentration range of components for the existing range of the single phase areas. The investigated surfactants were Tween® 80 and Kolliphor® RH 40. The Km values varied as 3:1, 2:1 and 1:1. The weight ratios of Plai oil and the surfactant mixtures varied from 1:9 to 9:1. Each pseudoternary phase diagram at a specific Km value was set up using the water titration method. Water was added drop by drop to each oily mixture under gentle agitation until the clear mixture became turbid at a certain point. The concentrations of components were recorded to construct the phase diagram.

**3.3.3 Optimization of the Plai oil concentration**

**3.3.3.1 Preparation of samples**

According to the single phase areas from the pseudoternary phase diagrams, the appropriate surfactant (Tween® 80) and the weight ratios of components were selected at two different Km values. Plai oil concentrations varied from 14 to 30% w/w. At a 50% the mixture of Tween® 80 and absolute ethanol, Tween® 80 and absolute ethanol were mixed at 2:1 and 1:1 weight ratios to obtain the surfactant and cosurfactant mixture. Thus, there were eight formulations of the

microemulsion preparation as shown in Table1. Microemulsion systems were obtained by mixing Plai oil with the mixture of Tween® 80 and absolute ethanol, and then adding water with gentle magnetic stirring at ambient temperature. Afterwards, the obtained systems were kept at ambient temperature to achieve equilibrium before further characterization.

**Table 1** Formulations of various amounts (%w/w) of Plai oil in the optimization study at two Km (surfactant:cosurfactant) values

| Materials | Km value at 1:1 | | | | Km value at 2:1 | | | |
|---|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 |
| Plai oil | 14.0 | 20.0 | 25.0 | 30.0 | 14.0 | 20.0 | 25.0 | 30.0 |
| Tween® 80 | 25.0 | 25.0 | 25.0 | 25.0 | 33.3 | 33.3 | 33.3 | 33.3 |
| Absolute ethanol | 25.0 | 25.0 | 25.0 | 25.0 | 16.7 | 16.7 | 16.7 | 16.7 |
| Water | 36.0 | 30.0 | 25.0 | 20.0 | 36.0 | 30.0 | 25.0 | 20.0 |

**3.3.3.2 Characterization of samples**

(1) Appearance observation

The physical appearances including color and clarity as well as the occurrence of phase separation and/or precipitation were visually observed.

(2) Polarized light microscopy

The optical isotropy of the obtained systems was investigated by using the cross-polarized light microscopy (Nikon Microscope, Eclipse 50i, Japan). A drop of sample was placed between a glass slide and a coverslip, and then investigated under cross-polarized light.

(3) Particle size measurements

The samples were used directly without dilution. The sizes of microemulsions were measured using a Delsa Nano™ C Particle Analyzer (Beckman Coulter, USA). The size measurements were performed in triplicate at 25°C. The results were recorded as mean ± S.D.

(4) pH measurements

The pH values of the systems were measured at 25°C by using a pH meter (Lab 850, Schott® Instrument, Germany). The measurements were run in triplicate at 25°C. The results were recorded as mean ± S.D.

(5) Electrical conductivity measurements

The electrical conductivity was measured at 25°C by using a conductivity meter (SevenEasy, Mettler Toledo, Germany) which was calibrated by using the standard solution of 1413 μS/cm before testing. The measurements were run in triplicate. The results were recorded as mean ± S.D.

(6) Viscosity measurements

The viscosities of the samples were determined by a Brookfield® Digital Rheometer (Model DV-II+ Visometer, Brookfield Engineering Laboratory, USA) using a S18 spindle at the speed of 30 rpm. The measurements were run in triplicate.

**3.3.4 Preparation and characterization of microemulsions with and without indomethacin**

**3.3.4.1 Experimental design for preparation of microemulsion samples**

The optimization study was held on the total experiments. According to the optimization results of the Plai oil concentration, a $2^3$ full factorial design was used for preparation of microemulsion samples to determine the effect of three variables. The factors were the Km, the concentration of the mixture of surfactant and cosurfactant and the presence of indomethacin. Each factor was tested at two levels designated as -1 and +1 as shown in Table 2. Consequently, eight formulations were designed as shown in Table 3 and their compositions as shown in Table 4.

**Table 2** The factors and levels of the $2^3$ factorial design

| Factors | Levels |
|---|---|
| (A) Km value | (+) 2:1<br>(-) 1:1 |
| (B) Indomethacin | (+) 0.75 %<br>(-) 0.00 % |
| (C) Concentration of the surfactant and cosurfactant mixture | (+) 50%<br>(-) 45% |

**Table 3** Illustrate the independent variables setting

| Combination* | Formulation | Composition** | | |
|---|---|---|---|---|
| | | A | B | C |
| (l) | F1 | - | - | - |
| A | F2 | + | - | - |
| B | F3 | - | + | - |
| AB | F4 | + | + | - |
| C | F5 | - | - | + |
| AC | F6 | + | - | + |
| BC | F7 | - | + | + |
| ABC | F8 | + | + | + |

*A, Km value; B, Indomethacin; C, the concentration of the mixture of surfactant and cosurfactant

**(-), Factor at low level; (+), factor at high level

**Table 4** Compositions of microemulsion samples (% w/w)

| Ingredients | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 |
|---|---|---|---|---|---|---|---|---|
| Tween®80 | 22.50 | 30.00 | 22.50 | 30.00 | 25.00 | 33.30 | 25.00 | 33.30 |
| Absolute ethanol | 22.50 | 15.00 | 22.50 | 15.00 | 25.00 | 16.70 | 25.00 | 16.70 |
| DI water | 41.00 | 41.00 | 40.25 | 40.25 | 36.00 | 36.00 | 35.25 | 35.25 |
| Indomethacin | 0.00 | 0.00 | 0.75 | 0.75 | 0.00 | 0.00 | 0.75 | 0.75 |
| Plai oil | 14.00 | 14.00 | 14.00 | 14.00 | 14.00 | 14.00 | 14.00 | 14.00 |

**3.3.4.2 Preparation of microemulsion samples**

According to Table 4, the compositions were weighted. Tween®80 and absolute ethanol were mixed under magnetic stirring to prepare the surfacatant and cosurfactant mixture. Plai oil was incorporated into the mixture to prepare the oil phase. Water was subsequently added under magnetic stirring to obtain the microemulsions. In case of indomethacin-loaded samples, indomethacin was dissolved in the surfacatant and cosurfactant mixture with magnetic stirring before incorporating Plai oil. All obtained samples were kept in the well-closed glass bottle at ambient temperature at least 24 h to achieve equilibrium before further investigation.

**3.3.4.3 Characterization of microemulsion samples**

(1) Appearance observation

See methods 3.3.3.2(1)

(2) Polarized light microscopy

See methods 3.3.3.2(2)

(3) Particle size measurements

See methods 3.3.3.2(3)

(4) pH measurements

See methods 3.3.3.2(4)

(5) Electrical conductivity measurements

See methods 3.3.3.2(5)

(6) Viscosity measurements

See methods 3.3.3.2(6)

(7) Centrifugation

Centrifugation was used as a tool to assess the physical stability of the samples. All samples were centrifuged by centrifugation (D-78532, Hettich, Germany) at speed of 20,000 rpm for 30 min at 25°C. The physical appearance including the occurrence of phase separation and/or precipitation was observed visually.

(8) Stability study

All samples were kept at ambient temperature for three months. After three months, the chemical and physical stability of microemulsion samples were observed for any change including clarity, colour, phase separation and/or precipitation, viscosity, electrical conductivity, pH, and the amount of the investigated compounds including terpinen-4-ol as a chemical marker of Plai oil and indomethacin.

**3.3.5 *In vitro* skin permeation study**

**3.3.5.1 Preparation of shed snake skin**

Shed snake skin of *Naja kaouthia* was used as a membrane in this study. It was obtained by the donation from the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand. The shed snake whole skin was kept at -10°C prior to use. After thawing, it was cut to a round piece with a diameter of 2 cm and then hydrated in deionized water overnight at room temperature before use.

**3.3.5.2 Preparation of 0.01 M phosphate buffer pH 7.4 containing 0.9% sodium chloride**

A 0.349 g of sodium dihydrogen orthophosphate, 1.102 g of di-sodium hydrogen orthophosphate anhydrous and 9.0 g of sodium chloride were dissolved in 980 ml of deionized water. The pH of solution was adjusted to the value of 7.4 by using sodium hydroxide solution. The volume of buffer solution was made to 1000 ml with deionized water.

**3.3.5.3 *In vitro* skin permeation study**

*In vitro* skin permeation study was carried out by using vertical Franz diffusion cells (PermeGear®, Germany). The effective diffusion area of each cell was 2.01 $cm^2$ and the receptor compartment volume was 15 ml. The diffusion cells were connected with a circulating water bath (Grant® Instruments, England) whose temperature was maintained at 34°C in order to maintain the membrane surface temperature at 32±1°C. The circular shed snake skin was placed upwards and clamped between the donor and the receptor compartment of the diffusion cell. The receptor compartment was filled with the receptor fluid and stirred at 600 rpm with a Teflon-coated magnetic bar. A 0.2 g of microemulsion samples was loaded into the donor compartment. The donor compartment and the sampling arm were covered with parafilm. At 0.5, 1, 2, 3, 4, 6, 8 h, a 0.3 ml of the receptor fluid was withdrawn from the center of the receptor compartment and replaced immediately with an equal volume of the receptor fluid. The samples were stored at 4°C until analysis. The permeated amounts of investigated substances were analyzed. The skin permeation experiment of each microemulsion formulation was run in triplicate. The cumulative amount of indomethacin and terpinen-4-ol was plotted against time, and the pseudo-steady state flux ($J_s$) was determined from the slope of the linear portion of the plot. The intercept on the X-axis was determined as the lag time ($T_L$).

**3.3.6 HPLC assay for indomethacin**

**3.3.6.1 HPLC conditions**

Indomethacin was analyzed by HPLC system (Shimadzu, Japan). HPLC with UV detection was chosen because it is simple and effective to separate indomethacin from other substances. The HPLC system consisted of a CBM-20A (system controller), a LC-20AD (solvent delivery unit), a DGU-20A5R (degassing unit), a SPD-20 A(UV-VIS detector). The reverse phase column is a SiliaChrom® XDB1 C8 column (4.6 mm x 250 mm, 5µm, Canada). The injection volume was 20 µl. The mobile phase was prepared by three solvents of A, B and C: solvent A was DI water pH 3.2 (adjusted with phosphoric acid), solvent B was acetonitrile and solvent C was methanol. The separation process followed a gradient elution procedure in which

concentration ratios of solvent A and B changed linearly. The run time program for HPLC step gradient method was shown in Table 5. The mobile phase composition (solvent A 35%, B 45%, and C 20%) was kept constant for 3 min, followed by a linear change from 45% to 60% v/v of solvent B and 35% to 20% v/v of solvent A (consumed 20 min), this condition was held constant for 5 min then the condition was set to revert to the initial concentration. Total run time was 35 min. The flow rate of the mobile phase was fixed at 0.8 ml/min and the detection wavelength was set at 320 nm. The peak area was performed with the LCsolution Program (Shimadzu, Japan).

**Table 5** The gradient elution procedure

| **Time (min)** | **DI water pH 3.2 (%v/v)** | **Acetonitrile (%v/v)** | **Methanol (%v/v)** |
|---|---|---|---|
| 3 | 35 | 45 | 20 |
| 20 | 20 | 60 | 20 |
| 25 | 20 | 60 | 20 |
| 30 | 35 | 45 | 20 |
| 35 | | Stop | |

**3.3.6.2 Preparation of indomethacin reference standard**

For analysis of indomethacin content, the stock solution of reference standard was prepared by dissolving 0.2 g of indomethacin in 100 ml of methanol.

For skin permeation studies, the stock solution of reference standard was prepared by dissolving 0.01 g of indomethacin in 100 ml of methanol.

**3.3.6.3 Sample preparation**

A 0.2 g of microemulsion sample was accurately weighted in 10-ml volumetric flask and then diluted to volume with methanol.

**3.3.6.4 Validation of HPLC analysis**

The developed method was validated by determining selectivity, linearity, accuracy, and precision.

(1) Specificity

The developed HPLC method was tested by injecting placebo with the same concentrations as those in the microemulsion formulations as well as reference standard solution of indomethacin. The peak of indomethacin at the specific retention time must be separated from other peaks.

(2) Range and linearity

For analysis of indomethacin content, the reference stock solution of indomethcin in methanol (2.0 mg/ml) was prepared. The calibration curve was constructed with five concentrations ranging from 25.0 to 200.0 µg/ml. Calibration solutions of indomethacin were prepared by separately transferring the stock reference standard solution of 0.12, 0.25, 0.5, 0.75 and 1.00 ml to 10-ml volumetric flasks and diluted to the volume with methanol to obtain the concentration range of 25.0-200.0 µg/ml (covering 70 to 130 percent of the finish product). The peak area of each concentration was the average of three determinations.

For skin permeation studies, the reference stock solution of indomethcin in methanol (0.1 mg/ml) was prepared. Calibration solutions of indomethacin were prepared by separately transferring the stock reference standard solution of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml to 10-ml volumetric flasks and diluted to the volume with methanol to obtain the concentration range of 0.2-1.2 µg/ml. The peak area of each concentration was the average of three determinations.

The calibration curve was constructed by plotting the peak areas (y-axis) as a function of indomethacin concentrations (x-axis). The regression equation was then calculated.

(3) Accuracy

To assay the known added amount of analyte in microemulsions, accuracy of the method indicated by the percent recovery was carried out by standard spiking method at three different concentration levels as

80%, 100% and 120%. According to the AOAC guideline, the limit on the percent recovery of indomethacin (0.75%) should be within the range of 90-108%. (39)

(4) Precision

The precision indicated by the percent relative standard deviation (RSD) was ascertained by determination of six replicate analysis of a fixed amount of indomethacin (150 µg/ml). The accepted value of the percent relative standard deviation should be not more than 2%.

(5) Detection limit

The detection limit was the lowest amount of the analyte in the sample that could be detected. Determination of the signal-to-noise ratio established the minimum concentration. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the detection limit.

(6) Quantitation limit

Determination of the signal-to-noise ratio established the minimum concentration at which the analyte can be reliably quantified. A signal-to-noise ratio was 10:1.

**3.3.7 GC analysis of terpinen-4-ol**

Terpinen-4-ol is one of the five active ingredients of Plai oil. In this study, terpinen-4-ol was used as a chemical marker of Plai oil components for the quantitative determination of Plai oil. Terpinen-4-ol was analyzed by the GC system (Shimadzu, Japan). GC with FID detection was chosen as it was a fast and effective separation method for determination of terpinen-4-ol. The GC system consisted of auto injector (AOC – 20i) and flame ionization detector (FID-2014, Shimadzu®, Japan). The column was a capillary column (BP 624, I.D. 0.53 mm, length 30 m, SGE Analytical Science, Australia) which is enable to separate the terpinene-4-ol within 12 min. Injection volume was 1.0 µl injected with auto injector (AOC – 20i, Shimadzu, Japan). Chromatography was performed using gradient temperature program controlled by software GC Solution®. The carrier gas was helium with a pressure of 75 kPa. Retention time and peak area were calculated by software GC Solution®. An injection port temperature was set at 220°C and FID detector temperature at

250°C. The oven temperature was programmed as follow: initial at 100°C for 3 min at the increasing rate of 35°C $min^{-1}$ to temperature of 150°C (2 min), and ending at 200°C (4 min).

**3.3.7.1 Preparation of terpinen-4-ol reference standard**

The stock reference standard solution was prepared by dissolving 0.1 ml (0.8513 g) of standard terpinen-4-ol in 100 ml of methanol and yielded 0.8513g/100ml.

**3.3.7.2 Sample preparation**

A 0.1 ml of microemulsion sample was weighted and dissolved in methanol and finally adjusted the volume to 10 ml.

**3.3.7.3 Validation of GC method**

(1) Specificity

The specificity was confirmed by injecting placebo with the same concentrations as those in the microemulsion formulations as well as reference standard solution of terpinen-4-ol. The peak of terpinen-4-ol at the specific retention time must be separated from other peaks.

(2) Range and linearity

For analysis of terpinen-4-ol content, the reference stock solution of terpinen-4-ol (3727 µg/ml) in methanol was prepared. The calibration curve was constructed with five concentrations ranging from 93.18 to 559.05 µg/ml. Calibration solutions for terpinen-4-ol were prepared by separately transferring the stock reference standard solution of 0.25, 0.50, 1.00, 1.25 and 1.50 ml to 10 ml volumetric flasks and diluted to the volume with methanol to furnish the concentration range of 93.18 – 559.05 µg/ml (covering 70 to 130 percent of finish product). The peak area of each concentration was the average of three determinations.

For skin permeation study, the reference stock solution of terpinen-4-ol in the methanol (902 µg/ml) was prepared. Calibration solutions of terpinen-4-ol were prepared by separately transferring the stock reference standard solution of 100, 250, 500, 750, 1,000 and 1,200 µl to 10-ml volumetric flasks and diluted to the volume with methanol to obtain the concentration range of

9.020 – 112.570 µg/ml. The peak area of each concentration was the average of three determinations.

The calibration curve was constructed by plotting the peak areas (y-axis) as a function of terpinen-4-ol concentrations (x-axis). The linear regression equation was then calculated.

(3) Accuracy

Accuracy of the analysis method indicated by the percent recovery was carried out by standard spiking method at three different concentration levels as 80%, 100% and 120%. According to the AOAC guideline, the limit on the percent recovery of terpinen-4-ol should be within the range of 85-110%. (39)

(4) Precision

Precision indicated by the percent relative standard deviation (RSD) was ascertained by determination of six replicate analysis of a fixed amount of known amount of analyte in microemulsion. The accepted value of the percent relative standard deviation should be not more than 2%.

(5) Detection limit

The detection limit was the lowest amount of the analyte in the sample that could be detected. Determination of the signal-to-noise ratio established the minimum concentration. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the detection limit.

(6) Quantitation limit

Determination of the signal-to-noise ratio established the minimum concentration at which the analyte can be reliably quantified. A signal-to-noise ratio was 10:1.

**3.3.8 Statistical analysis**

All experiments were carried out in triplicate. Data were expressed as the mean value ± standard deviation (S.D.). Statistical significance of difference was analyzed by one-way analysis of variance (ANOVA) using Minitab®17 software (free trial version). A *P* value of 0.05 was considered to be significant.

# CHAPTER 4
# RESULTS AND DISCUSSION

## 4.1 Determination of phase diagram construction

In this study we the phase diagram of Plai oil/Tween®80-absolute ethanol (1:1) was constructed by two methods: (i) titrating a mixture of two components with the third component and (ii) preparing a large number of samples of different compositions. It was found that the single phase regions constructed by methods (i) as shown in Diagram 1 and method (ii) as shown in Diagram 2 were identical. Diagram 3 showed the phase diagram constructed by such two methods in which their single phase areas were concomitant, suggesting that the studied system reached equilibrium rapidly. (1) Therefore, the titration method was used in the subsequent study.

**Diagram 1** The pseudoternary phase diagram of Plai oil/Tween®80-absolute ethanol(1:1)/ water system obtained from titration method.

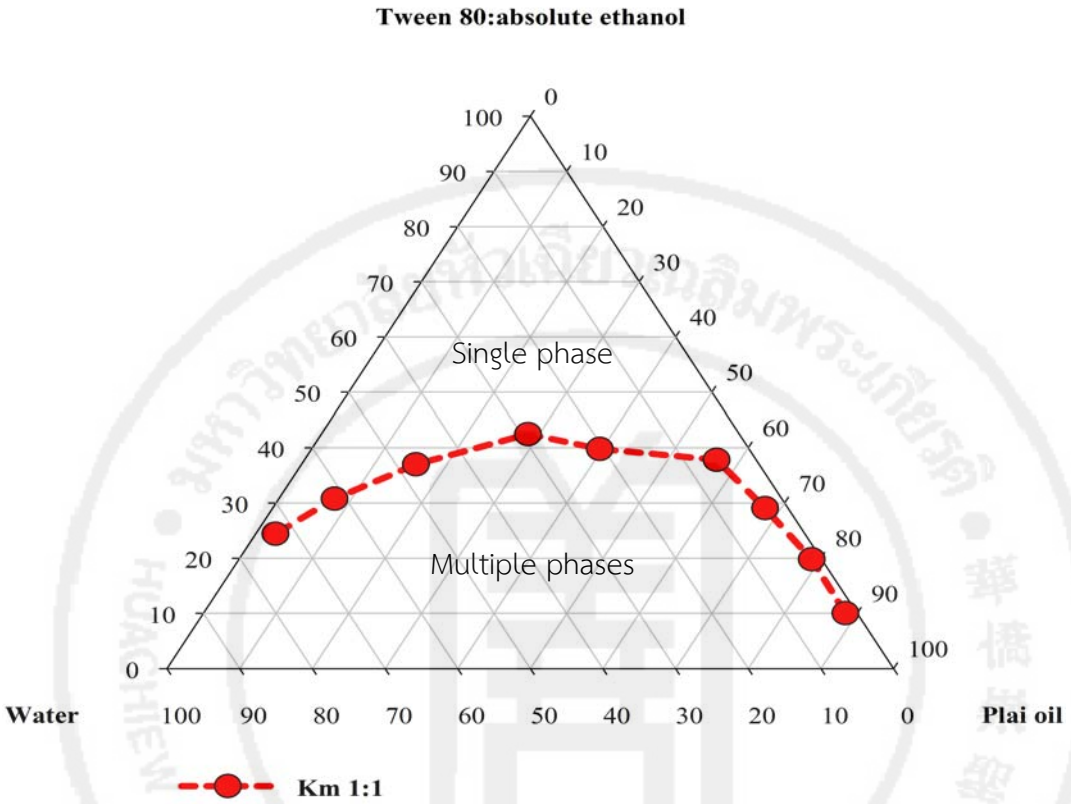


**Diagram 2** The pseudoternary phase diagram of Plai oil/Tween®80-absolute ethanol(1:1)/water system obtained from the preparing of a large number of samples of different compositions.

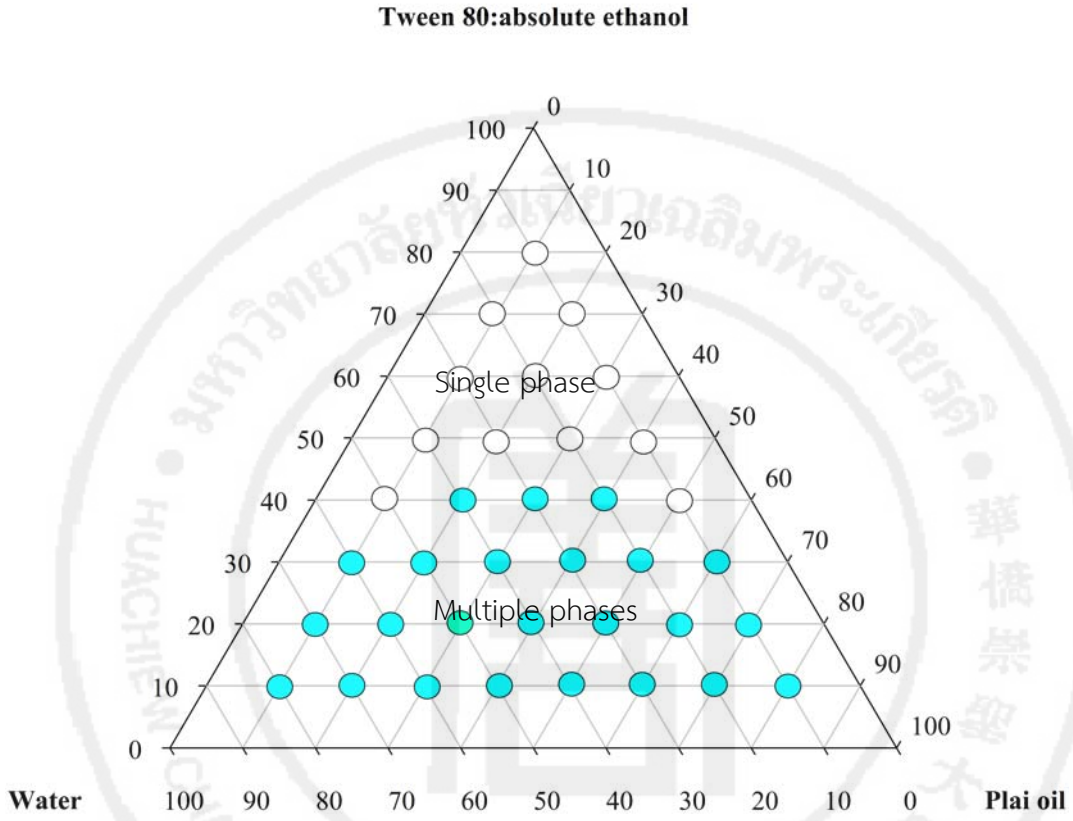
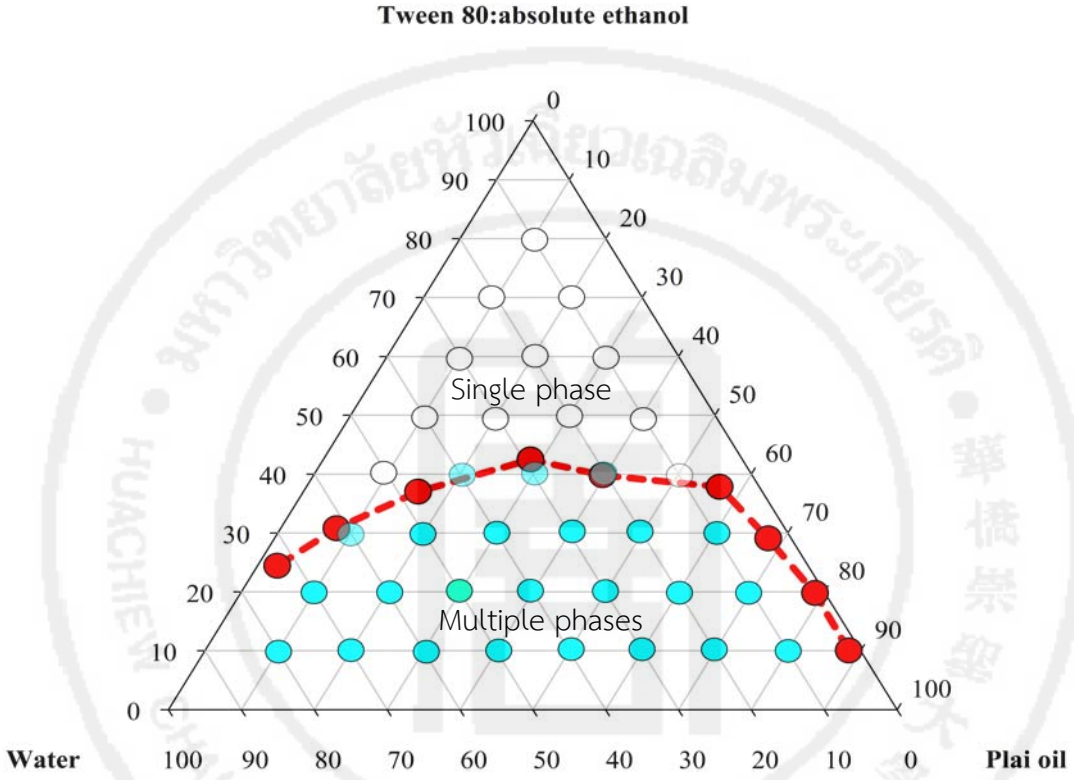


**Diagram 3** Comparison of the single phase areas on the pseudoternary phase diagram of Plai oil/Tween®80-absolute ethanol(1:1)/water system obtained from titration method (dotted line) and the preparing of a large number of samples of different compositions (filled circle).




## 4.2 Screening results of surfactants and cosurfactant weight ratio (Km)

The surfactants selected to investigate were Tween®80 and Kolliphor®RH40. Diagram 4 presented the pseudoternary phase diagram of the Plai oil/Tween®80-absolute ethanol/water system and Diagram 5 presented the pseudoternary phase diagram of the Plai oil/Kolliphor®RH40-absolute ethanol/water system. Each system was prepared at three different weight ratios of surfactant to cosurfactant. In comparison of the single phase area, it was found that the Km values at 1:1 and 2:1 of the system of Plai oil/Tween®80-absolute ethanol/water provided larger single phase areas than those of the system of the Plai oil/Kolliphor®RH40-absolute ethanol/water. This directly suggested that the system containing Tween®80 as surfactant could from microemulsion at the lower amount of surfactant to

cosurfactant. In contrast, the Km value at a 3:1 weight ratio of surfactant to cosurfactant of both systems produced the least single phase area. As a consequence, the systems containing Tween®80 as a surfactant and absolute ethanol as a cosurfactant at the Km value of 1:1 and 2:1 were considerately chosen for further investigation.

**Diagram 4** The pseudoternary phase diagram constructed from Plai oil/Tween®80-absolute ethanol/water at different Km values of 1:1, 2:1, and 3:1.

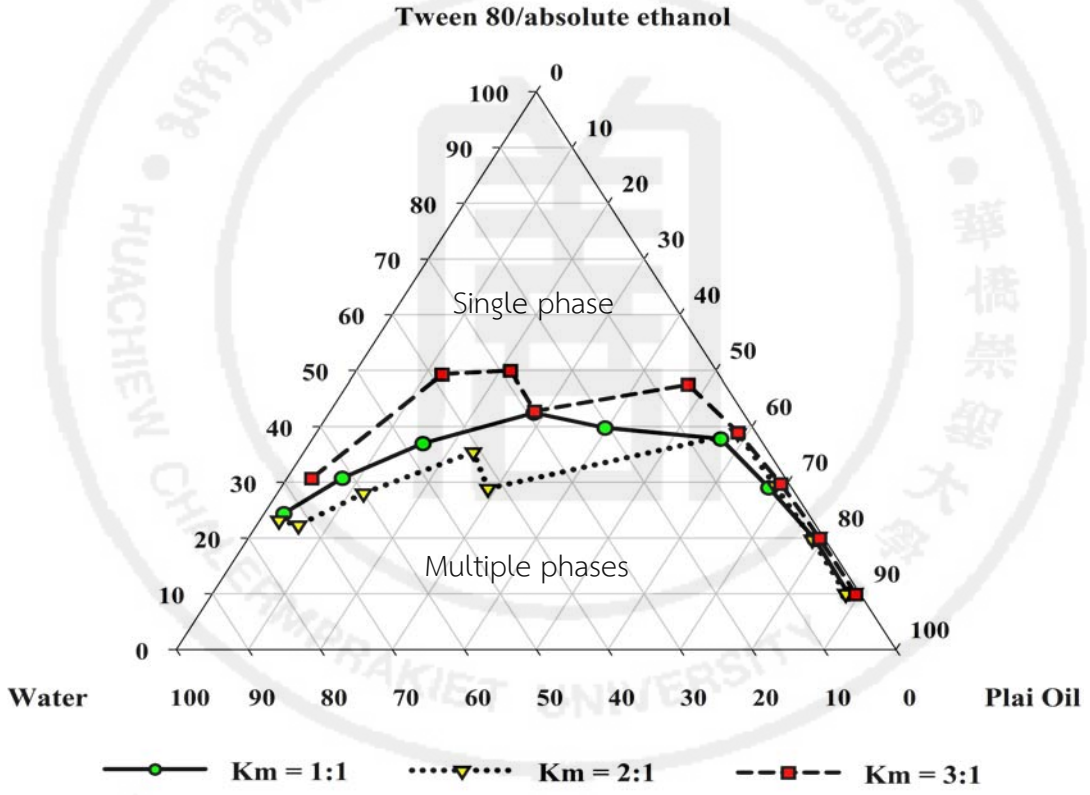
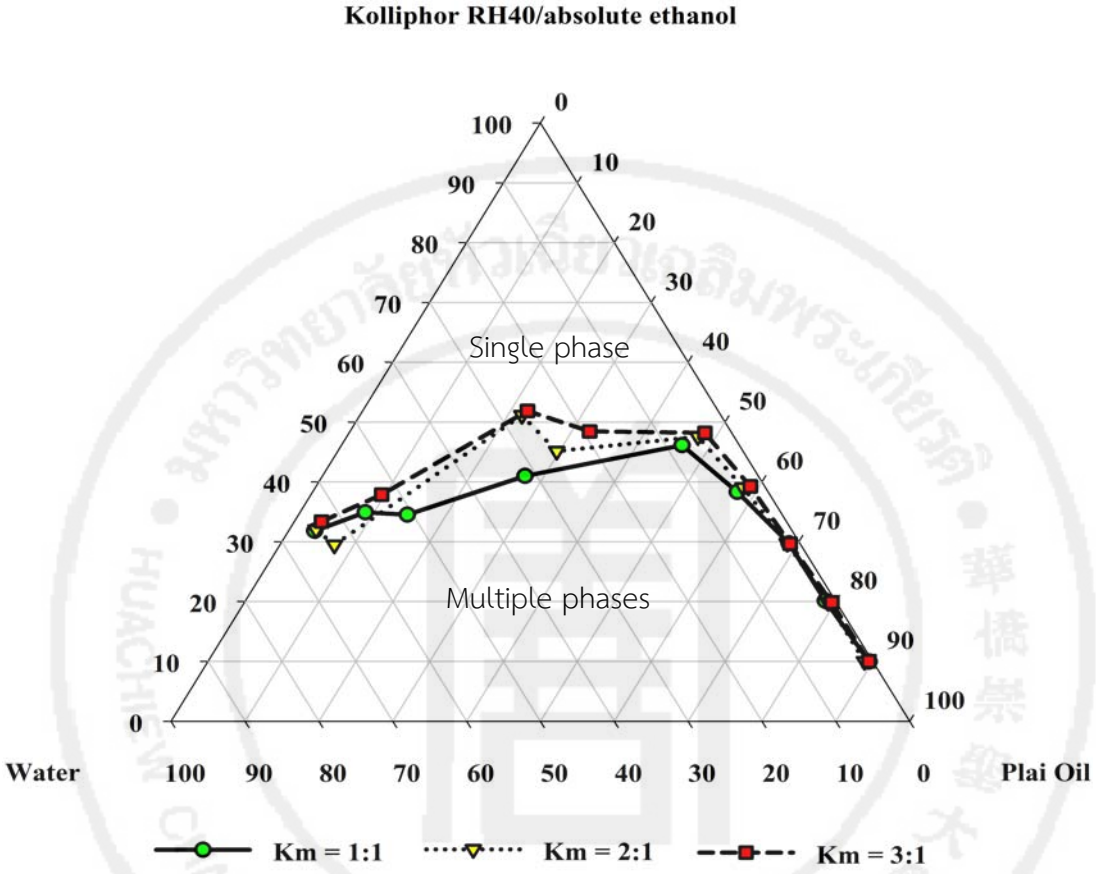


**Diagram 5** The pseudoternary phase diagram constructed from Plai oil/Kolliphor®RH40-absolute ethanol/water at different Km values of 1:1, 2:1, and 3:1.




## 4.3 Optimized Plai oil concentration as oil phase

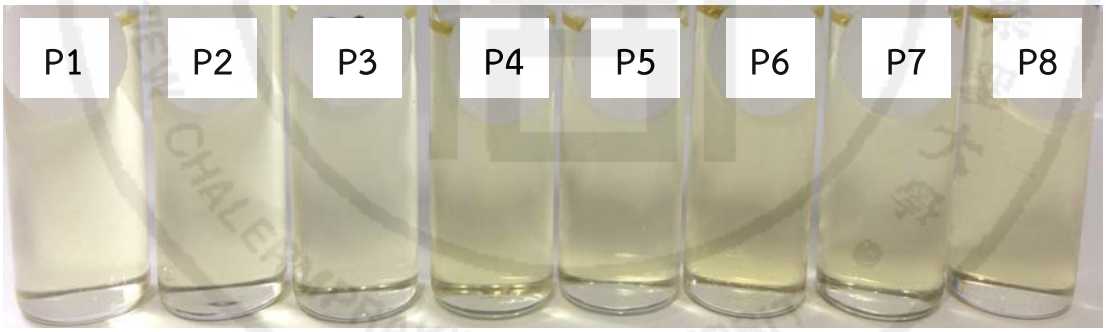
In accordance with the results of the single phase areas, the systems containing Tween®80 as surfactant and absolute ethanol as a cosurfactant at the Km values of 1:1 and 2:1 were selected to optimize the appropriate concentration of Plai oil which varied from 14% to 30% w/w. The concentration of the surfactant mixture of Tween®80 and absolute ethanol was kept constant at 50% w/w. Eight samples were prepared and fully evaluated for the microemulsion characteristics.

### 4.3.1 Characteristics of optimized samples

#### 4.3.1.1 Appearance

According to Table 1, formulations were prepared. The results showed that all samples were transparent homogenous yellowish liquid mixtures as shown in Figure 9. Samples P5-P8 were deeper yellowish than samples P1-P4 owing to the higher amount of Tween®80. In addition, the color of Plai oil is also yellow, thus samples P4 and P8 containing the highest concentration of Plai oil at 30% w/w appeared darker yellowish than samples P1 and P5 which contained only 14% w/w of Plai oil.

**Figure 9** The photograph of eight samples. Bottles P1-P4 had Km value at 1:1 and the concentrations of Plai oil were 14%, 20%, 25%, and 30%, respectively. Samples P5-P8 had Km value at 2:1 and the concentrations of Plai oil were 14%, 20%, 25%, and 30%, respectively.




#### 4.3.1.2 Polarized light microscopy

Figure 10 showed the photograph obtained from cross-polarized light microscope. The view of all samples presented dark field and no birefringence, indicating isotropic property. Isotropic characteristic is a typical property of microemulsion that can be investigated by this simple technique because naked eye cannot differentiate between various anisotropic liquid crystalline systems and isotropic microemulsion systems.

**Figure 10** An example of the photograph viewed under cross polarized light microscopy.

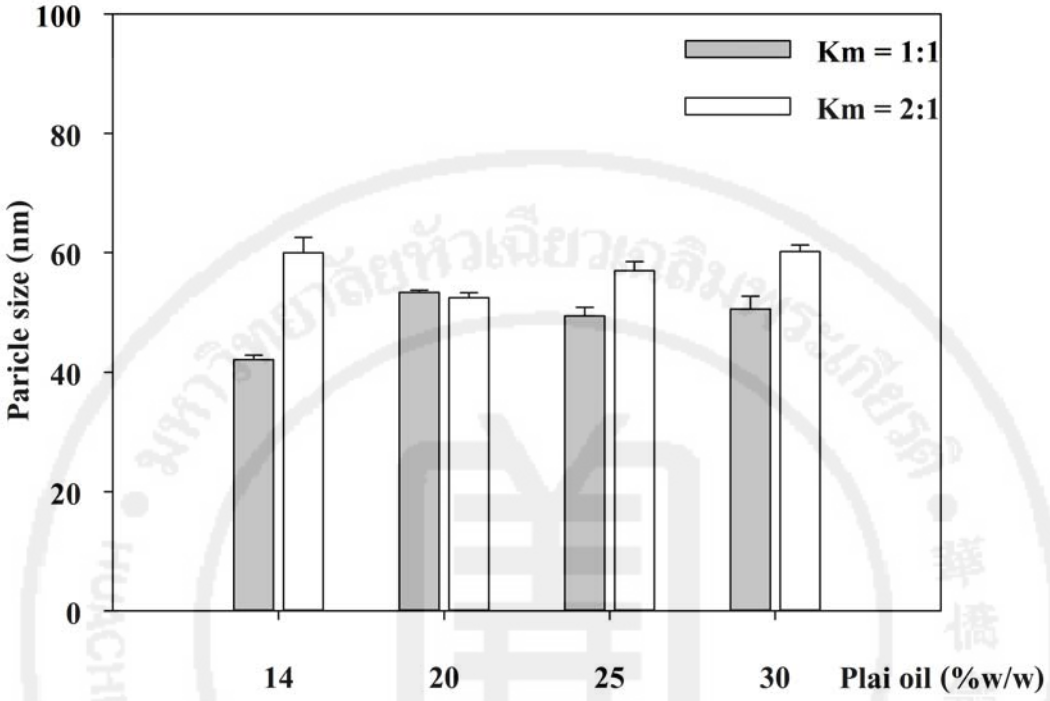
#### 4.3.1.3 Particle size and polydispersity index

The particle size of all formulations was determined. The average sizes were found in the range of 42.13-60.17 nm with polydispersity index (PI) values of 0.22 to 0.36 (Table 6). The particle size of all samples ranged from 10 to 100 nm; therefore they were nano-size particles. Polydispersity index is a measure of particle homogeneity and it varies from 0.0 to 1.0. (40) The closer to zero the homogenous are the particles. All samples showed narrow size distribution.

**Table 6** Particle size and polydispersity index of the optimized samples (mean±S.D., n=3)

| Formulation | Particle size (nm) | Polydispersity Index (PI) |
|---|---|---|
| P1 | 42.13±0.74 | 0.35±0.03 |
| P2 | 53.33±0.35 | 0.30±0.01 |
| P3 | 49.37±1.46 | 0.22±0.02 |
| P4 | 50.53±2.16 | 0.25±0.04 |
| P5 | 59.97±2.55 | 0.35±0.02 |
| P6 | 52.43±0.85 | 0.36±0.02 |
| P7 | 56.93±1.53 | 0.32±0.01 |
| P8 | 60.17±1.11 | 0.31±0.01 |

**Diagram 6** Particle sizes of all samples as a function of Plai oil concentrations (mean±S.D.; n=3).




#### 4.3.1.4 Physicochemical characteristics

Table 7 showed the physicochemical characteristics that were pH, conductivity, viscosity and correlation coefficient ($R_{xy}$) between shear rate (x) and shear stress (y) for determination of flow behavior.

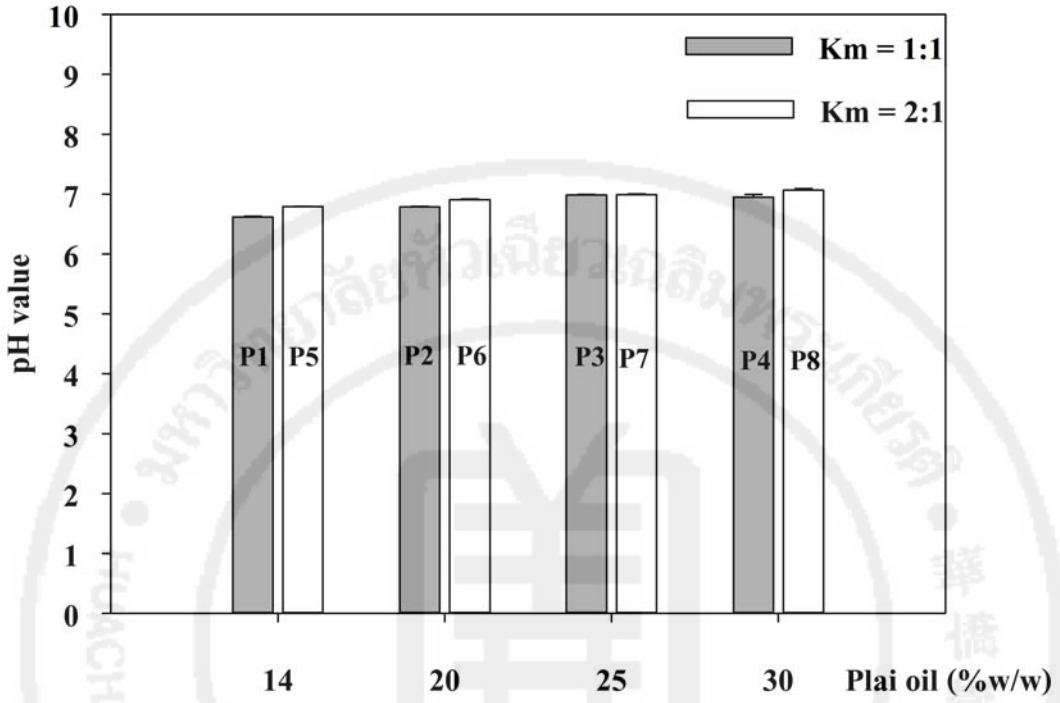
The pH values of all prepared samples were between 6.62 and 7.07 as shown in Table 7. It was found that the Km values and the concentrations of Plai oil had no influence on the pH of all samples as shown in Diagram 7.

**Table 7** The pH, conductivity, viscosity, and $R_{xy}$ values of all optimized samples (mean±S.D., n=3)

| Formulations | pH value | Conductivity (μS/cm) | Viscosity (cPs) | $R_{xy}$ |
|---|---|---|---|---|
| P1 | 6.62 ± 0.01 | 48.00 ± 0.10 | 33.37 ± 0.38 | 1.0000 |
| P2 | 6.79 ± 0.01 | 38.70 ± 0.00 | 19.53 ± 0.15 | 0.9936 |
| P3 | 6.99 ± 0.01 | 30.60 ± 0.00 | 16.67 ± 0.32 | 0.9999 |
| P4 | 6.95 ± 0.05 | 22.73 ± 0.06 | 14.57 ± 0.06 | 0.9999 |
| P5 | 6.79 ± 0.01 | 55.57 ± 0.06 | 63.60 ± 0.17 | 1.0000 |
| P6 | 6.91 ± 0.01 | 42.57 ± 0.06 | 45.30 ± 0.46 | 0.9987 |
| P7 | 6.99 ± 0.01 | 31.27 ± 0.06 | 36.23 ± 0.06 | 1.0000 |
| P8 | 7.07 ± 0.02 | 21.50 ± 0.00 | 30.00 ± 0.20 | 1.0000 |

$R_{xy}$ = correlation coefficient between shear rate (x) and shear stress (y)

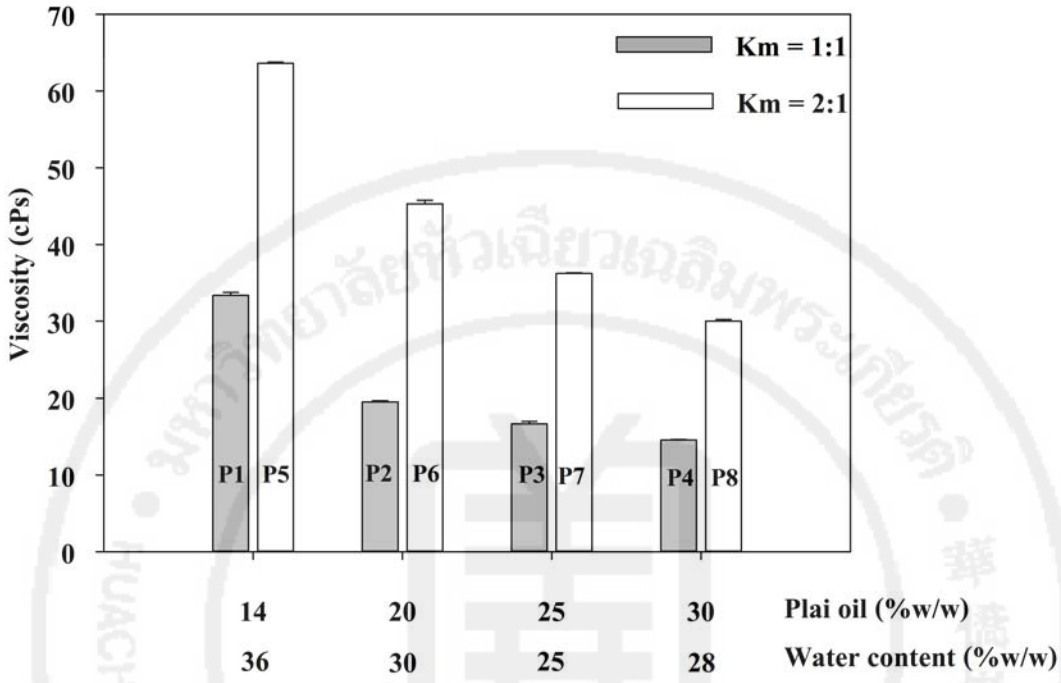
**Diagram 7** The pH value of all samples as a function of Plai oil concentrations (mean±S.D.; n=3).




For the viscosity values, it was found that the viscosity values of all samples were rather low. Diagram 8 illustrated the viscosity values as function of a percentage of Plai oil and water content.

The samples P5-P8 incorporating the surfactant to cosurfactant at Km value of 2:1 resulted in remarkably higher viscosity values compared to those at Km value of 1:1. Considering at Km value of 1:1, when the concentration of Plai oil increased from 14% to 30% w/w, the viscosity decreased from 33.37 to 14.57 cPs. In contrast, at the Km value of 2:1 the viscosity also decreased from 63.60 to 30.00 cPs with the increasing Plai oil concentrations and the decreasing of water content. It was noteworthy that the viscosity values decreased with the decrease of water content. The lower the water content, the less the interaction between the nonionic surfactant and water, resulting in the reduced viscosity value. (19) The results also showed that the amount of surfactant in this system clearly influenced the viscosity value because Tween® 80 appears as slightly viscous liquid.

**Diagram 8** Viscosity values of all samples as a function of Plai oil concentrations and water contents (mean±S.D.; n=3).

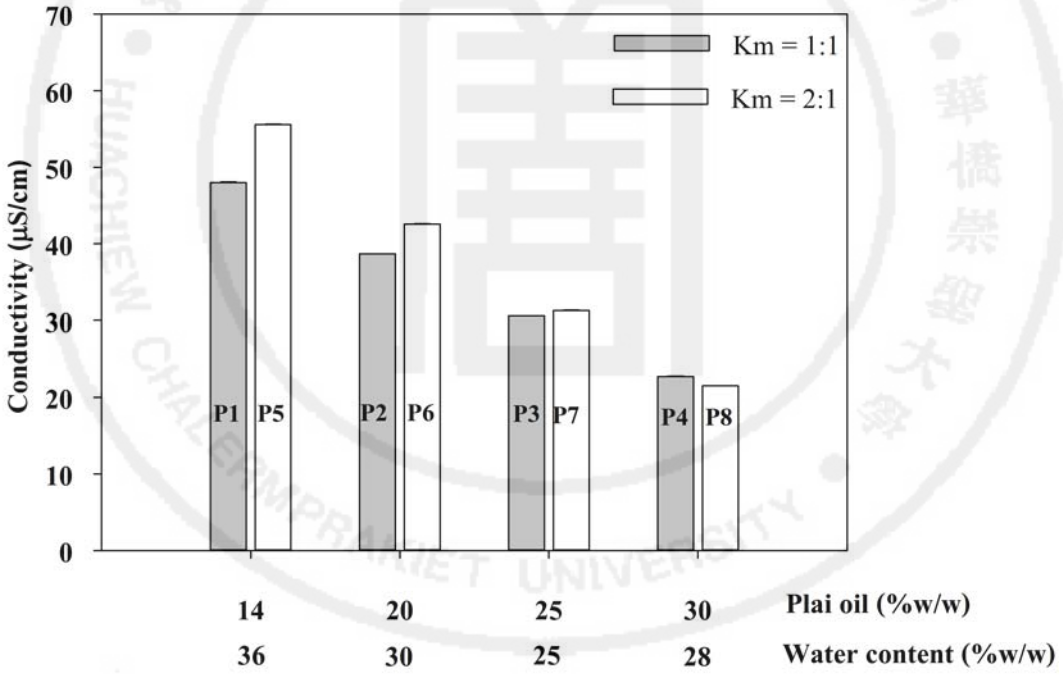



Rheological behavior of all samples was also determined by varying the speed of spindle from 10 to 50 rpm. Shear rate and shear stress were recorded and the correlation coefficients between shear rate and shear stress ($R_{xy}$) were calculated. As shown in Table 7, the $R_{xy}$ values ranged from 0.9936 to 1.0000 indicating that all the samples exhibited Newtonian flow. (41-42)

Conductivity measurement can be used to determine whether a microemulsion is oil-continuous or water-continuous and it is also used to monitor the phase inversion. The conductivity higher than 10 μS/cm always implies the formation of microemulsions with o/w type. (43-44) Diagram 9 showed the conductivity values of all prepared samples as function of Plai oil concentrations and water content. The conductivity values were in the range of 21.5-55.6 μS/cm as shown in Table 7. P1 and P5 showed the highest viscosity values. It was found that at higher water content, the increase in conductivity was observed. Above 30%w/w of water, systems prepared from the mixture of surfactant and cosurfactant at the Km

of 2:1 showed slightly higher conductivity values than those prepared at the Km of 1:1. A 30% w/w water content was a critical concentration point. Below 30% water content, the electrical conductivity of formulation having the surfactant mixture at a Km of 2:1 was comparable with that of formulation having the surfactant mixture at a Km of 1:1. This might be the result of high viscosity of the systems with high Km value. However, all samples had electrical conductivity higher than 10 μS/cm, indicating water as psuedoexternal phase.

**Diagram 9** Electrical conductivity value of all samples as a function of Plai oil concentrations and water contents (mean±S.D.; n=3).




In this study, the appropriate concentration of Plai oil was optimized. The microemulsion consisting 50% w/w of surfactant-cosurfactant mixture, the selected Km values, and Plai oil as oil phase which varied from 14% w/w to 30% w/w were successfully prepared and characterized. The resulting samples are oil in water microemulsions. The type of microemulsion was qualitatively determined by dilution test using water soluble dye. The results were in

good agreement with the conductivity values. All samples showed the microemulsion characteristics which had the small particle size and narrow size distribution, low viscosity with Newtonian flow behavior, and isotropic property. The pH of all samples was within the neutral range which was suitable for skin application.

From optimization study, the results indicated that all samples showed the microemulsion characteristics which had the small particle size and narrow size distribution, low viscosity with Newtonian flow behavior, and isotropic property. The pH of all samples was within the neutral range which was suitable for skin application. As a result, Plai oil at a concentration of 14% w/w was chosen to the further study because this lowest concentration also provided the acceptable microemulsion characteristic. In addition, the lower the Plai oil, the lower the cost.

### 4.4 Development of Plai oil microemulsion for transdermal delivery

In the previous section, Plai oil was successfully used as oil phase and could prepare microemulsions with excellent characteristics. Plai oil is not only as an oil phase but also has anti-inflammatory action. According to the National List of Essential Medicines, Plai oil is recommended for topical dosage forms at the concentration of 14% to 30% w/w. (45) In this study, Plai oil microemulsion was developed by fixing the concentration at 14% w/w. In order to synergist the anti-inflammatory effect of Plai oil, indomethacin was incorporated into the formulations prepared by using a $2^3$ full factorial design. The effect of the three factors including weight ratios (Km) of surfactant to cosurfactant, the concentration of the mixture of surfactant and cosurfactant and the presence of indomethacin on the physicochemical characteristics and the skin permeation of the obtained formulations was investigated.

### 4.5 Appearance and characteristics of Plai oil microemulsions

Eight formulations were prepared at the ambient temperature. All formulations were transparent, homogenous and yellowish. As shown in Figure 11, the formulations F3, F4, F7, and F8 containing indomethacin obviously appeared as deep yellow.

The photographs under cross-polarized light microscope showed dark field and no birefringence, indicating isotropic property. (2, 46) After centrifugation, no phase separation and drug precipitation were observed.

**Figure 11** The photograph of microemulsion formulations.

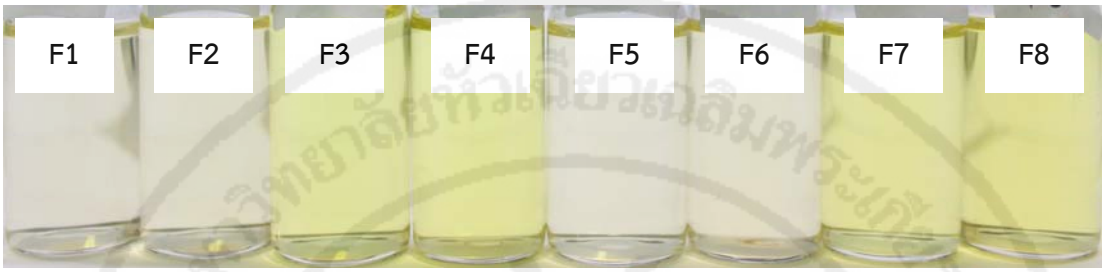



Table 8 showed the particle size, polydispersity index, viscosity, conductivity and pH value. The average particle sizes were found in the range of 44.57 to 92.70 nm and the polydispersity index values of all formulations ranged from 0.28 to 0.38. The results indicated that they were nano-size particles with the narrow size distribution.

Statistical of analysis of the data (Table 9-10) revealed that two factors including the Km value and the concentration of surfactant system ($X_1$ and $X_3$) significantly influenced on the particle size of microemulsions. When the Km value and the concentration of indomethacin were kept constant either at its low or high value, the particle size significantly decreased ($p<0.05$) with the increasing concentration of surfactant system from 45% to 50% w/w.

The viscosity of microemulsions was most influenced by the Km value. When the Km value changed from 1:1 to 2:1, the viscosity of microemulsion was significantly ($p<0.05$) higher about two times.

Considering conductivity, it was found that all formulations showed moderately high conductivity values. The conductivity value was significantly ($p<0.05$) increased when the Km value changed from 1:1 to 2:1. Although three factors significantly influenced the conductivity value, all formulations were of water as pseudoexternal phase.

The pH values of all formulations were between 5.45 and 6.68. This indicated that all formulations are appropriate for the transdermal application. From the regression equation the present of indomethacin reduced the pH value significantly ($p<0.05$), it was found that the presence of indomethacin was the most effective variable that significantly reduced the pH value.

Based on the obtained results mentioned before, all three factors matched to produce the acceptable microemulsions.

**Table 8** Experimental design and physicochemical properties of Plai oil microemulsions

| Run | Factors (code value) | | | Responses | | | | |
|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Particle size (nm) | Polydispersity Index | Viscosity (cPs) | Conductivity (μS/cm) | pH value |
| F1 | 1:1 | 0.00 | 45 | 52.43±0.40 | 0.37±0.02 | 38.70±0.12 | 62.03±0.23 | 6.43±0.01 |
| F2 | 2:1 | 0.00 | 45 | 88.73±4.50 | 0.38±0.01 | 92.50±0.15 | 78.27±0.06 | 6.52±0.02 |
| F3 | 1:1 | 0.75 | 45 | 54.67±0.47 | 0.34±0.00 | 32.20±0.15 | 57.30±0.10 | 5.45±0.01 |
| F4 | 2:1 | 0.75 | 45 | 92.70±1.78 | 0.38±0.03 | 81.70±0.25 | 68.70±0.10 | 5.52±0.01 |
| F5 | 1:1 | 0.00 | 50 | 44.57±2.14 | 0.33±0.02 | 25.00±0.00 | 55.70±0.00 | 6.59±0.01 |
| F6 | 2:1 | 0.00 | 50 | 74.37±3.76 | 0.28±0.02 | 61.60±0.30 | 65.73±0.21 | 6.68±0.01 |
| F7 | 1:1 | 0.75 | 50 | 46.57±1.86 | 0.31±0.02 | 24.20±0.12 | 51.33±0.06 | 5.62±0.01 |
| F8 | 2:1 | 0.75 | 50 | 70.20±4.67 | 0.31±0.01 | 59.90±0.12 | 59.00±0.17 | 5.76±0.01 |

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

**Table 9** Statistical probability value at a 95% confidence

| Source | *P*-Value | | | |
|---|---|---|---|---|
| | **Particle size** | **Viscosity** | **Conductivity** | **pH value** |
| $X_1$ | 0.000 | 0.000 | 0.000 | 0.000 |
| $X_2$ | 0.186 | 0.000 | 0.000 | 0.000 |
| $X_3$ | 0.000 | 0.000 | 0.000 | 0.000 |
| $X_1X_2$ | 0.162 | 0.000 | 0.000 | 0.039 |
| $X_1X_3$ | 0.000 | 0.000 | 0.000 | 0.000 |
| $X_2X_3$ | 0.218 | 0.000 | 0.000 | 0.000 |
| $X_1X_2X_3$ | 0.042 | 0.000 | 0.000 | 0.001 |

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

**Table 10** Summary of the regression equations

| Responses | Regression equation |
|---|---|
| Particle size | $65.829 + \mathbf{15.671X_1} - \mathbf{6.304X_3} - 2.912X_1X_3 - 1.288X_1X_2X_3$ |
| Viscosity | $51.9917 + \mathbf{21.9417X_1} - 2.4750X_2 - \mathbf{9.3000X_3} - 0.6583X_1X_2 - 3.8667X_1X_3 + 1.8667X_2X_3 + 0.4333X_1X_2X_3$ |
| Conductivity | $62.2583 + \mathbf{5.6667X_1} - 3.1750X_2 - 4.3167X_3 - 0.9000X_1X_2 - 1.2417X_1X_3 + 0.4000X_2X_3 + 0.3083X_1X_2X_3$ |
| pH value | $6.07042 + 0.04625X_1 - \mathbf{0.48625X_2} + 0.09042X_3 + 0.00458X_1X_2 + 0.00958X_1X_3 + 0.01042X_2X_3 + 0.00792X_1X_2X_3$ |

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

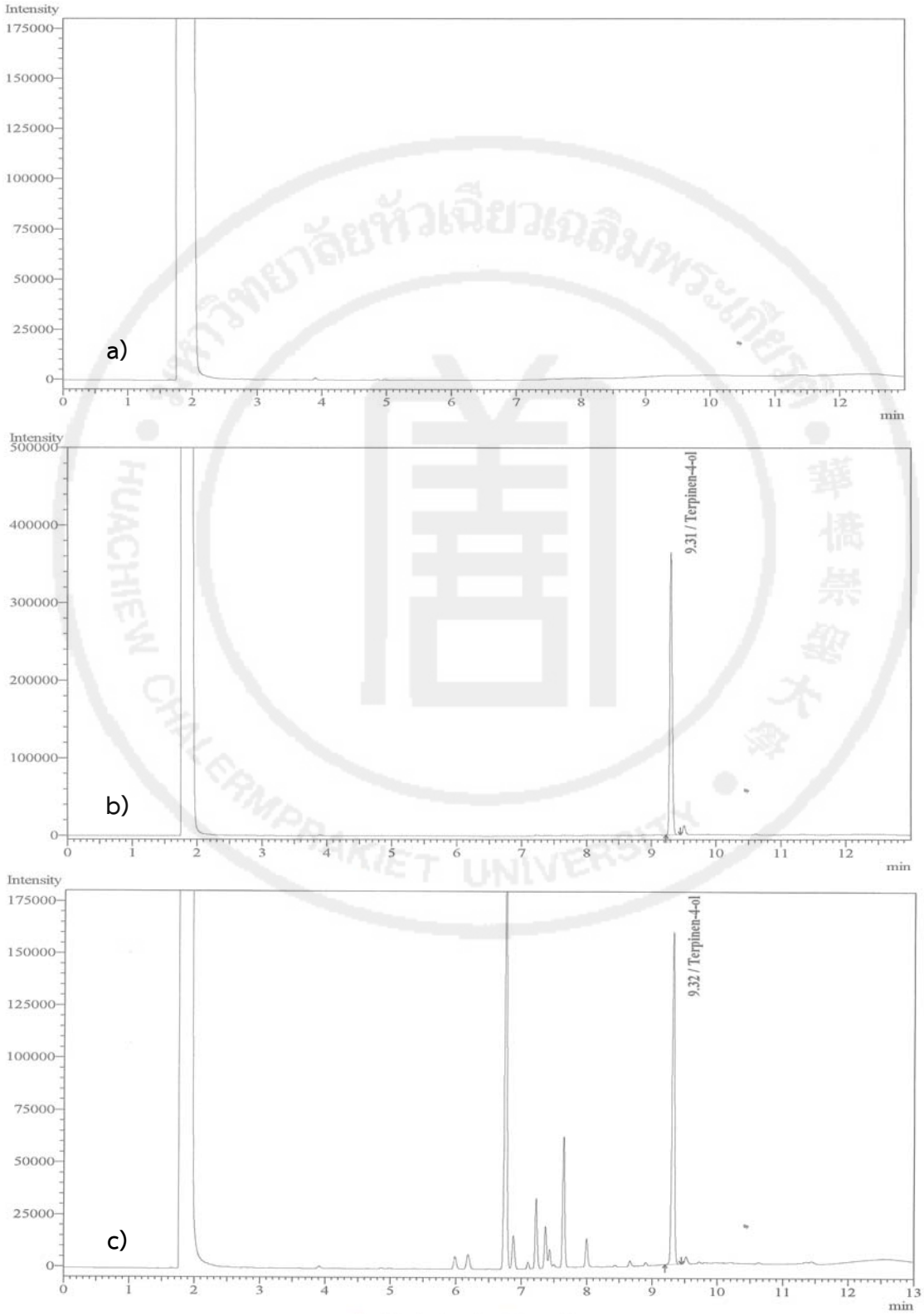
### 4.6 Validation of the chromatographic method: GC

The GC method was used to quantify the amount of terpinen-4-ol in the Plai oil microemulsions and in the terpinen-4-ol permeation study.

### 4.6.1 Specificity

The specificity of this method was demonstrated by the representative chromatograms as shown in Figure 12 of standard terpinen-4-ol solution and terpinen-4-ol in microemulsion samples. The retention time of terpinen-4-ol was about 9.31 min. No other peaks were observed at the retention time of tepinen-4-ol, indicating that interfering substances were absence.

**Figure 12** The GC-FID chromatogram for terpinen-4-ol assay: a); Placebo b); terpinen-4-ol solution standard (465.58 µg/ml) c); terpinen-4-ol in microemulsion formulation.



### 4.6.2 Linearity and range

The linearity of the method was carried out at five concentrations of standard terpinen-4-ol solution ranging from 93.18-559.05 µg/ml for analysis of terpinen-4-ol content and five concentrations ranging from 9.02-90.02 µg/ml for terpinen-4-ol permeation study. From a linear regression analysis, the results were shown in Table 11.

**Table 11** Linearity for the analysis of of terpinen-4-ol by GC method

| Types of assessment | Linear regression equation | Correlation coefficient (r) |
|---|---|---|
| terpinen-4-ol content | Y = 2143.403X – 45447.45 | 0.9997 |
| terpinen-4-ol permeation | Y = 1203.867X – 2263.11 | 0.9995 |

### 4.6.3 Accuracy

The accuracy of the method was determined by analyzing samples with the known amount of the terpinen-4-ol standard solution. An acceptance criterion for accuracy is that the %recovery will be in the range of 85-110%. (39) The mean recovery of terpinen-4-ol for assay of terpinen-4-ol content and terpinen-4-ol permeation study according to Table 12 was found to be 100.86% and 103.35%, respectively. It was evident that the %recovery was within the acceptance criteria.

### 4.6.4 Precision

The precision of the method is expressed as the percent relative standard deviation (%RSD). The precision was examined by analyzing six samples of terpinen-4-ol standard solution (n=6). An acceptance criterion for precision is that the %RSD will be not more than 2.0%. The %RSD values for assay of terpinen-4-ol content and terpinen-4-ol permeation study were 1.98% and 0.30%, respectively. The data were summarized in Table 13.

**Table 12** Accuracy of GC method for assay of terpinen-4-ol

| terpinen-4-ol content | | terpinen-4-ol permeation study | |
|---|---|---|---|
| **Concentration level spiked (terpinen-4-ol, µg/ml)** | **%Recovery (n=3)** | **Concentration level spiked (terpinen-4-ol, µg/ml)** | **%Recovery (n=3)** |
| 465.58 | 99.49±0.40 | 9.02 | 112.16±1.27 |
| 372.7 | 99.30±1.41 | 45.1 | 96.86±0.29 |
| 186.35 | 103.76±0.78 | 90.02 | 101.02±0.27 |
| mean | 100.86±0.86 | mean | 103.35±0.61 |

**Table 13** The repeatability of terpinen-4-ol assay obtained from six replicate injections of terpinen-4-ol standard solution at the concentration of terpinen-4-ol 213.21 µg/ml for assay of terpinen-4-ol content and 45.1 µg/ml for permeation study

| **Injection number** | **Terpinen-4-ol content** | | **Terpinen-4-ol permeation study** | |
|---|---|---|---|---|
| | **retention time (min)** | **Peak area** | **retention time (min)** | **Peak area** |
| 1 | 9.319 | 412282 | 9.320 | 50302 |
| 2 | 9.319 | 407532 | 9.320 | 50180 |
| 3 | 9.319 | 410952 | 9.320 | 50492 |
| 4 | 9.319 | 419099 | 9.321 | 50220 |
| 5 | 9.319 | 398478 | 9.321 | 50560 |
| 6 | 9.319 | 420959 | 9.320 | 50354 |
| mean±SD | 9.319±0.00 | 411550±8167 | 9.320±0.00 | 50351.33±149.92 |
| %RSD | 0.00 | 1.98 | 0.00 | 0.30 |

#### 4.6.5 Limits of detection and quantitation

The limit of detection (LOD) is defined as the lowest amount of analyte that can be detected above baseline noise (S/N =3:1). The limit of quantitation (LOQ) is defined as the lowest amount of analyte which can be quantitated above baseline noise that gives S/N =10:1. In the study, the LOD and LOQ for assay of terpinen-4-ol

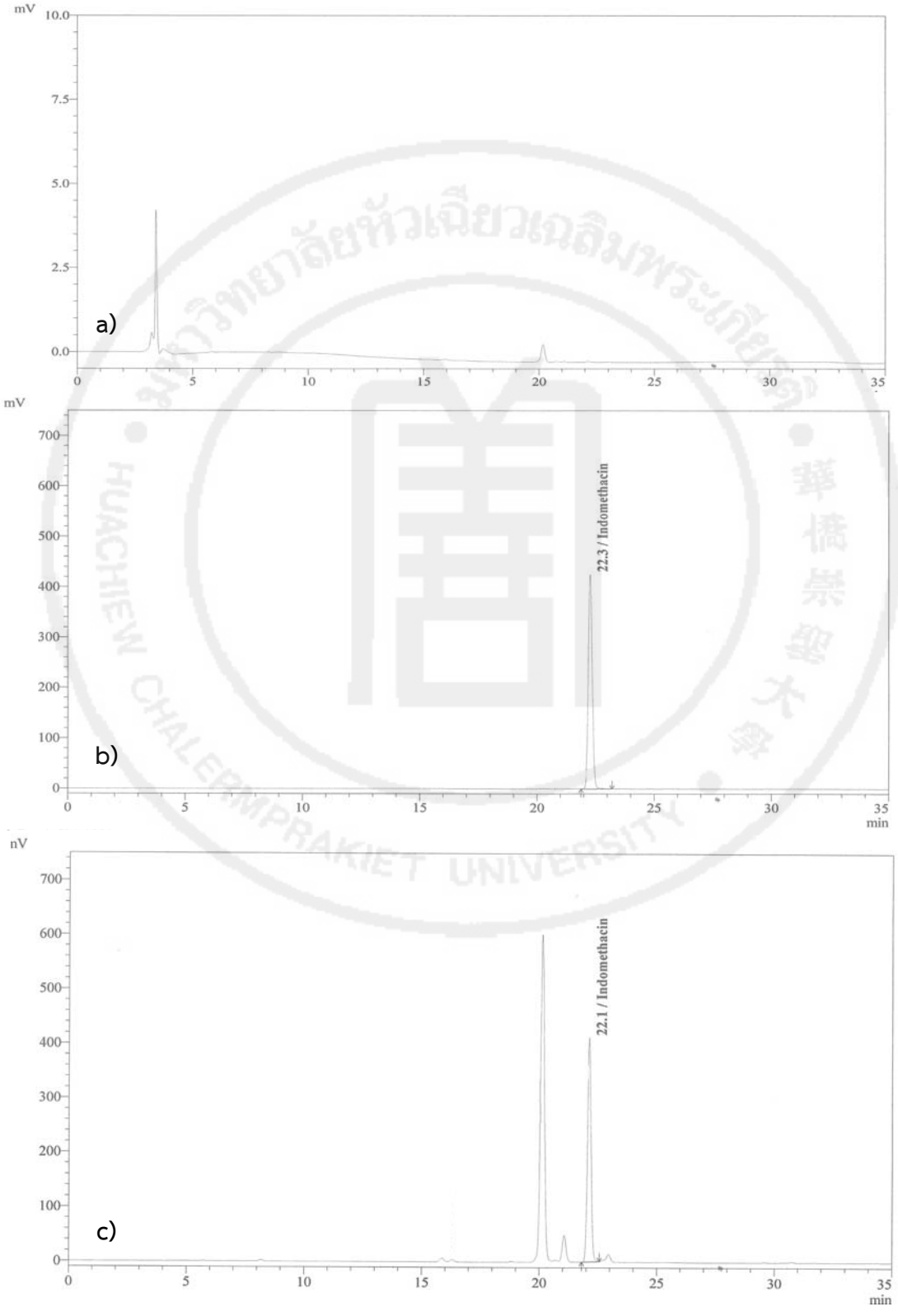
content was 16.27 µg/ml and 49.30 µg/ml, respectively. The LOD and LOQ for the permeation study was 3.31 µg/ml and 10.03 µg/ml, respectively.

## 4.7 Validation of the chromatographic method: HPLC

### 4.7.1 Specificity

The specificity of the HPLC method was confirmed by injecting placebo as well as standard solution as shown in Figure 13. The peak of indomethacin appeared at the retention time about 22 min. No other peaks were observed at the retention times of indomethacin, indicating that interfering substances were not present.

**Figure 13** The HPLC-UV chromatogram for indomethacin assay: a); Placebo b); indomethacin standard solution (150 µg/ml) c); indomethacin in microemulsions.



### 4.7.2 Linearity and range

The linearity of the method was carried out at five concentrations of standard indomethacin solution ranging from 25.0-200.0 µg/ml for analysis of indomethacin content and at six concentrations ranging from 0.20-1.20 µg/ml for indomethacin permeation study. From a linear regression analysis, the results were shown in Table 14.

**Table 14** Linearity for the analysis of indomethacin by HPLC method

| Types of assessment | Linear regression equation | Correlation coefficient (r) |
|---|---|---|
| indomethacin content | Y = 31533.81X – 163626.3 | 0.9984 |
| indomethacin permeation | Y = 34099.31X – 925.78 | 0.9979 |

### 4.7.3 Accuracy

The accuracy of the method was determined by analyzing the three concentrations of samples with the known amount of the indomethacin standard solution. An acceptance criterion for accuracy is that the %recovery will be in the range of 90-108%. (39) The mean recovery of indomethacin for assay of indomethacin content and indomethacin permeation study was found to be 96.63% and 102.48%, respectively, as shown in Table 15. It was evident that the %recovery was within the acceptance criteria.

### 4.7.4 Precision

The precision of the method is expressed as the percent relative standard deviation (%RSD). The precision was ascertained by determination of six replicate analysis of a fixed amount of indomethacin. An acceptance criteria for precision is that the %RSD will be not more than 2.0%. The %RSD values for assay of indomethacin content and indomethacin permeation study were 1.46% and 1.53%, respectively. The data was summarized in Table 16.

**Table 15** Accuracy of HPLC method for assay of indomethacin

| Indomethacin content | | Indomethacin permeation study | |
|---|---|---|---|
| Concentration level spiked (indomethacin, µg/ml) | %Recovery (n=3) | Concentration level spiked (indomethacin, µg/ml) | %Recovery (n=3) |
| 180 | 95.93±1.17 | 0.4 | 103.92±3.39 |
| 150 | 96.77±1.47 | 0.8 | 103.63±1.35 |
| 120 | 97.18±0.32 | 1.2 | 99.89±0.43 |
| mean | 96.63±0.99 | mean | 102.48±1.72 |

**Table 16** The repeatability data of indomethacin assay obtained from six replicate injections of indomethacin standard solution at the concentration of 150 µg/ml for assay of indomethacin content and of 0.6 µg/ml for permeation study

| Injection number | Indomethacin content | | Indomethacin permeation study | |
|---|---|---|---|---|
| | retention time (min) | Peak area | retention time (min) | Peak area |
| 1 | 22.45 | 4392595 | 22.07 | 18635 |
| 2 | 22.51 | 4435182 | 22.18 | 18506 |
| 3 | 22.50 | 4359998 | 22.18 | 18417 |
| 4 | 22.50 | 4532536 | 22.11 | 18721 |
| 5 | 22.54 | 4362745 | 22.25 | 18018 |
| 6 | 22.51 | 4435182 | 21.91 | 18809 |
| mean±SD | 22.50±0.03 | 4419706±64427.07 | 22.12±0.12 | 18517.67±282.82 |
| %RSD | 0.13 | 1.46 | 0.53 | 1.53 |

### 4.7.5 Limits of detection and quantitation

The limit of detection (LOD) is defined as the lowest amount of analyte that can be detected above baseline noise S/N =3:1. The limit of quantitation (LOQ) is defined as the lowest amount of analyte which can be quantitated above baseline noise that gives S/N =10:1. In this study, the LOD and LOQ for assay of indomethacin

content were 13.77 and 41.72 µg/ml, respectively while the LOD and LOQ for indomethacin permeation study were 0.08 and 0.25 µg/ml, respectively.

From validation data, the GC and HPLC used in this study showed good linearity, sensitivity, accuracy and precision. It suggested that the GC and HPLC were the appropriate instruments for quantity assay of terpinen-4-ol and indomethacin, respectively.

**4.8 Drug permeation**

The ability of microemulsions to increase skin permeation of active ingredient can be accounted by individual component such as surfactant type, cosurfactant type, Km value, components of oil phase and other incorporated substances. (3, 5, 7-8, 12-15, 21)

In the preliminary study, many fluid systems including phosphate buffer pH 7.4, phosphate buffer pH 7.4-PEG (1:1), and phosphate buffer pH 7.4-absolute ethanol (1:1) were investigated as the receptor fluid. It was found that when the phosphate buffer pH 7.4 and the phosphate buffer pH 7.4-PEG (1:1) were used as the receptor fluid, the permeated amounts of investigated substances were too low to detect by HPLC and GC. The phosphate buffer pH 7.4-absolute ethanol (1:1) was the optimum system and, therefore, used as a receptor fluid throughout this *in vitro* permeation study.

In this study the skin permeation of eight formulations obtained by $2^3$ full factorial design was investigated using Franz cell and shed snake skin of *Naja kaouthia* as a membrane. The three factors subjected to investigation were the Km value ($X_1$), the presence of 0.75% indomethacin ($X_2$) and the concentration of the mixture of surfactant and cosurfactant ($X_3$). Indomethacin was incorporated in the formulations for the synergistic effect on pain relief. Indomethacin might affect the permeation of terpinen-4-ol. Therefore, indomethacin was selected as one of three factors in this study. Both terpinen-4-ol and indomethacin skin permeation profiles were constructed. Multiple linear regression and ANOVA were performed to analyze the data.

Diagram 10 and Diagram 11 showed the permeation profiles of indomethacin and terpinen-4-ol, respectively. The cumulative amount of terpinen-4-ol and indomethacin was shown in Table 17. The cumulative amount of the terpinen-4-ol permeation could be ranked in the following descending order: F1> F5 > F2 > F3 > F4 > F7 > F6 > F8. The cumulative amount of indomethacin permeation was in the following descending order: F3 > F7 > F4 > F8. Fluxes and lag time of terpinen-4-ol and indomethacin were calculated and shown in Table 17.

The permeation rate or flux of the permeant was calculated from the slope of linear portion of the cumulative amount of permeant permeated through the membrane per unit area versus time plot.

**Diagram 10** Permeation profiles of terpinen-4-ol from Plai oil microemulsions (mean ± SD; n=3).

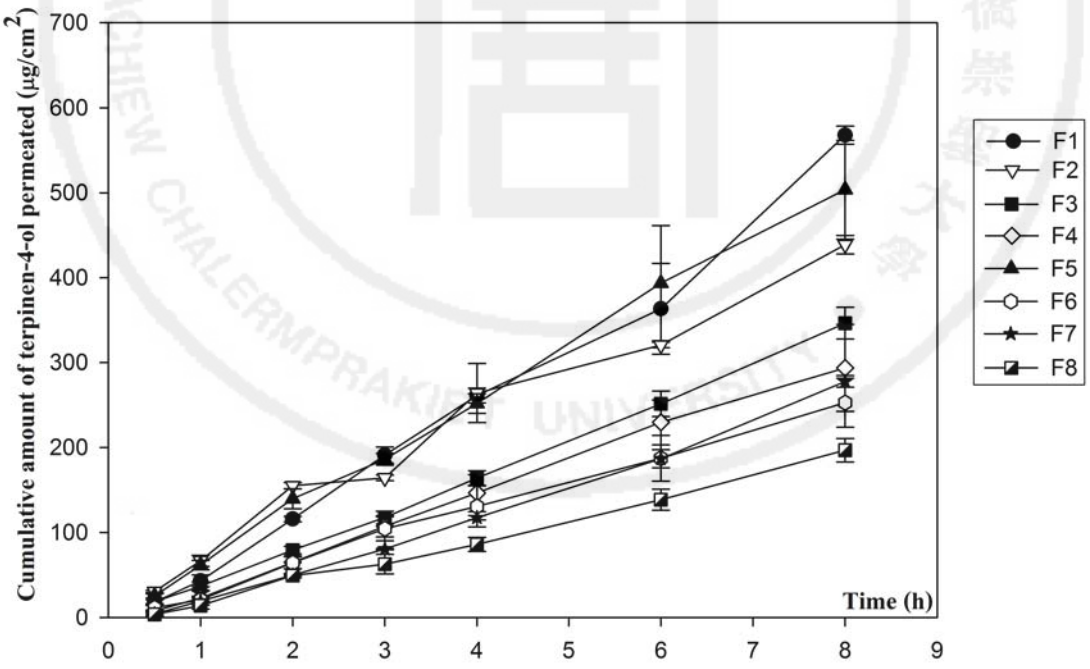


**Diagram 11** Permeation profiles of indomethacin from Plai oil microemulsions (mean ± SD; n=3).

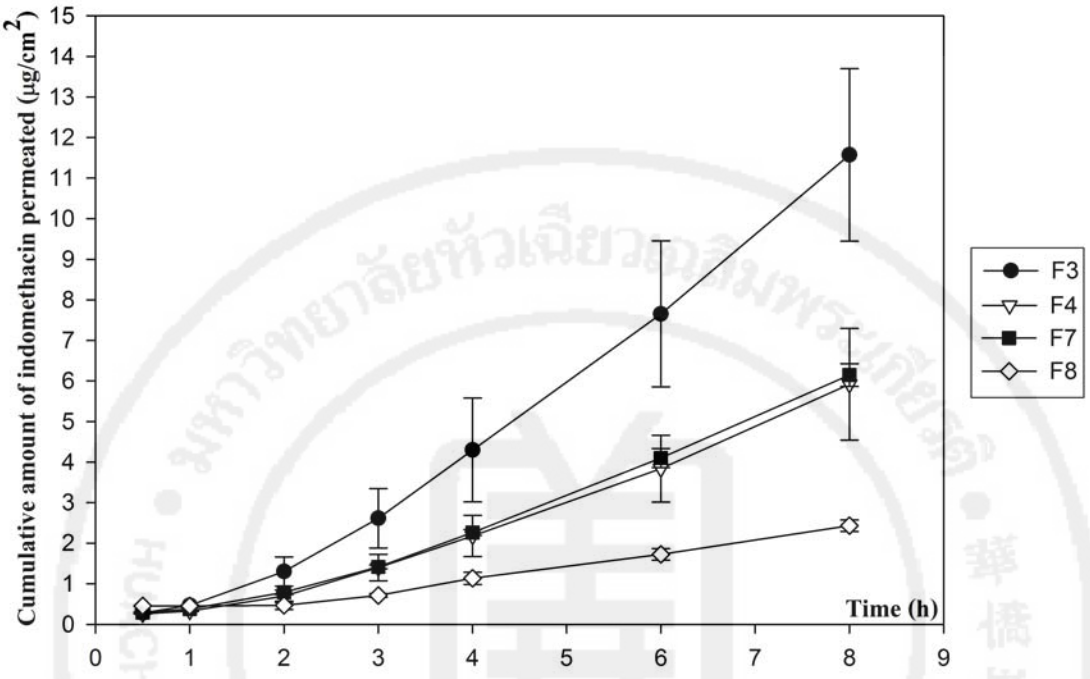


**Table 17** Permeation data of terpinen-4-ol and indomethacin from Plai oil microemulsions

| Formulations | Factors (code value) | | | Terpinen-4-ol | | | Indomethacin | | |
|---|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Cumulative amount (μg/cm$^2$) | Flux (μg/cm$^2$/h) | Lag time (h) | Cumulative amount (μg/cm$^2$) | Flux (μg/cm$^2$/h) | Lag time (h) |
| F1 | 1:1 | 0.00 | 45 | 567.81±10.65 | 72.90±3.56 | 0.40±0.13 | NA | NA | NA |
| F2 | 2:1 | 0.00 | 45 | 438.87±10.90 | 83.67±2.72 | 0.16±0.04 | NA | NA | NA |
| F3 | 1:1 | 0.75 | 45 | 346.52±18.67 | 45.62±2.61 | 0.43±0.08 | 11.57±2.13 | 1.82±0.21 | 1.69±0.48 |
| F4 | 2:1 | 0.75 | 45 | 293.75±51.22 | 41.30±5.49 | 0.43±0.14 | 5.92±1.38 | 0.93±0.22 | 1.74±0.23 |
| F5 | 1:1 | 0.00 | 50 | 503.63±58.19 | 63.83±2.22 | 0.02±0.04 | NA | NA | NA |
| F6 | 2:1 | 0.00 | 50 | 252.75±28.88 | 36.95±5.68 | 0.32±0.18 | NA | NA | NA |
| F7 | 1:1 | 0.75 | 50 | 277.75±6.87 | 32.53±0.92 | 0.45±0.05 | 6.14±0.28 | 0.97±0.06 | 1.70±0.07 |
| F8 | 2:1 | 0.75 | 50 | 196.90±13.79 | 27.73±1.44 | 0.94±0.18 | 2.43±0.14 | 0.32±0.00 | 0.56±0.49 |

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

NA = no assessment

For the permeation flux, the permeability of terpinen-4-ol of the formulations ranked in order from largest to smallest as F2> F1> F5> F3> F4> F6> F7> F8. The highest permeation flux was from formulation F2 which contained 45% the mixture surfactant and cosufactant at 2:1 weight ratio of surfactant to cosufactant without indomethacin. This might be as a result of both the appropriate components and the concentrations of components in the formulation F2. The lowest permeation flux was from formulation F8 which contained 50% the mixture of surfactant and cosurfactant at a 2:1 weight ratio of surfactant to cosufactant and 0.75% w/w of indomethacin. It should be noted that the first three running formulations were without indomethacin. When the Km value and the concentration of the surfactant mixture were kept constant, the presence of indomethacin in the formulations substantially reduced the flux of terpinen-4-ol.

Taking the concentration of the surfactant mixture in the microemulsion formulation into consideration, it was found that an increase in the concentration from 45% to 50% decreased not only the permeation flux of terpinen-4-ol in the presence and the absence of indomethacin, but also the permeation of indomethacin. This might be as a result of an increase in the thermodynamic activity as a driving force of permeants at a lower concentration of the surfactant mixture. (23, 47)

Considering formulations F1 and F2, their higher flux of terpinen-4-ol might be in part as a result of a higher water content (41%). The water content must be taken into consideration. Generally, water is involved in the hydration of the stratum corneum to a certain extent. Water hydrates the polar pathway that increases the interlamellar volume of the lipid bilayers and results in the disruption of the structure. In the same way, water may also swell the intercellular proteins and disturbs the lipid bilayers thus the lipophilic substance can then permeate more easily through the paracellular pathway. (48-49)

The effect of formulation variables on the fluxes of terpinen-4-ol was evaluated and shown in Table 18. It was found that all three factors significantly affected ($p< 0.05$) the flux of terpinen-4-ol from microemulsion. As was seen clearly in Figure 14, the

pareto chat showed the standardized main effect for the terpinen-4-ol flux. Indomethacin was the most interference with the permeation of the Plai oil component.

**Table 18** Analysis of variance for different variables on the permeation flux of terpinen-4-ol

| Source | *P*-Value |
|---|---|
| $X_1$ | 0.000 |
| $X_2$ | 0.000 |
| $X_3$ | 0.000 |
| $X_1X_2$ | 0.237 |
| $X_1X_3$ | 0.000 |
| $X_2X_3$ | 0.000 |
| $X_1X_2X_3$ | 0.000 |

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

**Figure 14** The Pareto chart showing the effects of Km value, the presence of indomethacin, and the concentration of the mixture of surfactant and cosurfactant on the flux of terpinen-4-ol.

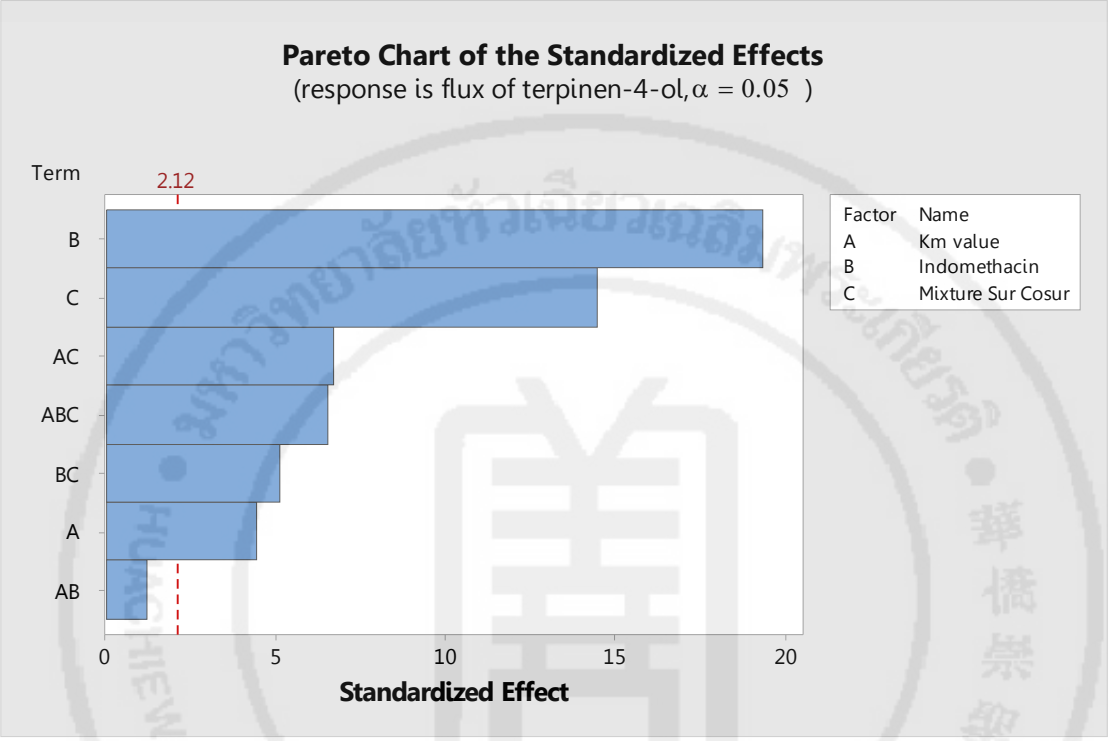
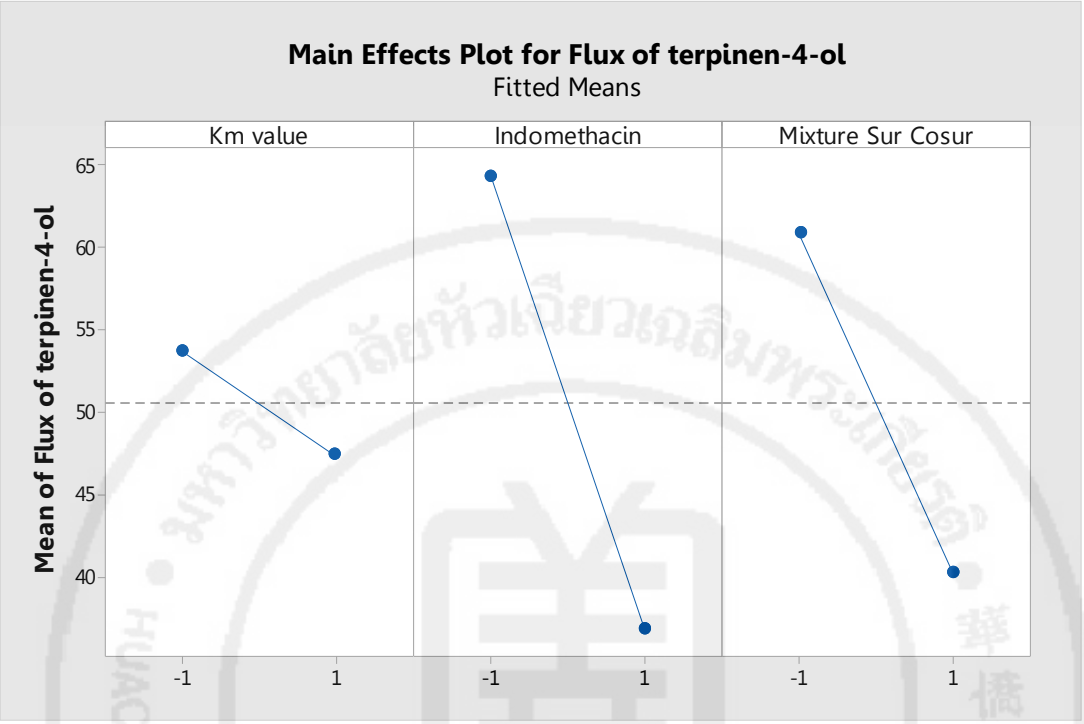



Figure 15 illustrated the main effect plot showing the main effects of the Km value ($X_1$), the presence of indomethacin ($X_2$), and the concentration of the mixture of surfactant and cosurfactant ($X_3$) on flux of terpinen-4-ol when the factors changed from low level to high level.

**Figure 15** Main effect plot for the flux of terpinen-4-ol.




Equation (3) showed the corresponding regression equation for the flux obtained by analyzing the experimentally determined input.

$$\text{Flux of terpinen-4-ol} = 50.567 - \mathbf{3.155X_1} - \mathbf{13.772X_2} - \mathbf{10.308X_3} - \mathbf{4.766X_1X_3} + 3.640X_2X_3 + 4.645\,X_1X_2X_3 \quad (3)$$

A positive sign of the coefficients in the regression equation indicated that the response increased with the increase of its variable factor while a negative sign indicated that the response decreased with the increase of its variable factor. The magnitude of the variable indicated the weight of each factor.

According to the regression equations (3) and Figure 26 clearly showed that the two main effects including the presence of indomethacin ($X_2$), and the concentration of the mixture of surfactant and cosurfactant ($X_3$) had more effect on the flux of terpinen-4-ol than the Km value ($X_1$). Three coupled factors ($X_1X_2$, $X_1X_3$, $X_2X_3$) and the together factors ($X_1X_2X_3$) also had effect, but to a lesser extent.

The regression equation for flux of terpinen-4-ol showed a negative sign for the Km value ($X_1$), the presence of indomethacin ($X_2$), and the concentration of the

mixture of surfactant and cosurfactant ($X_3$). The negative sign before each variable indicated that flux of terpinen-4-ol decreased when the variable changed at a time from its low to high value.

The presence of indomethacin reduced the permeation flux of terpinen-4-ol. The effect of indomethacin was obviously observed in the formulations (F7 and F8) containing high concentration of surfactant system. An decrease in permeation flux reflected a reduction in the permeation of Plai oil components. It was not clear by which mechanism it occurred. It was possible that there were the interaction between the Plai oil components with the indomethacin, the interaction between indomethacin with the surfactant which affected the permeation of Plai oil components, and the intense competition between terpinen-4-ol and indomethacin to penetrate through skin. The result was in agreement with the study of Wonglertnirant et al. who reported that the higher concentration of Tween 80 did not increase the permeation of the drug. (47) Similarly, Zhu et al reported a skin permeation study of penciclovir microemulsion using mice skin. They found that skin permeation was significantly increased when the ratio of surfactant to cosurfactant was close to 1:1. (51)

## 4.9 Stability

### 4.9.1 Appearance

Figure 16 showed physical appearances of eight microemulsion formulations after preparation (a) and after storage in dark place at ambient temperature for three months (b). After preparation, formulations F3, F4, F7 and F8 were darker yellowish. This was attributed to the property of indomethacin itself which appears as pale yellow to yellow-tan.

After storage at ambient temperature for three months, all microemulsion formulations were still transparent, homogenous, and yellowish. The appearance of all formulations visually remained unchanged. After centrifugation, no phase separation and drug precipitation were observed.

**Figure 16** The photograph of Plai oil microemulsions F1–F8 showing physical appearances. a); after preparing b); after storage ambient in dark place at ambient temperature for three months.


### 4.9.2 Polarized light microscopy

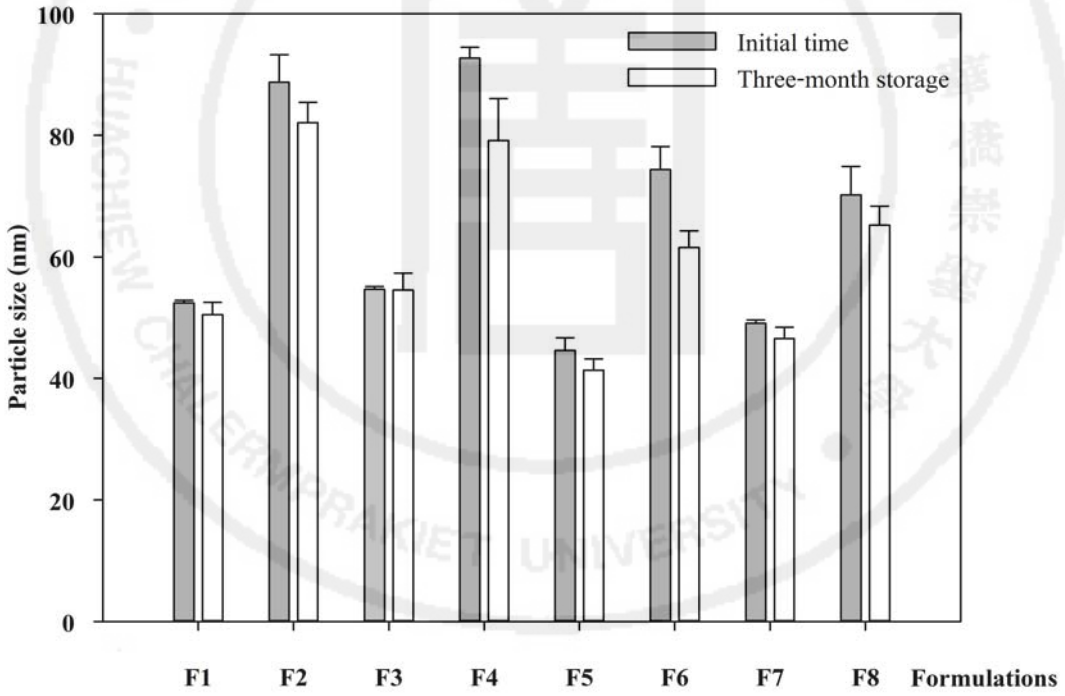
Figure 17 showed the photograph, for example, under cross-polarized light microscopy. The views of all microemulsion formulations after preparation and after storage for three months were similar. They remained dark and no birefringence was observed.

**Figure 17** A Photograph as an example of Plai oil microemulsions under cross-polarize light microscopy.

### 4.9.3 Particle size

Diagram 12 showed the average particle sizes of all microemulsion formulations after preparation in comparison with those after storage for three months. After storage, the average sizes of formulations F2, F4, F6 and F8 substantially reduced. Particles in the colloidal system have Brownian movement. Collision between microemulsion droplets occurred, resulting in either smaller or bigger particle droplets. Among formulations F2, F4, F6 and F8, the similarity was that they possessed the surfactant-cosurfactant mixture at Km value of 2:1.

**Diagram 12** Comparison of particle sizes of Plai oil microemulsions at initial time and after three-month storage (mean±S.D.; n=3).

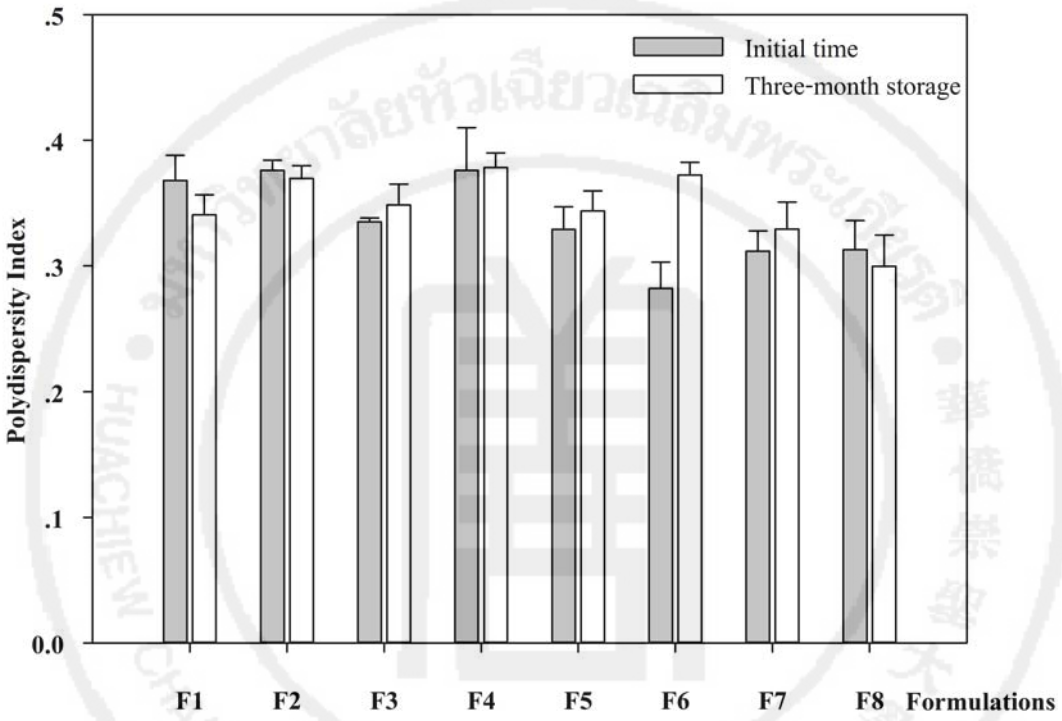



### 4.9.4 Polydispersity index

The polydispersity index of all microemulsion formulations after preparation and after storage for three months was illustrated in Diagram 13. It was found that upon storage the polydispersity index of most formulations was likely constant except for the formulation F6 whose polydispersity index became broader after storage. The polydispersity index is one method to indicate the physical stability

of the colloidal particles. An increase in the polydispersity index suggested the occurrence of droplet coalescence and/or aggregation.

**Diagram 13** Comparison of polydispersity index values of Plai oil microemulsions at initial time and after three-month storage. The values was mean±S.D.; n=3.

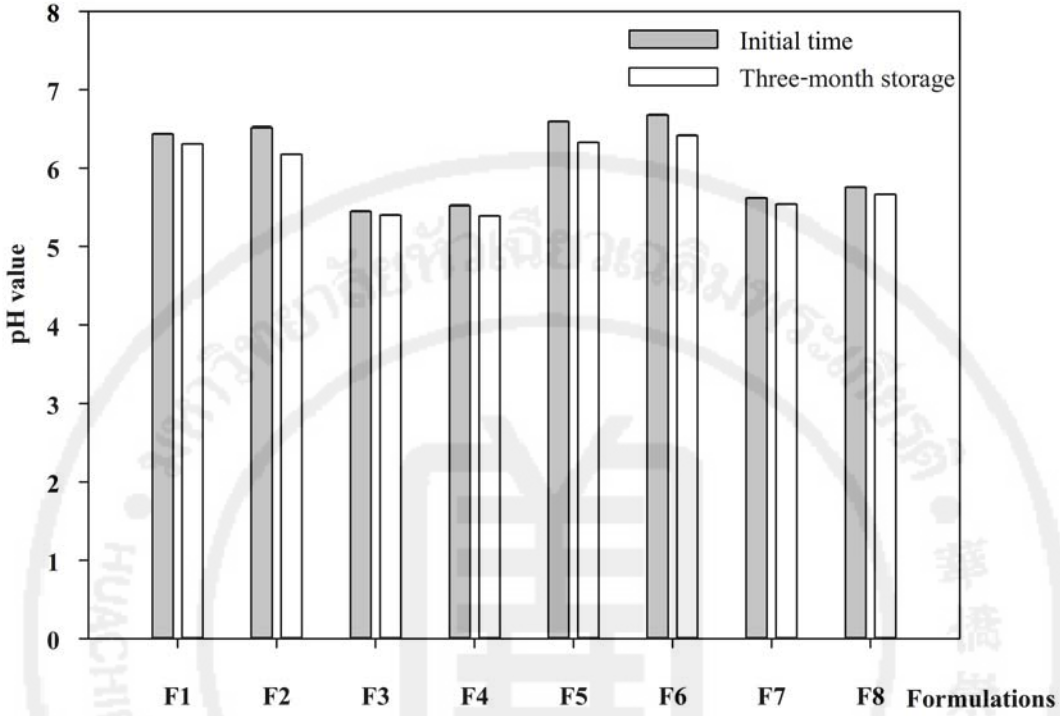



**4.9.5 pH**

The pH value of all formulations after preparation and after storage was shown in Diagram 14. After preparation, formulations F1, F2, F5, and F6 without indomethacin showed the pH above 6. It was obvious that a noticeable decrease in pH was observed in formulations F3, F4, F7, and F8 containing indomethacin. This was because indomethacin is a weak acid substance.

After storage at ambient temperature for three months, the pH of all microemulsions was likely comparable to that after preparation.

**Diagram 14** Comparison of the pH value of Plai oil microemulsions at initial time and after three-month storage. The values was mean±S.D.; n=3.

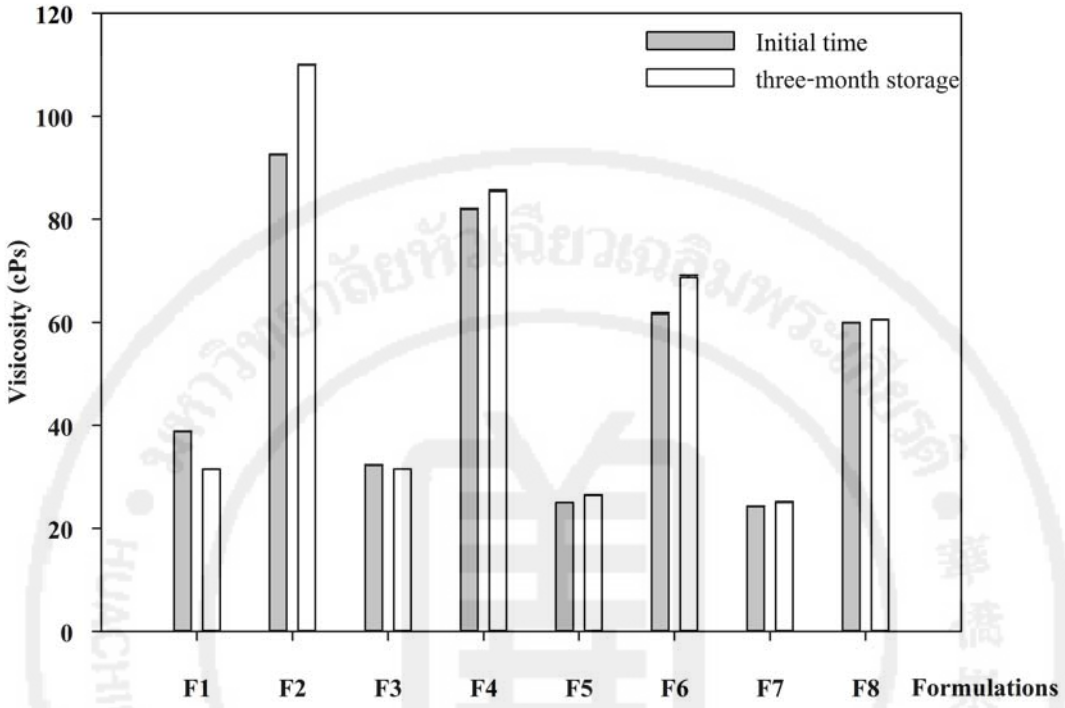



### 4.9.6 Viscosity

Diagram 15 showed the viscosity values of all microemulsion formulations after preparation in comparison with those after storage for three months. After preparation, formulations F2, F4, F6, and F8 composed of the surfactant to cosurfactant at Km value of 2:1 showed a remarkably higher viscosity values than those of formulations having Km value of 1:1. This might be as a result of the more interparticle interaction between droplets in the o/w systems. (19)

After storage at ambient temperature for three months, it was obvious that the viscosity of formulations F2 increased considerably, suggesting system instability.

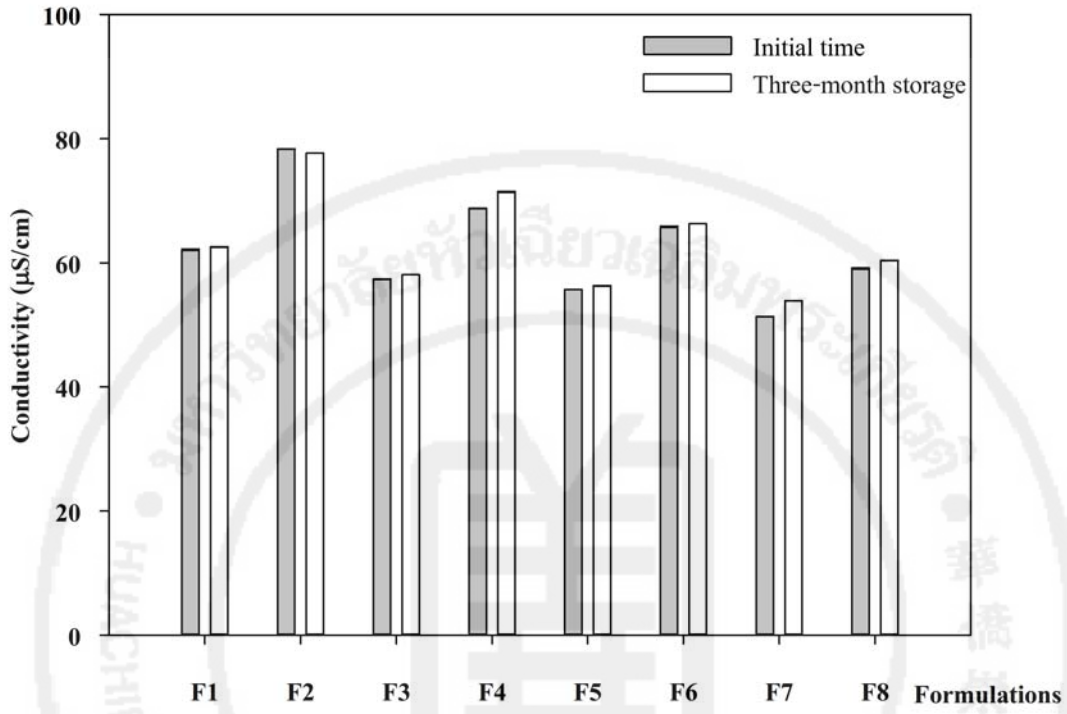
**Diagram 15** Comparison of viscosity values of Plai oil microemulsions at initial time and after three-month storage. The values was mean±S.D.; n=3.




### 4.9.7 Conductivity

The conductivity values of all formulations after preparation and after storage for three months were illustrated in Diagram 16. All formulations showed conductivity values higher than 10 μS/cm, suggesting that they were oil in water microemulsions. After storage, it was found that the conductivity value was comparable to that after preparation, suggesting that they still had water as pseudoexternal phase.

**Diagram 16** Comparison of conductivity values of Plai oil microemulsions at initial time and after three-month storage. The values was mean±S.D.; n=3.




### 4.9.8 Content of active ingredients of microemulsion formulations

Plai oil consists of five major components that are (E)-1(3,4 -dimethoxyphenyl) butadiene (DMPBD), terpinen-4-ol, sabinene, γ-terpinen, and α-terpiene. Terpinen-4-ol was used as a chemical marker for assay of Plai oil by GC.

The amount and a percentage of terpinen-4-ol and indomethacin in microemulsion samples after preparation and after three-month storage were shown in Table 19. After preparation, the terpinen-4-ol content was found in the range from 94.95 to 110.62% and indomethacin content from 95.14 to 97.05% at which it was used as an initial amount of the active ingredient in comparison with that after stability study.

After storage at ambient temperature for three months, the terpinen-4-ol content of all formulations F1 through F8 remained relatively constant, suggesting terpinen-4-ol was stable. In contrast, the indomethacin content of formulations F3, F4, F7, and F8 decreased. Formulations F3 and F7 had the indomethacin remaining about 88% of the initial amount while Formulations F4 and F8 had about 93% of the

initial amount, suggesting that indomethacin was stable to a certain extent. The decrease in indomethacin content was attributed to the degradation of indomethacin. Indomethacin underwent hydrolysis in aqueous environment. Its maximum stability in solution occurs near pH 3.75. The degradation increases with the increasing pH. Therefore, indomethacin was subject to hydrolysis in the circumstance pH of Plai oil microemulsion systems. (52)

**Table 19** Terpinen-4-ol and indomethacin contents in microemulsion formulations at initial time and after three-month storage (mean±S.D.; n=3)

| Formulations | %content of terpinen-4-ol | | %content of indomethacin | |
|---|---|---|---|---|
| | Initial time | Three-month storage | Initial time | Three-month storage |
| F1 | 96.74±0.18 | 106.66±0.20 | NA | NA |
| F2 | 96.11±0.35 | 104.99±0.87 | NA | NA |
| F3 | 94.95±0.79 | 109.02±0.44 | 95.56±0.77 | 84.56±0.49 |
| F4 | 110.62±0.29 | 107.84±0.65 | 95.94±1.56 | 85.99±0.23 |
| F5 | 104.08±1.31 | 104.15±1.05 | NA | NA |
| F6 | 102.97±0.25 | 103.08±0.36 | NA | NA |
| F7 | 104.32±0.46 | 107.09±1.32 | 95.14±0.92 | 86.90±0.46 |
| F8 | 96.26±0.40 | 108.27±0.76 | 97.05±0.37 | 86.30±0.59 |

NA = no assessment

# CHAPTER 5
# CONCLUSIONS AND RECOMMENDATION

Plai oil was successfully used as oil phase in the pseudoternary phase diagram study and in microemulsion preparation. In the pseudoternary phase diagram study, the system of Plai oil/Tween® 80-absolute ethanol (1:1 and 2:1)/water provided a larger single phase area than of that of the system of Plai oil/Kolliphor® RH40-absolute ethanol (1:1 and 2:1)/water. Plai oil at the concentration of 14% w/w was chosen to subsequently prepare Plai oil microemulsions in the presence and the absence of indomethacin. All eight Plai oil microemulsions in the presence and the absence of indomethacin exhibited oil in water microemulsion characteristics. They were isotropic transparent homogenous yellowish liquid mixtures.

Statistical data indicated that the presence of indomethacin had no effect on the particle size while the effects of all three main variables including the Km value, the concentration of indomethacin, and the concentration of the mixture of Tween® 80-absolute ethanol on the conductivity, viscosity, pH value, and the permeation of terpinen-4-ol through shed snake skin were significant. The effect varied from variable to variable. Obviously, the permeation flux of terpinen-4-ol considerably decreased with the presence of indomethacin. The first three rank order showing a higher cumulative amount and higher permeation flux of terpinen-4-ol were observed in the Plai oil microemulsion without indomethacin.

After storage at ambient temperature for three months, the physical appearance of all eight Plai oil microemulsions unchanged. No phase separation and drug precipitation were observed. They were still oil in water microemulsions. Terpinen-4-ol as a chemical marker was Plai oil component seemed to be stable while indomethacin was stable to some extent dependent on the formulation composition.

It should be concluded from the *in vitro* permeation study and the stability study that there were two optimum Plai oil microemulsion systems. One system in

the absence of indomethacin consisted of 14% Plai oil, 50% the mixture of Tween®80-absolute ethanol at a 1:1 weight ratio of Tween®80 to absolute ethanol, and water. Another system in the presence of 0.75% indomethacin was composed of 14%Plai oil, 45% the mixture of Tween®80-absolute ethanol at a 1:1 weight ratio of Tween®80 to absolute ethanol, and water. These two Plai oil microemulsion systems also showed an acceptable physicochemical characteristic for topical application.

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# APPENDIX

**Table 1** Analysis of variance for different responses on physicochemical properties of Plai oil microemulsions

| Particle size (nm) | | | | |
|---|---|---|---|---|
| **Source** | **Adj sum of square** | **Adj mean of square** | ***F*-value** | ***P*-Value** |
| $X_1$ | 5893.80 | 5893.80 | 725.06 | 0.000 |
| $X_2$ | 15.52 | 15.52 | 1.91 | 0.186 |
| $X_3$ | 953.82 | 953.82 | 117.34 | 0.000 |
| $X_1X_2$ | 17.51 | 17.51 | 2.15 | 0.162 |
| $X_1X_3$ | 203.58 | 203.58 | 25.04 | 0.000 |
| $X_2X_3$ | 13.35 | 13.35 | 1.64 | 0.218 |
| $X_1X_2X_3$ | 39.78 | 39.78 | 4.89 | 0.042 |
| Error | 130.06 | 8.13 | | |
| Total | 7267.43 | | | |
| S = 2.85110 R-sq = 98.21% R-sq (adj) = 97.43% | | | | |
| **pH value** | | | | |
| **Source** | **Adj sum of square** | **Adj mean of square** | ***F*-value** | ***P*-Value** |
| $X_1$ | 0.05134 | 0.05134 | 513.37 | 0.000 |
| $X_2$ | 5.67454 | 5.67454 | 56745.37 | 0.000 |
| $X_3$ | 0.19620 | 0.19620 | 1962.04 | 0.000 |
| $X_1X_2$ | 0.00050 | 0.00050 | 5.04 | 0.039 |
| $X_1X_3$ | 0.00220 | 0.00220 | 22.04 | 0.000 |
| $X_2X_3$ | 0.00260 | 0.00260 | 26.04 | 0.000 |
| $X_1X_2X_3$ | 0.00150 | 0.00150 | 15.04 | 0.001 |
| Error | 0.00160 | 0.00010 | | |
| Total | 5.93050 | | | |
| S = 0.01 R-sq = 99.97% R-sq (adj) = 99.96% | | | | |
| $X_1$ = the Km value | | | | |
| $X_2$ = the amount of indomethacin (%w/w) | | | | |
| $X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w) | | | | |

**Table 1** (cont) Analysis of variance for different responses on physicochemical properties of Plai oil microemulsions

| Viscosity (cPs) | | | | |
|---|---|---|---|---|
| **Source** | **Adj sum of square** | **Adj mean of square** | ***F*-value** | ***P*-Value** |
| $X_1$ | 11554.5 | 11554.5 | 385149.39 | 0.000 |
| $X_2$ | 147.0 | 147.0 | 4900.50 | 0.000 |
| $X_3$ | 2075.8 | 2075.8 | 69192.00 | 0.000 |
| $X_1X_2$ | 10.4 | 10.4 | 2346.72 | 0.000 |
| $X_1X_3$ | 358.8 | 358.8 | 11960.89 | 0.000 |
| $X_2X_3$ | 83.6 | 83.6 | 2787.56 | 0.000 |
| $X_1X_2X_3$ | 4.5 | 4.5 | 150.22 | 0.000 |
| Error | 0.5 | 0.0 | | |
| Total | 14235.1 | | | |
| S = 0.173205 R-sq = 100.00% R-sq (adj) = 100.00% | | | | |
| **Conductivity (μS/cm)** | | | | |
| **Source** | **Adj sum of square** | **Adj mean of square** | ***F*-value** | ***P*-Value** |
| $X_1$ | 770.67 | 770.667 | 40208.70 | 0.000 |
| $X_2$ | 241.94 | 241.935 | 12622.70 | 0.000 |
| $X_3$ | 447.21 | 447.207 | 23332.52 | 0.000 |
| $X_1X_2$ | 19.44 | 19.440 | 1014.26 | 0.000 |
| $X_1X_3$ | 37.00 | 37.002 | 1930.52 | 0.000 |
| $X_2X_3$ | 3.84 | 3.840 | 200.35 | 0.000 |
| $X_1X_2X_3$ | 2.28 | 2.282 | 119.04 | 0.000 |
| Error | 0.31 | 0.019 | | |
| Total | 1522.68 | | | |
| S = 0.138444 R-sq = 99.98% R-sq (adj) = 99.97% | | | | |

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

**Table 2** Analysis of variance for different responses on permeation flux of terpinen-4-ol

| Source | Adj sum of square | Adj mean of square | *F*-value | *P*-Value |
|---|---|---|---|---|
| $X_1$ | 238.85 | 238.85 | 19.10 | 0.000 |
| $X_2$ | 4551.73 | 4551.73 | 363.99 | 0.000 |
| $X_3$ | 2549.93 | 2549.93 | 203.91 | 0.000 |
| $X_1X_2$ | 18.34 | 18.34 | 1.51 | 0.237 |
| $X_1X_3$ | 545.22 | 545.22 | 44.91 | 0.000 |
| $X_2X_3$ | 318.06 | 318.06 | 26.20 | 0.000 |
| $X_1X_2X_3$ | 517.91 | 517.91 | 42.66 | 0.000 |
| Error | 194.25 | 12.14 | | |
| Total | 8934.29 | | | |

S = 3.48435 R-sq = 97.83% R-sq (adj) = 96.87%

$X_1$ = the Km value

$X_2$ = the amount of indomethacin (%w/w)

$X_3$ = the concentration of the mixture of surfactant and cosurfactant (%w/w)

**Table 3** The pH, particle size, polydispersity index, viscosity, and conductivity values of Plai oil microemulsions at initial time and after three-month storage

| Formulations | pH value | | Particle size (nm) | | Polydispersity Index | | Viscosity (cPs) | | Conductivity (μS/cm) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Initial time | 3-month storage | Initial time | 3-month storage | Initial time | 3-month storage | Initial time | 3-month storage | Initial time | 3-month storage |
| F1 | 6.43±0.01 | 6.31±0.01 | 52.43±0.40 | 50.5±2.01 | 0.37±0.02 | 0.34±0.02 | 38.70±0.12 | 31.40±0.06 | 62.03±0.23 | 62.53±0.06 |
| F2 | 6.52±0.02 | 6.18±0.00 | 88.73±4.50 | 82.1±3.32 | 0.38±0.01 | 0.37±0.01 | 92.50±0.15 | 109.9±0.15 | 78.27±0.06 | 77.70±0.00 |
| F3 | 5.45±0.01 | 5.4±0.00 | 54.67±0.47 | 54.53±2.82 | 0.34±0.00 | 0.35±0.02 | 32.20±0.15 | 31.50±0.00 | 57.30±0.10 | 58.10±0.00 |
| F4 | 5.52±0.01 | 5.39±0.00 | 92.70±1.78 | 79.18±6.88 | 0.38±0.03 | 0.38±0.01 | 81.70±0.25 | 85.40±0.35 | 68.70±0.10 | 71.37±0.15 |
| F5 | 6.59±0.01 | 6.33±0.00 | 44.57±2.14 | 41.33±1.88 | 0.33±0.02 | 0.34±0.02 | 25.00±0.00 | 26.40±0.15 | 55.70±0.00 | 56.27±0.06 |
| F6 | 6.68±0.01 | 6.42±0.01 | 74.37±3.76 | 61.53±2.75 | 0.28±0.02 | 0.37±0.01 | 61.60±0.30 | 68.70±0.46 | 65.73±0.21 | 66.30±0.00 |
| F7 | 5.62±0.01 | 5.54±0.01 | 46.57±1.86 | 46.57±1.86 | 0.31±0.02 | 0.33±0.02 | 24.20±0.12 | 25.00±0.17 | 51.33±0.06 | 53.90±0.00 |
| F8 | 5.76±0.01 | 5.66±0.00 | 70.20±4.67 | 65.2±3.15 | 0.31±0.02 | 0.30±0.02 | 59.90±0.12 | 60.50±0.12 | 59.00±0.17 | 60.37±0.06 |

# BIOGRAPHY

| | |
|---|---|
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| **Work experience** | |
| 2007 - 2009 | OPD Pharmacist<br>Paolo Hospital Sumutprakan |
| 2009 - present | Radio Pharmacist<br>Bangkok Hospital |