การพัฒนาโซลิดลิพิดนาโนพาร์ทิเคิลที่บรรจุสารสกัดจากแก่นมะหาด (ปวกหาด) เพื่อผลทำให้ผิวขาวและชะลอริ้วรอย

นางสาวสุรัชวดี อมรเดชาวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

DEVELOPMENT OF SOLID LIPID NANOPARTICLES CONTAINING *ARTOCARPUS LAKOOCHA* HEARTWOOD EXTRACT (PUAG-HAAD) FOR WHITENING AND ANTI-WRINKLE EFFECTS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutics
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2008

Thesis Title	DEVELOPMENT OF SOLID LIPID NANOPARTICLES CONTAINING *ARTOCARPUS LAKOOCHA* HEARTWOOD EXTRACT (PUAG-HAAD) FOR WHITENING AND ANTI-WRINKLE EFFECTS
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สุรัชวดี อมรเดชาวัฒน์: การพัฒนาโซลิดลิพิดนาโนพาร์ทิเคิลที่บรรจุสารสกัดจากแก่นมะหาด (ปวกหาด) เพื่อผลทำให้ผิวขาวและชะลอริ้วรอย. (DEVELOPMENT OF SOLID LIPID NANOPARTICLES CONTAINING *ARTOCARPUS LAKOOCHA* HEARTWOOD EXTRACT (PUAG-HAAD) FOR WHITENING AND ANTI-WRINKLE EFFECTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ภาคภูมิ เต็งอำนวย, 247 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาโซลิดลิพิดนาโนพาร์ทิเคิล (เอสแอลเอ็น) ซึ่งเป็นระบบตัวพาสำหรับสารสกัดจากแก่นมะหาด (ปวกหาด) เพื่อเพิ่มความคงตัว และประเมินประสิทธิศักย์ในการทำให้ผิวขาวและชะลอริ้วรอยในอาสาสมัครเพศหญิง เอสแอลเอ็นที่บรรจุปวกหาดประสบความสำเร็จในการพัฒนาโดยวิธีอิมัลชันซ้อนชนิดน้ำในน้ำมันในน้ำและวิธีโฮโมจีไนเซชันแบบเย็น เมื่อใช้กลีเซอริลโมโนสเตียเรต โพลีออกซีเอทิลีน 40 สเตียเรต โพล็อกซาเมอร์ 188 เป็นไขมัน สารลดแรงตึงผิว และสารร่วมลดแรงตึงผิว ตามลำดับ ขนาดเฉลี่ยและประจุอนุภาคของสูตรตำรับเมื่อวัดโดยวิธีโฟตรอน คอรีเรชัน สเปคโทรสโคปี (พีซีเอส) มีขนาด 150 – 190 นาโนเมตร สำหรับวิธีอิมัลชันซ้อนชนิดน้ำในน้ำมันในน้ำ และ มีขนาด 250 – 320 นาโนเมตร สำหรับวิธีโฮโมจีไนเซชันแบบเย็น เอสแอลเอ็นที่บรรจุปวกหาดมีขนาดอนุภาคเป็นทรงกลมที่สม่ำเสมอ และขนาดใกล้เคียงกันเมื่อตรวจสอบด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่าน (ทีอีเอ็ม) การทำให้เอสแอลเอ็นที่บรรจุปวกหาดบริสุทธิ์โดยการกรองละเอียดยิ่งยวด และวิเคราะห์ปริมาณออกซีเรสเวอร์ราทอลซึ่งเป็นส่วนประกอบหลักในปวกหาดโดยวิธีไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี (เอชพีแอลซี) แสดงให้เห็นประสิทธิภาพการกักเก็บสูงประมาณ 85% สำหรับวิธีอิมัลชันซ้อนชนิดน้ำในน้ำมันในน้ำ และ 70% สำหรับวิธีโฮโมจีไนเซชันแบบเย็น การศึกษาการปลดปล่อยแบบนอกกายของออกซีเรสเวอร์ราทอลพบว่า การปลดปล่อยเป็นไปอย่างช้า ๆ และสัมพันธ์อย่างดีกับสมการของฮิกูชิ ภายหลังการเก็บเอสแอลเอ็นที่บรรจุปวกหาด (0.25%) ที่อุณหภูมิห้อง พ้นแสงเป็นเวลา 4 เดือน พบว่าความคงตัวทางกายภาพและเคมีดีกว่าสารละลายปวกหาด การมีสารลดแรงตึงผิว (โพลีออกซีเอทิลีน 40 สเตียเรต) ร่วมกับซิเตรทบัฟเฟอร์พีเอส 5.5 ให้ผลเสริมกันในการเพิ่มความคงตัวของปวกหาดทั้งทางกายภาพและเคมี อาสาสมัครเพศหญิงจำนวน 26 คน เข้าร่วมการศึกษาเป็นเวลา 8 สัปดาห์ แต่ละคนจะได้รับเอสแอลเอ็นที่บรรจุปวกหาด (0.25%) ที่แขนข้างหนึ่ง เปรียบเทียบกับสารละลายไมเซลล์ของปวกหาดที่แขนอีกข้างหนึ่ง ความเข้มของสีผิว ความหยาบกร้าน ความเต่งตึง และความชุ่มชื้นของผิวหนัง จะถูกวัดทุก 2 สัปดาห์ รีพีส เมสเซอร์ อาโนวา เป็นสถิติที่ใช้ในการประเมินผลของความแตกต่างโดยรวมระหว่างสองสูตรตำรับกับเวลา ที่ความแตกต่างอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่น 0.05 เอสแอลเอ็นที่บรรจุปวกหาดให้ผลในการชะลอริ้วรอย เพิ่มความเต่งตึงและความชุ่มชื้นของผิวหนังดีกว่าสารละลายไมเซลล์ ในทางตรงข้ามสารละลายไมเซลล์ให้ผลในการทำให้ผิวขาวได้ดีกว่าเอสแอลเอ็นเล็กน้อย อาสาสมัครทุกคนเข้าร่วมจนจบการศึกษา โดยไม่พบอาการไม่พึงประสงค์ใด ๆ หรือถอนตัวจากการศึกษา ดังนั้น เอสแอลเอ็นที่บรรจุ ปวกหาดมีแนวโน้มพัฒนาเป็นเครื่องสำอางที่มีประสิทธิศักย์ในการทำให้ผิวขาวและชะลอริ้วรอยต่อไป

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชกรรม ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2551

4976606933 : MAJOR PHARMACEUTICS
KEYWORDS:*ARTOCARPUS LAKOOCHA* / PUAG-HAAD / OXYRESVERATROL / SOLID LIPID NANOPARTICLES / WHITENING / ANTI-WRINKLE

SURATCHAWADEE AMORNDECHAWAT: DEVELOPMENT OF SOLID LIPID NANOPARTICLES CONTAINING *ARTOCARPUS LAKOOCHA* HEARTWOOD EXTRACT (PUAG-HAAD) FOR WHITENING AND ANTI-WRINKLE EFFECTS. THESIS ADVISOR: ASSOC. PROF. PARKPOOM TENGAMNUAY, Ph.D., 247 pp.

The main purpose of this study was to develop solid lipid nanoparticles (SLN), an alternative carrier system, for *Artocarpus lakoocha* heartwood extract (Puag-Haad) to improve Puag-Haad stability and evaluate the whitening and anti-wrinkle efficacies in woman volunteers. At optimum formulations, SLN containing Puag-Haad were successfully developed by w/o/w double emulsion and cold homogenization method using glyceryl monostearate, polyoxyethylene 40 stearate and poloxamer 188 as lipid, surfactant and cosurfactant, respectively. The mean particle sizes and zeta potentials of formulations, when measured by photon correlation spectroscopy (PCS), were 150 – 190 nm for w/o/w double emulsion method and were 250 – 320 nm for cold homogenization method. The particles showed regular, spherical and uniform nanospheres when investigated by transmission electron microscope (TEM). Purification of SLN containing Puag-Haad was achieved by ultrafiltration. Analysis of the amount of oxyresveratrol, a main constituent in Puag-Haad, by high performance liquid chromatography (HPLC) showed high entrapment efficiency of about 85% for w/o/w double emulsion method and 70% for cold homogenization method. In vitro release studies showed sustained release profiles of oxyresveratrol and well fitted the Higuchi equation. After 4 months storage at room temperature and protected from light, the physical and chemical stabilities of SLN containing Puag-Haad (0.25%) were superior to the aqueous solutions of Puag-Haad. The presence of surfactant (polyoxyethylene 40 stearate) and citrate buffer pH 5.5 had additive effect in stabilizing Puag-Haad both physically and chemically. Twenty-six woman volunteers participated in an 8-week study. Each applied the SLN containing Puag-Haad (0.25%) on one of the forearm in comparison with the micellar solution of Puag-Haad on another forearm. The melanin index, skin roughness, skin elasticity and skin hydration were measured every 2 weeks. Repeated measures ANOVA was employed to evaluate the difference in the overall effect between the two formulations over time at the significance level (α) of 5%. SLN containing Puag-Haad gave significantly better anti-wrinkle, skin elasticity and skin hydration than the micellar solution. On the other hand, the solution showed slightly greater whitening effect than SLN. All the subjects completed the study without developing any unwanted side effects or withdrawal. Therefore, the SLN containing Puag-Haad appeared to have a very promising potential as a novel and effective skin whitening / anti-wrinkle cosmetic delivery system.

Department : Pharmaceutics and Industrial Pharmacy Student's Signature .....

Field of Study : Pharmaceutics Advisor's Signature .....

Academic Year : 2008

ACKNOWLEDGEMENTS

I would never have undertaken this thesis without the dedicated, warm encouragement and hospitality, as well as valuable guidance and assistance from a number of people.

Firstly, I am deeply grateful to my advisor, Associate Professor Dr. Parkpoom Tengamnuay for his continual guidance, valuable suggestion, extremely valuable encouragement and understanding throughout the course of my study.

I would like to express my appreciation to Associate Professor Dr. Uthai Suvanakoot, chairman of my thesis examination committee, as well as other committee members, Dr. Uracha Ruktanonchai, Dr. Vipaporn panapisal and Assistant Professor Nontima Vardhanabhuti, for their kind and valuable comments, enlightening discussions and make this thesis complete.

I am very grateful to Associate Professor Dr. Suchada Chutimaworapan for DSC pan and Assistant Professor Warangkana Warisnoicharoen for valuable suggestion and kindness.

I would like to express my thank to Namsiang International Co., Ltd, Thailand for providing Visioscan® VC 98 and Associate Professor Dr. Ubonthip Nimmannit for providing Derma-Spectrometer®, Dermalab® and Skin diagnostic® SD 27.

I would like to express my thank to The Government of Pharmaceutical Organization for allowing the use of Zetasizer.

A special acknowledgement is extended to the Faculty of Pharmaceutical Sciences, Chulalongkorn University, for the support of equipment and laboratory space in this study, I also want to express special thanks to my friends for their support and warm encouragement during my study. Sincere thanks are also given to all staff members of the Department of Pharmaceutics and Industrial Pharmacy and other people whose names nave not been mentioned for their assistance and great helpful support.

Finally, I would like to thank and dedicate this work to my family for their love, understanding, encouragement and support which make me a person whom I am today.

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LIST OF ABBREVIATIONS

%	=	percentage
µg	=	microgram
ANOVA	=	analysis of variance
°C	=	degree celsius
conc	=	concentration
cm	=	centimeter
CV	=	coefficient of variation
df	=	degree of freedom
DSC	=	differential scanning calorimetry
et al.	=	*et alii*, 'and others'
F-68	=	Poloxamer® 188
g	=	gram
GMS	=	glyceryl monostearate
h	=	hour
HPLC	=	high performance liquid chromatography
J	=	joule
k	=	release rate constant
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	milimeter
MW	=	molecular weight
n	=	sample size
nm	=	nanometer
No.	=	number
pH	=	the negative logarithm of the hydrogen ion concentration
PI	=	polydispersity index
R^2	=	coefficient of determination
rpm	=	round per minute

s	=	second
S-40	=	polyoxyethylene 40 stearate
SD	=	standard deviation
SLN	=	solid lipid nanoparticles
SS	=	sum of square
TEM	=	Transmission Electron Microscopy
USP/NF	=	The United States Pharmacopoeia/National Formulary
UV	=	ultraviolet
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Nowadays, Asian women especially in Thailand take care of their skin extensively. Perfect white skin without wrinkle is highly desirable. Cosmetic products are necessary to enhance the beauty of their appearance. Natural extracts are often used for this reason since it is believed that they are less skin-irritating than the synthetic compounds.

Artocarpus lakoocha Roxb. (family Moraceae) is a tropical tree in Thailand, which is locally called Ma-Haad. The local people have been using the dried aqueous extract of *A. Lakoocha* heartwood or Puag-Haad in the traditional treatment of parasites such as tapeworms. The main constituent in Puag-Haad is 2, 4, 3′, 5′-tetrahydroxystilbene or oxyresveratrol (Mongkolsuk, Robertson, and Towers, 1957).

Recently, Puag-Haad has been studied for its whitening and anti-wrinkle effects in woman volunteers. It was found that Puag-Haad had the most whitening effect when compared with kojic acid and licorice extract (Parkpoom Tengamnuay et al., 2006), and had the most anti-wrinkle effect when compared with epigallocatechin gallate (EGCG) and vitamin C (Manatchaya Wanawatanakun, 2006). For this reason, Puag-Haad appeared to have promising potential for further development as a safe, effective and economical whitening and anti-wrinkle agent in cosmetic products. Unfortunately, the physical and chemical stability of Puag-Haad is relatively poor in aqueous solutions (Khumkwan Pengrungruangwong, 2001). Therefore, development of a suitable carrier system has to be investigated in order to protect Puag-Haad stability.

Solid lipid nanoparticles (SLN) are an alternative carrier system for topical delivery of drug and cosmetic products. They are also used in other routes of administration such as oral, parenteral, ocular administration and targeting effect on brain (Müller, Radtke, and Wissing, 2002; Bunjes and Siekmann, 2006). SLN are interesting because they can protect incorporated drug against chemical and physical degradation such as tocopherol (Dingler et al., 1999), retinol (Jenning, Schäfer-

Korting, and Gohla, 2000; Jenning et al., 2000; Jenning and Gohla, 2001), coenzyme Q10 (Wissing et al., cited in Schäfer-Korting, Mehnert, and Korting, 2007) and curcuminoids (Waree Tiyaboonchai, Watcharaphorn Tungpradit, and Pinyupa Plianbangchang, 2006). Furthermore, they can modify drug release and improve skin penetration (Jenning et al., 2000; Pople and Singh, 2006; Liu et al., 2007a). SLN are also an effective carrier for cosmetic products because they are intended to increase skin hydration. (Wissing and Müller, 2003). Thus, to enhance its stability and skin penetration efficiency, SLN containing Puag-Haad were developed in this research. In general, SLN can be prepared by various methods such as a high pressure homogenization (hot or cold) and a warm microemulsion method. The selection of method depends on various factors including the properties of the incorporated drug especially the drug solubility in lipid or water (lipophilic or hydrophilic). Since Puag-Haad contained oxyresveratrol, a semipolar compound, which is soluble in hot water, methanol, ethanol as well as in 20% propylene glycol aqueous solution. Therefore, a cold homogenization and a w/o/w double emulsion method were selected as method to prepare SLN of Puag-Haad. Both techniques are suitable for soluble substances. The cold homogenization, a basic technique, can prevent the partition of drug between a melted lipid and an aqueous phase during homogenization (Almeida, Runge, and Müller, 1997). Recently, the w/o/w double emulsion method has been developed (Morel et al., 1996; Ma et al., 2007), which has an advantage that it does not require special manufacturing equipment or use of organic solvent. Moreover, it also generates smaller particles than other methods. Therefore, both methods are chosen in this study to prepare SLN containing Puag-Haad.

Therefore, this study focuses on the development of SLN containing Puag-Haad to improve its stability. The whitening and anti-wrinkle efficacies of SLN containing Puag-Haad were also investigated in woman volunteers in comparison with Puag-Haad aqueous solution.

The specific objectives of this investigation were as follows:

1. To compare the characteristics of SLN containing Puag-Haad prepared by the cold homogenization and the w/o/w double emulsion methods.
2. To study the effect of various ingredients in the SLN formulation on the characteristics of SLN containing Puag-Haad.
3. To evaluate physical and chemical stability of SLN containing Puag-Haad.
4. To evaluate the whitening and anti-wrinkle effects of SLN containing Puag-Haad in woman volunteers.

The overall aim of this study was to assess the potential of SLN containing Puag-Haad for use as a novel whitening and anti-wrinkle cosmetic delivery system.

CHAPTER II

LITERATURE REVIEW

A. *Artocarpus lakoocha Roxb.* and Puag-Haad

Artocarpus lakoocha Roxb. (family Moraceae) is a tropical tree in Thailand, which is locally called Ma-Haad. Morphology and medicinal use of Ma-Haad are shown in the details below.

Morphology of *Artocarpus lakoocha Roxb.* (Chanai Sambhandharaksa, Payorm Thantivatana, and Tongchai Ratanachai, 1962)

Family : Moraceae

A large, deciduous tree

Bark dark colored with milky sap

Wood hard, sapwood large, white and soft

Fresh heartwood yellow, dark brown after standing for a long tine, hard, shining mottled

Branchlets densely grey or rusty tomentose

Leaves ovate or obvoate, 3 ½ – 12 inches by 2 – 6 inches

Apex shortly, finely acuminate or cuspidate

Buse truncate or subcordate

Margin entire, sometimes serrate or subundulated in young leaves, coriaceous, glabrous, shining above, densely grey-downy beneath

Lateral nerves 8 – 12 pairs, prominent and with a fine, distinct reticulation between beneath

Petioles ½ – 1 long

Stipules small, pubescent, carducous

Flowers in shortly pedunculate or sub-sessile, axillary, globose heads ½ – 1 inch diameter

Bractioles peltate

Male flowers: - Sepals on sub-sessile, receptacles, 3 – 4 triangular, truncate, pubescent, 2 – 3, says Trimen. Stamen 1, filament broad at base, tapering upward Another exserted, broad 2 celled.

Female flowers on shortly peduncled receptacles

Anthrocarps flat, smooth, at apices, completely united

Fruits oblong, irregularly globose, 2 – 3 inches diameters, minutely velvety, yellow when ripe, edible

Seeds oblong, 1 inch thick, flat

Mature root outer bark has separated layers of cork in orange red or brown.

Inner bark narrow, brown

Sapwood wide, pale brown

Heartwood with pale yellow, mottled

Figure 1 A mature tree of *Artocarpus lakoocha* (Joshee, Bastola, and Yadav, 2002)

Figure 2 A developing fruit of *Artocarpus lakoocha* (Joshee, Bastola, and Yadav, 2002)

Medicinal use

The claimed efficacies in Thailand traditional textbooks are as follows (Farnsworth and Bunyapraphatsara, 1992):

Roots: as an antipyretic, antihelmintic; for allervation of toxic symptoms and treatment of urinary stones.

Wood: an antiflatulence, carminative and laxative; treatment of skin rash; chronic gastrointestinal ailments of children between the ages of 5 – 13 charactezied by marked malnutrition and usually associated with intestinal parasitism; round and tape worm infestation; menstrual disorders; fainting; and any disorders or diseases which cause cachexia, disorder of flatulence and tendomyopathy.

Bark: as an antipyretic.

Pith: treatment of menstrual disorders; any disorders or diseases which cause cachexia; nephropathy; distension of abdomen due to peritonitis or paralytic ileus; insomnia; malnutrition syndrome in children due to intestinal parasitism; splenomegaly; eye irritation; dissipate hematoma; oropharyngeal symptom from gastroenteric disease; dyspepsia caused by wind element; cramps; clouded mind; incontinent urination; as antidiarrheal, antihelmintic, taenifuge, antituberculosis, analgesic and for increasing appetite.

In Thai traditional medicine, the local people have been using the dried aqueous extract of *A. lakoocha* heartwood or Puag-Haad in the treatment of parasites such as tapeworms and as antipruretic. The main constituent in Puag-Haad is 2, 4, 3′, 5′ - tetrahydroxystillbene or oxyresveratrol up to 70 – 90% depending on batch (Mongkolsuk, Robertson, and Towers, 1957).

Figure 3 *Artocarpus lakoocha* heartwood extract (Puag-Haad)

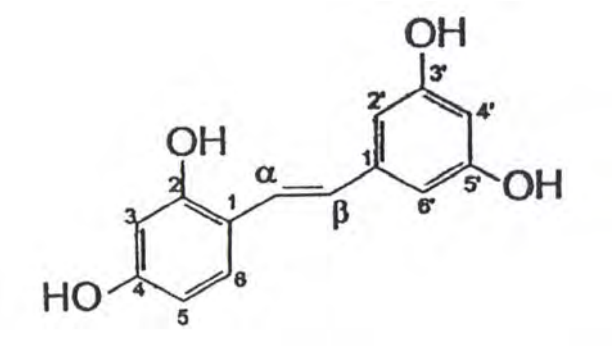


Figure 4 Chemical structure of 2, 4, 3′, 5′ - tetrahydroxystillbene (oxyresveratrol) (Boonchoo Sritulalak, 1998)

***In vitro* and *in vivo* efficacy test of oxyresveratrol and Puag-Haad**

La-iad Tiptabiankarn (1967) reported that oxyresveratrol from Puag-Haad was considered to be an effective antioxidant by delaying rancidity of lard as compared to Tenox II (Tenox II contained 20% BHA, 6% propyl gallate, 4% citric acid and 70% propylene glycol).

Oxyresveratrol has been reported to exert an antihelmintic activity (Preecha Charoenlarp et al., 1981; Preuksaraj et al., 1983).

Oxyresveratrol has been reported to exhibit good safety profile in cytotoxicity test. (Nilvises et al., 1985; Wantana Ngamwat et al., 1987)

Boonchoo Sritulalak (1998) have screened a large number of plants for their *in vitro* anti-tyrosinase activity. It was found that oxyresveratrol from Puag-Haad exhibited the highest activity when comparing with kojic acid and norartocarpetin (the main constituent in the root of *Artocarpus gomezianus*). Furthermore, oxyresveratrol had a concentration causing 50% enzyme inhibition (IC_{50}) of tyrosinase about 1.5 μM, which was 17.9 times higher than kojic acid. This result agreed with the previous studies of Shin et al. (1998) and Kim et al. (2002) who reported that IC_{50} of oxyresveratrol was 1.0 and 1.2 μM, respectively.

Kanjana Wachiranuntasin (2005) has reported that oxyresveratrol and Puag-Haad were effective in scavenging the reactive oxygen species (ROS). Some of their effects were comparable or even superior to epigallocatechin gallate (EGCG) or pine bark extract, the well-known potent antioxidants in commercial use.

Parkpoom Tengamnuay et al. (2006) have reported that Puag-Haad was able to reduce melanin formation in human volunteers. Comparing to other tyrosinase inhibitors commonly used in whitening products like kojic acid and licorice extract. Puag-Haad produced a faster onset of significant whitening effect, requiring only 2 – 4 weeks of application depending on the type of formulation and area of application.

Manatchaya Wanawatanakun (2006) has reported that Puag-Haad had an anti-wrinkle activity. Its effect was more rapid and superior to EGCG and vitamin C at the same concentration. Moreover, it also possesses a skin whitening effect with low concentration (0.25 – 0.50%).

Stability of oxyresveratrol and Puag-Haad

Oxyresveratrol from Puag-Haad decomposes rapidly under alkaline conditions (Nitaya Poopyruchpong et al., 1978).

Khumkwan Pengrungruangwong (2001) has studied physical and chemical property of Puag-Haad solution. It was found that the solutions of Puag-Haad was changes in color upon storage at room temperature for 24 weeks without adding antioxidants and the anti-tyrosinase activity was decreased up to 50%.

Nowadays, no reports were clearly described the degradation mechanism of oxyresveratrol. But, oxidation process might be involved in Puag-Haad degradation. Observing the changes in color, it correlated to decrease in the amount of oxyresveratrol and anti-tyrosinase activity when adding antioxidant such as sodium metabisulfite with butylated hydroxyl anisole (BHA).

Furthermore, the degradation of oxyresveratrol can be increased under alkaline condition. The condition that could stabilize both the physical and chemical stabilities of Puag-Haad solutions was a slightly acidic pH using citrate buffer pH 4.0 – 5.5 Manatchaya Wanawatanakun (2006).

However, no studies especially the pH – rate profile have been elucidated for the mechanisms of oxyresveratrol degradation.

Solubility and partition coefficients of oxyresveratrol (Nitaya Poopyruchpong et al., 1978)

The solubilities of oxyresveratrol in various solvents have been determined by UV absorption and are as follows:

Water, 0.640 mg/ml (2.285 mM)

Methanol, 406.6 mg/ml (1.450 M)

Ethanol, 328 mg/ml (1.171 M)

1, 4 dioxane, 415.6 mg/ml (1.484 M)

Diethyl ether, 355.5 mg/ml (1.269 M)

Isobutanol, 173.5 mg/ml (0.62 M)

1-hexanol, 114.1 mg/ml (0.41 M)

1-octanol, 69.76 mg/ml (0.25 M)

These values were obtained at 25°C expect in the case of diethyl ether where the study was made at 5°C. The solubility of oxyresveratrol in carbon tetrachloride is extremely low and could not be determined accurately even by fluorescence technique. Starting with 10 μg/ml of oxyresveratrol in organic solvents the partition coefficients of oxyresveratrol (the ratio of oxyresveratrol concentration in organic solvent to oxyresveratrol concentration in water at equilibrium, K_D) are as follows:

Chloroform, 0

Isobutanol, 5.6

1-hexanol, 7.8

1-octanol, 48

It was found that diethyl ether quantitatively extracted all the oxyresveratrol from the aqueous phase.

B. Solid lipid nanoparticles

Solid lipid nanoparticles (SLN), a submicron-size and pearl-like particle, are alternative colloidal carrier system for drug delivery such as topical, parenteral, oral, ocular, etc. This system is the first generation consisting of solid matrix which is produced from solid lipid. It was developed at the beginning of the 1990's. SLN combine the advantages of the traditional colloidal system (eg. liposomes and polymeric micro- or nanoparticles) but avoid some of their major disadvantages. The storage stability of liposomes is limited in comparison with SLN. The solid-like state of lipid structure is expected to provide better stability and control drug release. Furthermore, the preparation techniques of liposomes are relatively difficult, need special techniques and take a long time. Moreover, the suitable large scale production method need to be required. The manufactures of SLN are relatively easy and can be produced in large scale with production technology. The used lipids to prepare SLN are non-toxic, biodegradable and biocompatible. In contrast, the cytotoxicity of polymers and the use of organic solvent to produce polymeric micro- or nanoparticles should be considered. There are a lot of techniques to prepare SLN which depend on facility and properties of drug entrapped. There are a lot of advantages and rational to prepare SLN. SLN can protect the entrapped drug from physical and chemical degradation. Furthermore, it can improve skin penetration and modify drug release. Recently, SLN is interesting to develop as topical drug delivery and cosmetics because it can improve skin hydration and can be incorporated into cream and gel or other formulations for topical application.

Preparation techniques of SLN

These techniques can be divided into three groups.

1. High pressure homogenization technique
2. Microemulsion technique
3. Other techniques such as solvent emulsification-evaporation, solvent emulsification-diffusion

1. The high pressure homogenization technique

The two basic methods of high pressure homogenization are the hot and cold methods. For both methods, the drug is dissolved or solubilized in melted lipid at about 5-10°C above melting temperature. Figure 5 shows the preparation of SLN by high pressure homogenization technique (hot and cold).

Figure 5 The preparation of SLN by high pressure homogenization technique (hot and cold) (Bunjes and Siekmann, 2006)

The difference between both methods is environmental temperature that lipid is dispersed into aqueous surfactant solution.

If temperature > melting point of lipid, it is the hot method.

If temperature < melting point of lipid, it is the cold method.

For this reason, there are some different characteristics of SLN preparations. The hot process is suitable for lipophilic drugs because the drugs are solubilized in melted lipid. So, the loading and entrapment efficiency of drugs depend on their solubility in lipid. SLN by the hot process are stable dispersions, good reproducibility and smaller particle size and size distribution than the cold process. In contrast, SLN by the cold process are larger mean particle size and broader size distribution. However, the cold process is suitable for highly temperature-sensitive drugs. It is also necessary to use this method when formulating hydrophilic drugs because they would partition between the melted lipid and the water phase during the hot process. This process avoids or minimizes the loss of hydrophilic drugs to the water phase. Considering the homogenization process, the heat may be raised. Therefore, the

difference between the melting point of lipid and homogenization temperature needs to be large enough to avoid melting of lipid during homogenization process.

For the high pressure homogenization technique, there is a need to have the special equipment, high pressure homogenizer. The mean particle size of SLN depends on the composition of the formulation eg. lipid and surfactant as well as temperature, pressure and cycle number of homogenization process.

2. The microemulsion technique

Microemulsion is clear or transparent formulation. It is composed of lipid and water phase with surfactant (and co-surfactant). For this technique, microemulsion is formed with lipid being solid at room temperature and need to be produced at a temperature about 5-10°C above melting point of lipid. This microemulsion is then dispersed into a aqueous surfactant solution under mild stirring. After the temperature of this mixture below the melting point of lipid, the lipid is recrystallized to solid to form SLN. Figure 6 shows the preparation of SLN by microemulsion technique.

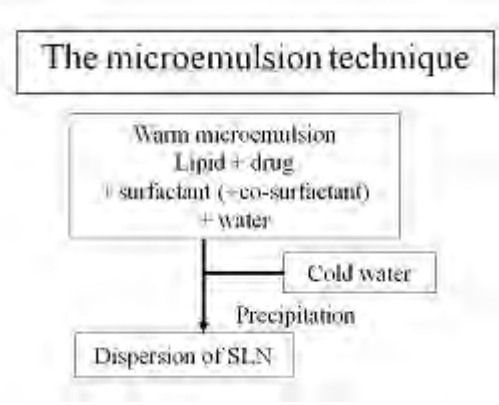


Figure 6 Preparation of SLN from the microemulsion technique (Bunjes and Siekmann, 2006)

The solid lipids that are usually used are stearic acid and glyceryl monostearate because of their moderate melting point. The microemulsion technique is suitable for both lipophilic and hydrophilic drugs.

If the drug is lipophilic, the o/w microemulsion is formed and the drug is dissolved or solubilized in lipid phase.

If the drug is hydrophilic, the w/o/w double emulsion is formed and the drug is dissolved or solubilized in aqueous internal phase.

SLN by this technique is relatively small because they are generated from microemulsion. This technique is simple process and does not require special equipment. There are no risks of damaging shear sensitive drugs. The technique point of this method is precipitation of the lipid particles in water or a dilution system. It leads to a reduction of solid content of SLN dispersion. Table 1 shows the advantages and disadvantages of different techniques.

3. Other techniques

Other techniques such as solvent emulsification-evaporation, solvent emulsification-diffusion are developed to prepare SLN because each technique has different advantages and disadvantages. Each technique is suitable for different drugs depend on their properties. So, no details are mentioned in this part.

Table 1 The comparison of difference techniques (High pressure homogenization and microemulsion techniques) (Bunjes and Siekmann, 2006)

Technique	Advantages	Disadvantages	Suitable for:
The high pressure homogenization (the hot method)	- Stable dispersions - Good reproducibility - High concentration of lipid - No organic solvents required - Established technology	- Use of heat and shear forces - Special manufacturing equipment needed	- Highly lipophilic, thermally stable drugs

Table 1 (Cont.) The comparison of difference techniques (High pressure homogenization and microemulsion techniques) (Bunjes and Siekmann, 2006)

Technique	Advantages	Disadvantages	Suitable for:
The high pressure homogenization (the cold method)	- Thermal stress limited - Limited partitioning of drug during homogenization - No organic solvents required	- Use of shear forces - High energy input required - Yields comparatively coarse dispersions - Special manufacturing equipment needed	- Heat sensitive, lipophilic (and hydrophilic) drugs
The microemulsion	- Simple process, conventional equipment - No shear forces involved	- Often very low initial lipid concentration (i.e., low drug load) - Use of heat - Particle growth upon storage - Usually requires further processing	- Shear sensitive, lipophilic drugs (highly potent drugs in laboratory environment)

Drug incorporation and loading capacity

In general, the highly ordered and tightly packed crystalline particle matrix should be expected to represent a rather unfavorable localization for the incorporation of at least larger amounts of drugs because the drug will disturb the order of the crystal lattice. The incorporation capacity will depend on the physicochemical properties of the drug, but also on the type of matrix material (e.g. pure triglycerides are assumed to have a lower incorporation capacity than complex lipids) and the matrix state (in particular the degree of crystallinity and polymorphic form). Drug molecules that can not be accommodated within the crystalline matrix may adsorb to the nanoparticle surface or separate from the particles. This may lead to the formation of drug crystals or droplet or to distribution into the aqueous phase or additional colloidal structures present in the dispersion (e.g. micelles or phospholipid vesicles) (Bunjes and Siekmann, 2006).

A very important point to judge the suitability of a drug carrier system is its loading capacity. The loading capacity is generally expressed in percent related to the lipid phase. Factors determining the loading capacity of drug in the lipid are, for example:

1. Solubility of drug in melted lipids
2. Miscibility of drug melt and lipid melt
3. Chemical and physical structure of solid lipid matrix
4. Polymorphic state of lipid material

To enhance the solubility in the lipid melt one can add solubilizers. In addition, the presence of mono- and diglycerides in the lipid used as matrix material promotes drug solubilization.

The chemical nature of the lipid is also important because lipids which form highly crystalline particles with a perfect lattice (e.g. monoacid triglycerides) lead to drug expulsion (Westesen, Bunjes, and Koch, 1997). More complex lipids being mixtures of mono-, di- and triglycerides and also containing fatty acids of different chain length form less perfect crystals with many imperfections offering space to accommodate the drug.

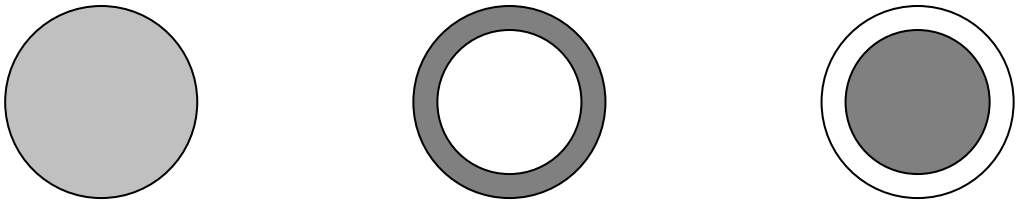
Crystalline structure, related to the chemical nature of the lipid, is a key factor to decide in determining whether a drug will be expelled or firmly incorporated in the long term.

The polymorphic form is also a parameter determining drug incorporation. With increasing formation of the more stable modifications the lattice is getting more perfect and the number of imperfections decreases resulting in the promotion of drug expulsion (Müller, Mäder, and Gohla, 2000). The transformation is slower for long chain than for short chain triglycerides (Bunjes, Westesen, and Koch, 1996).

The drug incorporation models (Müller,Radtke, and Wissing, 2002)

There are basically three different models for the incorporation of active ingredients into SLN.

1. Homogeneous matrix model
2. Drug-enriched shell model
3. Drug-enriched core medel



a) Homogeneous matrix b) Drug-enriched shell c) Drug-enrich core

Figure 7 Models for incorporation of active compounds into SLN

The structure obtained is a function of the formulation composition (lipid, active compound, surfactant) and of the production conditions.

A homogeneous matrix with molecularily dispersed drug or drug being present in amorphous clusters is thought to be mainly obtained when applying the cold homogenization method and when incorporating very lipophilic drugs in SLN with the hot homogenization method. In the cold homogenization method, the bulk lipid

contains the dissolved drug in molecularily dispersed form, mechanical breaking by high pressure homogenization leads to nanoparticles having the homogeneous matrix structure (figure 7a). The same will happen when the oil droplet produced by the hot homogenization method is being cooled, crystallized and no phase separation between lipid and drug occurs during this cooling process. This model is assumed to be valid for incorporation of, e.g. the drug prednisolone, which can show release from 1 day up to weeks (Muhlen and Mehnert, 1998)

An outer shell enriched with active compound can be obtained (figure 7b) when phase separation occurred during the cooling process from the liquid oil droplet to the formation of a solid lipid nanoparticle. According to the diagram, the lipid can precipitate first forming a practically compound-free lipid core. At the same time, the concentration of active compound in the remaining liquid lipid increases continuously during the forming process of the lipid core. Finally, the compound-enriched shell crystallizes comparable to the eutecticum in the diagram. This model is assumed, for example, for coenzyme Q10 which the enrichment leads to a very fast release.

A core enriched with active compound can be formed when the opposite occurs, which means the active compound starts precipitating first and the shell will have distinctly less drug (figure 7c). This leads to a membrane controlled release governed by the Fick law of diffusion. The three models presented each represent the ideal type. There can also be mixed types which can be considered as a fourth model.

Drug release (Müller, Radtke, and Wissing, 2002)

The release profile characterization of SLN may play a significant role on the application. The effect of formulation parameters and production conditions on the release profile from SLN was intensively investigated by Muhlen, Schwarz, and Mehnert (1998). For example, they investigated the release profile as a function of production temperature. It can be summarized that the release was followed by a prolonged release. The burst release was highest when producing at high temperatures and applying the hot homogenization method. It decreased with decreasing temperature and was almost non-existent when applying the cold homogenization method. The extent of burst release could also be controlled by the amount of surfactant used in the formulation. High surfactant concentration leads to high burst

release, low surfactant concentration to minimization of the burst. This was explained by redistribution effects of the compound between the lipid and the water phase during the cooling down process after production of the hot oil in water emulsion during the hot homogenization process. Heating the lipid and water mixture leads to an increased solubility of the active compound in the water phase, the compound partitions from the melted lipid droplet to the water phase. After homogenization, the oil in water emulsion is cooled, the lipid core starts crystallising with still a relatively high amount of active compound in the water phase. Further cooling leads to supersaturation of the compound in the water phase, the compounds tries to partition back into the lipid phase; a solid core has already started forming leaving only the liquid outer shell for compound accumulation. The production condition and composition of formulation affected to model of incorporation drug and the release profile.

Chemical stabilization of incorporated ingredients

Incorporation of active substances into solid matrix of SLN can protect them against chemical degradation by other species, e.g. water or oxygen. This is of relevance for topical agents used for anti-aging and mild acne. SLN improve the chemical stability of tocopherol (Dingler et al., 1999), retinol (Jenning, Schäfer-Korting, and Gohla, 2000; Jenning et al., 2000; Jenning and Gohla, 2001) and coenzyme Q10 (Schäfer-Korting, Mehnert, and Korting, 2007) as compared to an aqueous dispersion. Furthermore, the cream containing curcuminoids loaded SLN can improve stability as comparing to cream base (Waree Tiyaboonchai et al., 2006).

Lightening effect

According to market, appearance is an important factor of a cosmetic to be sold. Not only the packaging should be attractive but also appearance of the product itself. White products are preferred by the consumers. Coenzyme Q10 are yellow color leading to a yellowish appearance of the cream or formulation. The color can be weakened by incorporation of drug into the SLN.

This whitening effect is interesting for active compounds which are degraded and turns to more color such as vitamin C. Not only the incorporation to SLN can be weakened the color but also the improvement of physical and chemical stability.

Adhesiveness, occlusion and skin hydration

Submicron-sized particles show adhesiveness when in contact with surfaces. It has been reported that approximately 4% of lipid nanoparticles with diameter of about 200 nm should form theoretically a monolayer film when 4 mg of formulation is applied per cm^2 (Wissing, Lippacher, and Müller, 2001). Being hydrophobic in character, this mono-layered film has an occlusive action on the skin retarding the loss of moisture caused by evaporation. The different degree of occlusion depended on the size of the applied particles (Wissing and Müller, 2003).

When applying lipid particles onto the skin, a film layer will be formed, having the surface area which is dependent on the particle size.

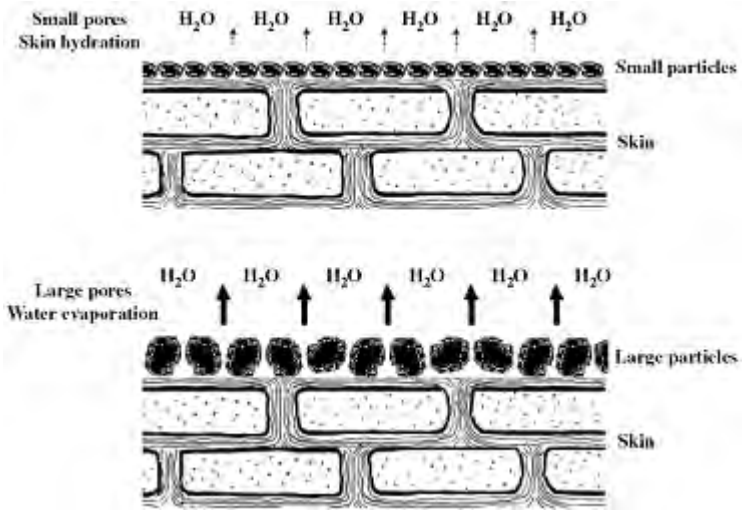


Figure 8 Occlusion effects of lipid particles depending on their size. An aqueous solid lipid nanoparticles dispersion (diameter 500 nm, upper) compared with a solid lipid microparticle dispersion (diameter 1 μm, lower) (Souto and Müller, 2008).

Comparing a layer of nanoparticles (figure 8, upper) to a microparticles (figure 8, lower), the dimensions of the air channels will be much smaller. Thus, the

hydrodynamic evaporation of water will decrease. On the contrary, the larger pores will facilitate the water loss from the surface of the skin and enhance the moisture loss.

Under aging the dermis loses much of its elasticity. The skin stretched by muscular movement then fails to shrink back to its normal smoothness and wrinkles are formed. Because of their hydration properties, it can be assumed that lipid nanoparticles may enhance skin elasticity (Wissing and Müller, 2003).

A consequence of the occlusive properties, SLN may also increase skin penetration of active ingredients.

Skin penetration

It is necessary to consider the function of topically applied cosmetic actives. A majority of those substances are not intended for deeper skin penetration and absorption. Cosmetic actives are intended to have a predominantly local effect, thus it is undesired to have an absorption into the blood.

Penetration of actives when applying common topical base ingredients does not occur to any large extent, although such materials may become enmeshed or entrained in the outer regions of the horny layer when massage is applied. Modulation of release and active penetration into certain layers of the skin can be achieved as a consequence of e.g. the creation of supersaturated systems (Müller, Radtke, and Wissing, 2002). These systems can be created by incorporation of lipid nanoparticles into topical formulation (creams, ointments, emulsions, gels). The increase in saturation solubility will lead to an increased diffusion pressure of the active into the skin. During shelf life, the active remains entrapped into the lipid matrix because this latter preserves its polymorphic form. After application of supersaturated cream onto the skin, and because of an increase in temperature and water evaporation, increasing the thermoactivity, the lipid matrix transforms from a more unstable polymorph into a more ordered polymorph leading to the release of active into a system already saturated with the same active, and thus creating a supersaturation effect.

SLN as novel UV sunscreen system

It was discovered that highly crystalline SLN can also act as particulate UV blockers by scattering the light efficiently. To enhance the UV protection by SLN, a molecular sunscreen was incorporated into the SLN matrix. Incorporation was performed in a way that the release prolonged. The fixation of the molecular sunscreen inside the solid matrix minimizes side effects due to penetration of the molecular sunscreen into the skin. It was found that incorporation of the molecular sunscreen into the SLN matrix led to a synergistic protective effect. This means the total amount of molecular sunscreen in the formulation can be reduced, thus further minimizing the side effects in addition to the already achieved reduction by firm incorporation of the sunscreen into the particle matrix (Müller, Radtke, and Wissing, 2002).

C. The property of material used in this study

1. Glyceryl monostearate (Rowe, Sheskey, and Owen, 2006)

Synonyms : monostearin, GMS, glycerin monostearate, glycerol monostearate

Empirical formula : $C_{21}H_{42}O_4$

Molecular weight : 358.6

Structural formula

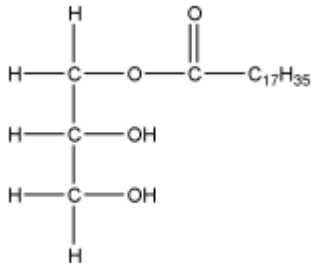


Figure 9 Structure formula of glyceryl monostearate

HLB value : 3.8

Melting point : 55 – 60°C

Glyceryl monostearate is consisted of not less than 90% of monoglycerides, chiefly glyceryl monostearate and glyceryl monopalmitate (USPNF 20) or a mixture of monoacylglycerols, mostly monostearoylglycerol, together with quantities of di- and triacylglycerols. It contains 40 – 55% of monoacylglycerols, 30 – 45% of diacylglycerols, and 5 – 15% of triacylglycerols (PhEur 2002).

Glyceryl monostearate is a white to cream-colored, waxlike solid in the form of beads, flakes, or powder. It is waxy to the touch and has a slight fatty odor and taste.

Glyceryl monostearate is widely used in cosmetics, foods, oral and topical pharmaceutical formulations and is generally regarded as a nontoxic and nonirritant material.

2. Glyceryl behenate (Rowe, Sheskey, and Owen, 2006)

Synonyms : Compritol 888 ATO, glycerol dibehenate

Structural formula

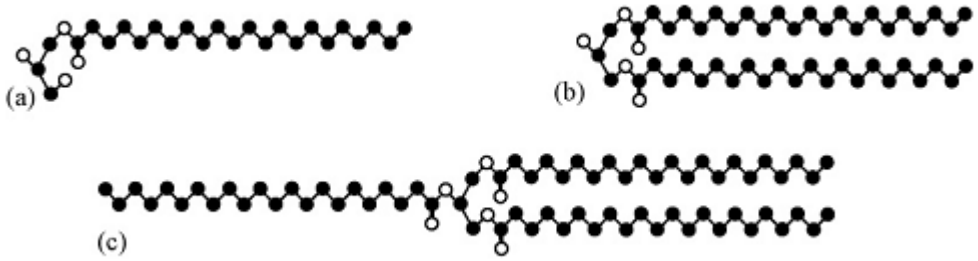


Figure 10 Structural formula of compritol 888 ATO (Brubach et al., 2007)
(a) is a monobehenate, (b) is a dibehenate and (c) is a tribehenate

HLB value : 2

Melting point : 65 – 77°C

Glyceryl behenate is a mixture of glycerides of fatty acids, mainly behenic acid. It specifies that the content of 1-monoglycerides should be 12.0 – 18.0% (USPNF 20) or a mixture of diacylglycerols, mainly dibehenoylglycerol, together with variable quantities of mono- and triacylglycerols (PhEur 2002).

Glyceryl behenate is used in cosmetics, foods and oral pharmaceutical formulations. In cosmetics, it is mainly used as a viscosity-increasing agent in emulsions.

Glyceryl behenate occurs as a fine white powder or hard waxy mass with faint odor.

Glyceryl behenate is used in cosmetics, foods and oral pharmaceutical formulations and is generally regarded as a relatively nonirritant and nontoxic material.

3. Gelucire® 50/13

Chemical name : Stearoyl macrogolglycerides (polyoxylglycerides)

Structural formula

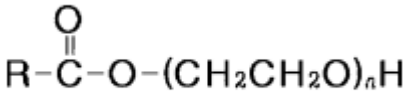


Figure 11 Structural formula of Gelucire® 50/13 (n = 32, R = fatty acid, mixed glycerides of C16 is 40 – 50%, C18 is 48 – 58% and C16 + C18 is 90%)

Appearance : Waxy solid pellets

HLB value : 13

Melting point : 50°C

Gelucires are formed by esterification of PEG 1500 and hydrogenated palm oil. They are composed of partial glycerides and esters of PEG 1500. Gelucires are a family of glyceride-based excipients which may be used in the manufacture of controlled release dosage forms. These materials consist of mixtures of mono-, di- and triglycerides with polyethylene glycol (PEG) stearates, the nature and proportion of these components determining the hydrophobicity and drug release properties of the corresponding dosage forms. Each Gelucire is described by two numbers, the first referring to the melting point of the base and the second to the HLB number, hence the nomenclature adopted for these materials gives some indication as to their physical properties (Sutananta, Craig, and Newton, 1996). Gelucire 50/13 contains a higher proportion of the hydrophillic PEG stearates (Sutananta, Craig, and Newton, 1994). Gelucire® 50/13 is an excipient that can greatly improve the bioavailability of poorly-soluble drugs. It can be used in a variety of formulation techniques such as capsules, granules and tablets. When formulated in capsules, it confers a slow release profile due to its drop point. Gelucire® 50/13 provides an immediate release due to its high HLB when used in granulation or other combined approaches.

4. Polyoxyethylene 40 stearate (Rowe, Sheskey, and Owen, 2006)

Synonyms : Myrj 52, macrogol stearate 2000, PEG-40 stearate, polyoxyethylene glycol 2000 monostearate, polyoxylethylene (40) monostearate

Empirical formula : $C_{98}H_{196}O_{42}$

Molecular weight : 2046.61

Structural formula

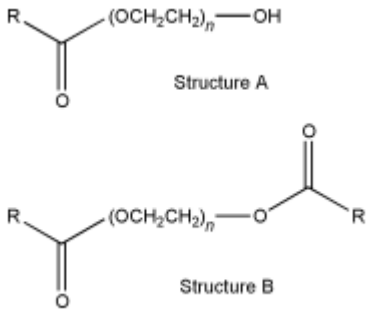


Figure 12 Structural formula of polyoxyethylene 40 stearate

Structural A applies to the monostearate;

where the average value of n is 40 for polyoxyl 40 stearate.

Structural B applies to the distearate;

where the average value of n is 40 for polyoxyl 40 distearate.

In both structures, R represents the alkyl group of the parent fatty acid.

With stearic acid, R is $CH_3(CH_2)_{16}$.

HLB value : 16.9

Melting point : about 38°C

Polyoxyethylene 40 stearate is nonionic surfactant produced by polyethoxylation of stearic acid.

Polyoxyethylene 40 stearate is waxy solid, with a faint, bland, fatlike odor, off-white to light tan in color.

Polyoxyethylene 40 stearate is used as emulsifying agent in topical pharmaceutical formulations. It has also been used in intravenous injection and oral preparation. It is generally regarded as essentially nontoxic and nonirritant material.

5. Poloxamer 188 (Rowe, Sheskey, and Owen, 2006)

Synonyms : F-68, Lutrol, Pluronic, polyethylene-propylene glycol copolymer, polyoxyethylene-polyoxypropylene copolymer

Empirical formula : $HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$

Molecular weight : 7680 – 9510

Structural formula

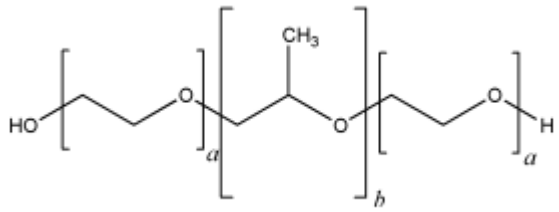


Figure 13 Structural formula of poloxamer 188

For poloxamer 188, a is 80 and b is 27.

HLB value : 29

Melting point : 52 – 57°C

Poloxamer 188 is a nonionic emulsifying or solubilizing agent. It generally occurs as white, waxy, free-flowing prilled granules, or as cast solids. It is practically odorless and tasteless.

Poloxamer 188 is used in a variety of oral, parenteral and topical pharmaceutical formulations and is generally regarded as nontoxic and nonirritant materials.

D. Methods of measuring skin conditions

1. Skin color (Park and Lee, 2005)

Several methods are available for assessing skin colors. Recently, a hand-held microprocessor controlled reflectance spectrophotometer such as Derma-Spectrophotometer® (figure 14) (Cortex technology, Hudsund, Denmark) began to used in many dermatological studies. This instrument provides a readout of the erythema and melanin indices as a function of the absorbance characteristic of human skin. Each index increases as the skin becomes more erythematous and more pigmented, respectively, so the melanin index (M-index) can be regarded as a parameter which is mainly influenced by the melanin content.

The reflectance spectrophotometer is a narrow-band spectrophotometer designed for measuring specific colors due to two major chromophores, hemoglobin and melanin. The light sources are two light-emitting diodes with selected narrow bands of emitted wavelengths. The peaks of the two bands are centered at 568 nm (green light) and 655 nm (red light). They emit light in sequence, and the reflected light from skin is detected with a photodetector. After being converted into digital form with a built-in microcomputer, the reflectance in the two bands are transformed into the erythema index (E-index) and the melanin index (M-index).

Figure 14 Derma-Spectrophotometer® (Cortex technology, Hudsund, Denmark)

2. Skin roughness (CK electronic GmbH, 2005)

Skin roughness is measured by Visioscan VC 98 (figure 15). A measurement where the skin can be monitored optically using an image digitalization process without using replica is a great process in scientific research, done by Prof. Tronnier from the University of Witten in Germany. This new method is called SELS (Surface Evaluation of the Living Skin). It is based on a graphic depiction of the living skin under special illumination and the electronic processing and evaluation of this image according to four clinical parameters. The parameters correspond quantitatively and qualitatively to the physiological condition of the skin surface. Those parameters are, according to Prof. Tronnier, the skin smoothness (SEsm), which is the calculation from the average width and depth of the wrinkles. Evidently, the tenser the skin is, the better (e.g. in its youth). The second parameter is skin roughness (SEr), which seems to be the opposite parameter first. The scaliness (SEsc) which shows the level of dryness of the stratum corneum, and the wrinkles (Sew) calculated from the proportion of horizontal and vertical wrinkles, are the third and fourth parameters, respectively.

Figure 15 Visioscan® VC98 (Courage and Khazaka, Cologne, Germany)

Roughness (R1-R5) were another important parameters to evaluate the skin surface. These parameters derive originally from the metal industry and are leaned on to the directive DIN 4762-4768 as Ra-Rz. Choose if the lines should be arranged horizontally, vertically or circularly. The advantage of circularly arranged lines is that an influence from the direction of the wrinkles is compensated.

Skin roughness R1: The roughness R1 is the distance between the highest mountain and the lowest value, referred to as a reference length l. In the DIN norm this parameter is known as Rt.

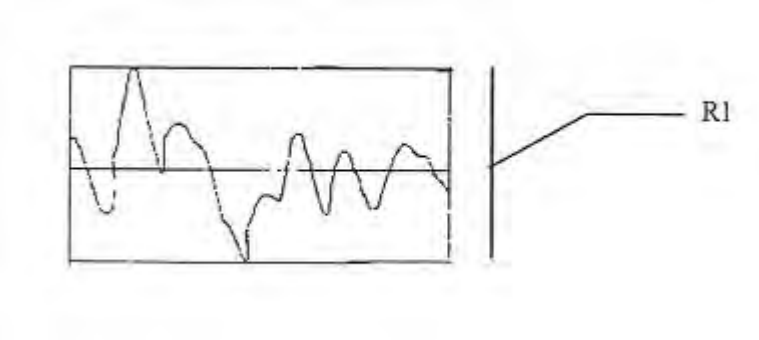


Figure 16 Skin roughness R1

Skin roughness R2: R2 is the biggest roughness of the different segment roughnesses. In DIN norm this parameter is known as Rm or Rmax.

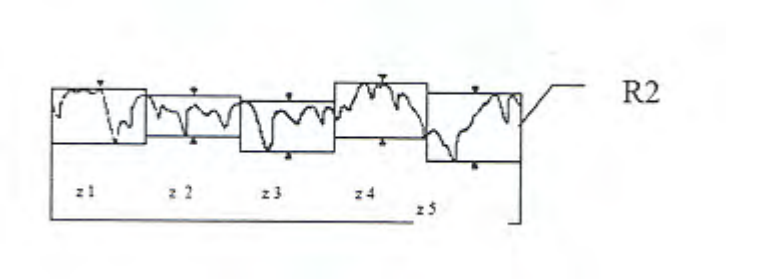


Figure 17 Skin roughness R2

Average roughness R3: The average roughness is the arithmetic average of the different segment roughnesses calculated from 5 succeeding measurement segments of the same length. In contrast to R1, R3 is not that much influenced by artifacts due to calculating the average. In the DIN norm this parameter is known as Rz.

Smoothness Depth R4: This parameter describes the area above the real profile and a line drawn above the highest mountain (reference profile). This area is divided by the average height of the profile, therefore R4 is distance. In the DIN norm this parameter is known as Rp.

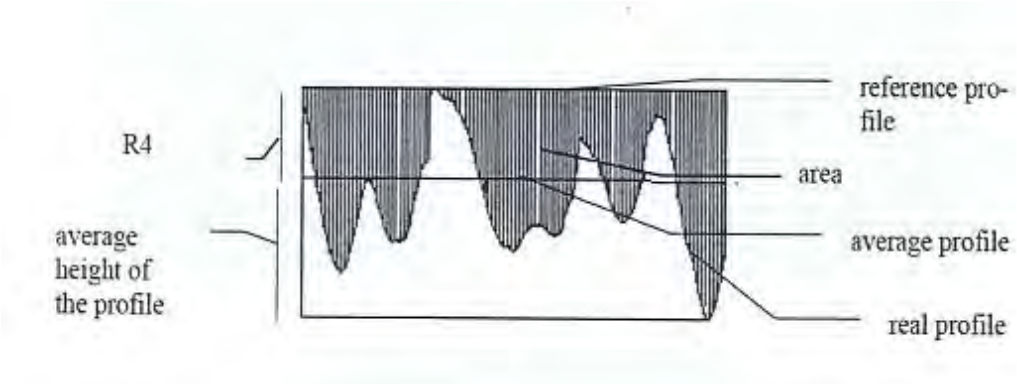


Figure 18 Smoothness depth R4

Arithmetic average roughness R5: The area surrounded by the profile and the average profile is calculated. The average profile is the profile on the average height between the real profile and the reference profile drawn on the top of the highest mountain. The area is divided by the average height, thus R5 is a distance: the distance describing the standard deviation of the reference profile to R4. In the DIN norm this parameter is known as Ra.

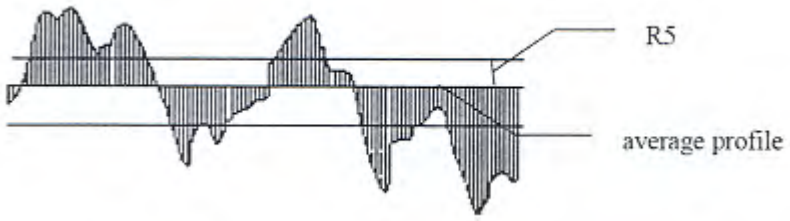


Figure 19 Arithmetic average roughness R5

In this study, the average roughness R3 was chosen as a key parameter. Circular arranged lines (circular roughness) and horizontal + vertical lines (mean roughness) were used as roughness parameters.

3. Skin elasticity (Wilhelm, Cua, and Maibach, 1991; Takema et al., 1994; Harnisch et al., 1999)

The mechanical properties of the skin have always been a very important subject for dermatologists and biophysicists. The biomechanical parameters of the skin like

the elasticity are characteristic for different body site. There vary very mach during the aging process or certain diseases of the skin.

Physiologic skin aging starts at the age of thirty. Indications for that are flabbiness, wrinkles, dehydration, blotchy pigmentation and loss of elasticity.

Our skin is viscoelastic, i.e. elastic as well as plastic properties are presented. The following examples explain these characteristics:

An inflated balloon is fully elastic. It is deformed by pressure applied to the surface, but it regains its original shape, when the pressure is stopped. Depending on how much the balloon is inflated the deforming pressure results in different amplitudes. If the surface is tight due to high inflation the amplitude is small, but the deformation is elastic in any case.

Modeling clay, e.g. plasticine is completely plastic. Pressure deforms plasticine easily. The deformation stays when the pressure is stopped. The deformation can be continued by applying pressure again.

A young skin with good blood circulation is very elastic, but does not resemble a balloon. The skin does not regain its original state immediately after application of force, but stays slightly deformed. This phenomenon is called hysteresis. In old skin with bad blood circulation the plastic deformation dominates.

Different parts of the body do not only have different degrees of elasticity and plasticity but in elastic skin also the amplitudes differ. The room conditions (temperature and humidity) also influence skin elasticity.

The measuring principle is based on suction and elongation. The device generates negative pressure, which can be varied between 20 and 500 mbar. The skin area to be measured is drawn into the aperture of the probe due to the negative pressure. The penetration depth of the skin into the aperture is determined without contact by an optical measuring system.

This system consists of a light emitter and light acceptor. Two opposing glass prisms transmit the light from emitter to acceptor. The light ratio changes proportionally to the penetration depth of the skin.

High losses of the elastic properties of the skin are generally due to anomalies in the collagen biosynthesis. In vivo measurements of the elasticity are great interest for the quantification of the stiffness of the skin and finding a correlation with the course

of the illness. Elasticity measurements also lead to important results in case of psoriasis, especially if the psoriasis plaques, which are characterized by thickening of the skin, are measured. Measurement of elasticity on keloids gives information on the healing process.

Elasticity measurements are not only indispensible in the field of clinical and practical dermatology but also for testing the efficiency of cosmetic and pharmaceutical products.

The measurement of the skin elasticity for the determination of different parameters of the mechanical properties of the skin must have its place beside the biochemical characterization of the skin in dermatological diagnosis, therapy, research and development.

In order to ensure un-biased readings, the Dermalab® (figure 20) (Cortex technology, Denmark) features a light weight probe which, when glued to the skin using a double adhesive sticker, eliminates movement artifacts from holding the probe. With the probe in place, negative pressure will elevate the skin, and the differential negative pressure needed to lift the skin a predetermined distance is used as input to calculate Young´s modulus.

Figure 20 Dermalab® (Cortex technology, Denmark)

4. Skin hydration and sebum (Mitsui, 1997; Wilhelm, Cua, and Maibach, 1991)

4.1 Skin hydration

The measurement of the skin moisture is based on the worldwide acknowledged Corneometer® CM 825, a capacitance method.

This measurement is based on the completely different dielectric constant of water and other substances. The measuring capacitor shows changes of capacitance according to the moisture content of the samples. A glass lamina separates the metallic tracks (gold) in the probe head from the skin in order to prevent current conduction in sample. An electric scatter field penetrates the skin during the measurement and the dielectricity is determined. One trick builds up a surplus of electrons (minus charge) the other a lack of electrons (plus charge). An electric field between the tracks with alternating attraction develops. During the measurement the scatterfield penetrates the very first layer of the skin and determines the dielectricity.

In contrary to the impedance measurement no galvanic relation between the device and the measuring object and no polarization effects exist.

4.2 Sebum

The measurement of sebum on the skin as well as on the hair and scalp is based on the worldwide acknowledged Sebumeter® method. It is a direct measurement of the sebum secretion on skin, hair and scalp.

The measurement principle is the photometric method, the grease spot photometer. This method is indifferent to moisture.

The supplied sebumeter-cassette contains a mat synthetic tape which is 0.1 mm thick. The measuring head of the cassette exposes a 64 mm^2 measuring section of the tape. For the next measuring the tape is transported by a trigger at the side of the cassette so that a new measuring section is exposed. The used tape is rewound inside the cassette.

On cassette can be used for approximately 450 measurements. The scale from 1-0 on the trigger shows how much of the tape is still unused. The complete cassette is exchanged for hygienic reasons.

A mirror under the measuring section of the tape protrudes approximately 1 mm from the measuring head. This mirror is linked with the cassette by a 3 N spring. This makes sure that the tape is pressed onto the measuring area with constant pressure by the mirror. The measuring time of 30 seconds is controlled by the clock in the device.

For determination of the sebum, the measuring head of the cassette is inserted into the aperture of the device, where a photocell measures the transparency. The light transmission represents the sebum content on the surface of the measuring area. A

microprocessor calculates the result, which is shown on the display in µg sebum/cm^2 of the skin.

Figure 21 shows Skin Diagnostic® SD 27, portable device with Corneometer® and Sebumeter®.

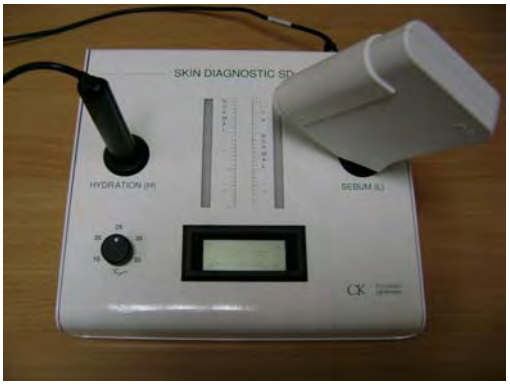


Figure 21 Skin Diagnostic® SD 27, portable device with Corneometer® and Sebumeter® probe to measure the skin hydration and sebum content.

CHAPTER III

MATERIALS AND METHODS

The experiments were divided into five parts :

A. Development and validation of UV-Visible spectrophotometric and HPLC methods for determination of Puag-Haad and oxyresveratrol content

B. Development of SLN containing Puag-Haad

C. Characterization of SLN containing Puag-Haad

D. Physical and chemical stability study of SLN containing Puag-Haad formulations in comparison with aqueous solutions of Puag-Haad

E. Evaluation of whitening and anti-wrinkle efficacy of SLN containing Puag-Haad in human volunteers

Crude Drug

1. Dried aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad), a local herbal medicine store, Chiangmai, Thailand, Lot no. 10/47

Materials

1. Acetonitrile, AR grade, Lab Scan Co., Ltd., Thailand, Lot no. 07121153
2. Chloroform, AR grade, Lab Scan Co., Ltd., Thailand, Lot no. 08041023
3. Citric acid monohydrate, Ajax Finechem, Australia, Lot no. AF411021
4. Furazolidone was a gift from Miss Manatchaya Wanawatanakun
5. Glyceryl behenate (Compritol® 888 ATO), Gattefosse', France, Lot no. 103100
6. Glyceryl monostearate (Nikkol®), S Tong Chemicals Co., Ltd., Thailand, Lot no. 2/2144744
7. Macrogol glyceryl stearate (Gelucire® 50/13), Gattefosse', France, Lot no. 102880
8. Methanol, AR grade, Lab Scan Co., Ltd., Thailand, Lot no. 08091114
9. Methanol, HPLC grade, Lab Scan Co., Ltd., Thailand, Lot no. 08080221

10. Oxyresveratrol Standard (244 g/mol), purified from Puag-Haad (purity > 95%), with courtesy of Associate Professor Dr. Rutt Suttisri
11. Poloxamer 188 (Lutrol® F-68), BASF, Germany, Lot no. WPAB524C
12. Polyoxyethylene 40 stearate (Myrj®52), Sigma, USA, Lot no. 028K0119
13. Propylene glycol, Srichand United dispensary Co., Ltd., Thailand, Lot no. 10215
14. Tri-sodium citrate dihydrate, Merck, Germany, Lot no. A327248 133

Apparatuses

1. Analytical balance (Model AX105, Mettler Toledo, Switzerland)
2. Centrifuge (Model Universal 320R, Hettich, Germany)
3. Dermalab® (Cortex technology, Denmark)
4. Derma-Spectrometer (Cortex technology, Denmark)
5. Differential scanning calorimeter (DSC822^{e}, Mettler Toledo, Switzerland)
6. Freeze dryer (Model FD-6-850MPO, Dura-Dry™, FTS System Inc., USA)
7. High performance liquid chromatography system
   - Automatic sample injector (SIL-10A, Shimadzu, Japan)
   - Communications bus module (CBM-10A, Shimadzu, Japan)
   - Column (Luna Phenomenex® C18 (2), 5 µm, 250 x 4.6 mm, USA, Lot no. 5291-60)
   - Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
   - Precolumn (µBondapack C18, 10 µm, 125 Aº, Water Corporation, Ireland, Lot no. W2336B1)
   - UV-VIS detector (SPD-10A, Shimadzu, Japan)
8. High pressure homogenizer (Model EmulsiFlex C5, Avestin, Canada)
9. High speed stirrer (WiggenHouser, Germany)
10. Micropipette (Biohit, Finland)
11. Modified Franz Diffusion cells (Crown Glass Company, USA)
12. Magnetic stirrer (Model RCT basic, KIKA® Works Guangzhou, China)
13. Photon correlation spectrometer (Zetapals, Brookhavern Instrument, USA)
14. pH meter (Orion model 420A, Orion Research Inc., USA)
15. Skin diagnostic SD27 (CK Cologne, Germany)

16. Stability cabinet (Eurotherm Axyos, Germany)
17. Stopwatch (Heuer, Switzerland)
18. Transmission electron microscope (Model JEM-1230, Jeol, Japan)
19. Ultrasonicator (Crest Ultrasonics, Model 275DAE, Malaysia)
20. UV-Visble Spectophotometer (UV-1601, Shimadzu, Japan)
21. Vacuum pump (CB 169 Vacuum System, Buchi, Switzerland)
22. Video Digitizer® VD300, Visioscope BW30 and Visioscan® VC98 (Courage and Khazaka, Cologne, Germany)
23. Vortex mixer (Vortex Ginies-2, Scientific Industries, USA)
24. Water bath (Model WB22, Becthai Co., Ltd., Thailand)

Accessories

1. Centrifugal filter device, MWCO = 50 K (Centriprep®, Millipore corporation, USA) Lot no. PVA025
2. Disposable syringe filter nylon 13 mm, 0.45 µm (Chrom Tech, USA)
3. Double sticking rings
4. Medfix cloth tape, latex free product (Medline) Lot no. 41163010
5. Parafilm (American National Can TM, USA)
6. Regenerated cellulose membrane, MWCO 12,000-14,000 (CelluSep®T4, Canada) Lot no. 8764
7. Sebum cassette SM810
8. Whatman filter paper No.1, 150 mm (Whatman International Ltd., England)

Methods

Part A. Development and validation of UV-Visible Spectrophotometric and HPLC method for determination of Puag-Haad and oxyresveratrol content

1. Determination of Puag-Haad content in SLN containing Puag-Haad by UV-Visible Spectrophotometric method (for initial evaluation of SLN)

The determination of Puag-Haad was performed by UV-Visible Spectrophotometric method due to its rapidity and convenience, especially during the initial evaluation phase. The amount of Puag-Haad was determined from its maximum absorption at 327 nm.

1.1 Standard solutions of UV-Visible Spectrophotometric method

A stock solution of blank SLN (blank SLN contained about 5% w/w of lipid) was prepared by weighing 1 g of blank SLN into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to the final volume. The final concentration of lipid was 2 mg/ml.

A stock solution of Puag-Haad was prepared by accurately weighing 25 mg of Puag-Haad into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to the final volume. After filtering, this stock solution was clear and had a concentration of 1 mg/ml. Standard solutions of Puag-Haad were prepared by pipeting 20, 40, 60, 80, 100 and 120 μl of the above solution into a respective 10-ml volumetric flask filled with 0.5 ml of the stock solution of blank SLN as matrix. The solutions were adjusted to volume with methanol so that the concentrations of Puag-Haad were 2, 4, 6, 8, 10 and 12 μg/ml, respectively. The concentration of lipid was 100 μg/ml. A solution of blank SLN in methanol (no Puag-Haad) which had the same concentration of lipid was used as blank. These standard solutions were prepared for each run. As a result, the standard curve of Puag-Haad between concentration and absorbance were plotted.

1.2 Preparation of sample solution (for assay of total Puag-Haad in SLN)

The sample stock solution was prepared by weighing 1 g of SLN containing Puag-Haad (about 0.25% w/w of Puag-Haad) into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to the final volume. The concentration of Puag-Haad in this solution was 0.1 mg/ml. Then, 0.5 ml of this stock solution was transferred into a 10 ml volumetric flask. The solution was adjusted to volume with methanol to give the final concentration of Puag-Haad of 5 μg/ml. A solution of blank SLN in methanol (no Puag-Haad) which had the same concentration of lipid was used as blank.

1.3 Validation of UV-Visible Spectrophotometric method

The analytical parameters used in the assay validation for UV-Visible Spectrophotometric method were specificity, linearity, accuracy and precision.

1.3.1 Specificity

The wavelength of maximum absorbance of Puag-Haad must not be interfered by the absorbance of other components in the sample.

1.3.2 Linearity

Three sets of six standard solutions were prepared and analyzed. Linear regression analysis of the absorbance versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

Acceptance criteria:

The coefficient of determination should be more than 0.9990.

1.3.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of analytical recovery. Five sets of three concentrations of Puag-Haad at 3, 7 and 11 μg/ml with blank SLN (the concentration of lipid was 100 μg/ml) were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100

Acceptance criteria:

The percentage of analytical recovery should be within 98.0-102.0% of each nominal concentration.

1.3.4 Precision

a) Within-run precision

The within-run precision was determined by analyzing five sets of three concentrations of Puag-Haad at 3, 7 and 11 μg/ml with blank SLN (the concentration of lipid was 100 μg/ml) in the same day. The percent coefficient of variation (%CV) of Puag-Haad of each concentration was determined.

b) Between-run precision

The between-run precision was determined by analyzing three concentrations of Puag-Haad at 3, 7 and 11 μg/ml with blank SLN (the concentration of lipid was 100 μg/ml) on five different days. The percent coefficient of variation (%CV) of Puag-Haad of each concentration was determined.

Acceptance criteria:

The percent coefficient of variation for both within-run and between-run precision should be less than 2%.

2. Determination of Puag-Haad content in Puag-Haad-entrapped pellets by UV-Visible Spectrophotometric method

During the selection of appropriate formulations, the percentage of entrapment efficiency of Puag-Haad was calculated. The determination of Puag-Haad was performed by UV-Visible Spectrophotometric method during the initial evaluation phase due to its rapidity and convenience. The amount of Puag-Haad was determined from its maximum absorption at 327 nm.

2.1 Standard solutions of UV-Visible Spectrophotometric method

The following topic was performed as the same concentrations and same conditions as topic 1.1.

2.2 Preparation of sample solution

With the starting amount of 1 g SLN, the Puag-Haad-entrapped pellets were transferred from the centrifugal filter device into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to volume. Then, the next processes were performed as the same concentrations and same conditions as topic 1.2.

3. Determination of Puag-Haad content in Puag-Haad-unentrapped filtrate by UV-Visible Spectrophotometric method

The amount of free Puag-Haad in the filtrate after separation was also analyzed using the following steps:

3.1 Standard solutions of UV-Visible Spectrophotometric method

The following topic was performed as the same concentrations and same conditions as topic 1.1. But, there was no need to add a stock solution of blank SLN into a set of standard solutions. Water was used to adjust the final volume to 10.0 ml instead of methanol and was also used as a blank.

3.2 Preparation of sample solution

The filtrates were collected from centrifugal filter device into a 25 ml volumetric flask. Water was added to dilute and adjust to the final volume. Then, 2 ml of this stock solution was transferred into a 10 ml volumetric flask. The solution was adjusted to volume with water. In addition, water was used as a blank.

4. Determination of oxyresveratrol content in SLN containing Puag-Haad by HPLC method

The determination of active constituent (oxyresveratrol) was performed by HPLC method due to its specificity and high sensitivity.

4.1 HPLC condition

The HPLC conditions as adapted from Manatchaya Wanawatanakun (2006)

Column	:	Luna Phenomenex® C18 (2) (5 μm, 250 x 4.6 mm)
Mobile phase	:	methanol : water (40:60)
Injection volume	:	100 μl
Flow rate	:	1 ml/min
Detector	:	UV detector at 329 nm
Temperature	:	ambient
Run time	:	23 min
Internal standard	:	Furazolidone

The mobile phase was prepared by using methanol and water with the ratio of 40:60 %v/v. The mixture solution was thoroughly mixed, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 minutes prior to use.

Standard solutions of HPLC method

Furazolidone was used as an internal standard due to its appropriate retention time and potential optimal resolution from oxyresveratrol peak. A stock solution of furazolidone was prepared by accurately weighing 15 mg of furazolidone into a 100 ml volumetric flask. 25 ml of acetonitrile was added to dissolve and the mobile phase was added to adjust the final volume. The final concentration of furazolidone was 0.15 mg/ml.

A stock solution of blank SLN (blank SLN contained about 5% w/w of lipid) was prepared by weighing 1 g of blank SLN into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to the final volume. The final concentration of lipid was 2 mg/ml.

A stock solution of oxyresveratrol was prepared by accurately weighing 12.5 mg of oxyresveratrol into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to the final volume. This stock solution had a concentration

of 0.5 mg/ml. Standard solutions of oxyresveratrol were prepared by pipeting 10, 100, 200, 300, 400 and 500 μl of the above solution into a respective 10-ml volumetric flask filled with 0.5 ml of the stock solution of blank SLN as matrix. Then, 1 ml of furazolidone stock solution was added into each volumetric flask. The solutions were adjusted to volume with mobile phase so that the concentrations of oxyresveratrol were 0.5, 5, 10, 15, 20 and 25 μg/ml, respectively. The concentration of furazolidone was 15 μg/ml and the concentration of lipid was 100 μg/ml. These standard solutions were prepared for each HPLC run. As a result, the standard curve of oxyresveratrol between concentration and peak area ratio were plotted.

4.3 Preparation of sample solution (Total concentration of oxyresveratrol in SLN)

The sample stock solution was prepared by weighing 1 g of SLN containing Puag-Haad (about 0.25% w/w of Puag-Haad) into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to the final volume. The concentration of Puag-Haad in this solution was 0.1 mg/ml. Then, 0.5 ml of this stock solution and 1 ml of furazolidone stock solution were transferred into a 10 ml volumetric flask. The solution was adjusted to volume with mobile phase to give the final concentration of Puag-Haad and furazolidone of 5 and 15 μg/ml, respectively.

4.4 Validation of HPLC method

The analytical parameters used in the assay validation for HPLC method were specificity, linearity, accuracy and precision.

4.4.1 Specificity

Under the chromatographic conditions used, the peak of oxyresveratrol must be completely separated from and not be interfered by the peaks of other components in the sample.

4.4.2 Linearity

Three sets of six standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

Acceptance criteria:

The coefficient of determination should be more than 0.9990.

4.4.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of analytical recovery. Five sets of three concentrations of oxyresveratrol at 1, 12 and 24 μg/ml with blank SLN (the concentration of lipid was 100 μg/ml) were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100

Acceptance criteria:

The percentage of analytical recovery should be within 98.0-102.0% of each nominal concentration.

4.4.4 Precision

a) Within-run precision

The within-run precision was determined by analyzing five sets of three concentrations of oxyresveratrol at 1, 12 and 24 μg/ml with blank SLN (the concentration of lipid was 100 μg/ml) in the same day. Peak area ratios of oxyresveratrol to furazolidone were calculated and the percent coefficient of variation (%CV) at each concentration was determined.

b) Between-run precision

The between-run precision was determined by analyzing three concentrations of oxyresveratrol at 1, 12 and 24 μg/ml with blank SLN (the concentration of lipid was 100 μg/ml) on five different

days. The percent coefficient of variation (%CV) at each concentration was determined.

Acceptance criteria:

The percent coefficient of variation for both within-run and between-run precision should be less than 2%.

5. Determination of oxyresveratrol content in Puag-Haad-entrapped pellets by HPLC method

In the step of characterization of SLN containing Puag-Haad, the percentage of entrapment efficiency was calculated. So, the amount of entrapped oxyresveratrol in pellets had to be analyzed. The determination of oxyresveratrol was performed by HPLC method due to its specificity and high sensitivity.

5.1 HPLC condition

The following topic was performed as the same conditions as topic 4.1.

5.2 Standard solutions of HPLC method

The following topic was performed as the same concentrations and same conditions as topic 4.2.

Preparation of sample solution

Starting from 1 g SLN sample, Puag-Haad-entrapped pellets were transferred from centrifugal filter device into a 25 ml volumetric flask. Methanol was added to dissolve and adjust to volume. Then, the next processes were the same as topic 4.3.

6. Determination of oxyresveratrol content in Puag-Haad-unentrapped filtrate by HPLC method

To determine the amount of unentrapped Puag-Haad, the amount of free oxyresveratrol in the filtrate was analyzed.

6.1 HPLC condition

The following topic was performed as the same conditions as topic 4.1.

6.2 Standard solutions of HPLC method

The following topic was performed as the same concentrations and same conditions as topic 4.2. However, there was no need to add a stock solution of blank SLN into a set of standard solutions.

6.3 Preparation of sample solution

The filtrates were collected from centrifugal filter device into a 25 ml volumetric flask. Water or citrate buffer pH 5.5 was added to dilute and adjust to the final volume. Then, 2 ml of this stock solution and 1 ml of furazolidone stock solution were transferred into a 10 ml volumetric flask. The solution was adjusted to volume with mobile phase. The final concentration of furazolidone was 15 μg/ml.

Part B. Development of SLN containing Puag-Haad

1. Preparation of blank SLN

1.1 Preparation of blank SLN by w/o/w double emulsion method (modified from Morel et al., 1996; Ma et al., 2007)

To understand easily, the diagram shows the scope of this study in the step of preparation of blank SLN by w/o/w double emulsion method as in figure 22.

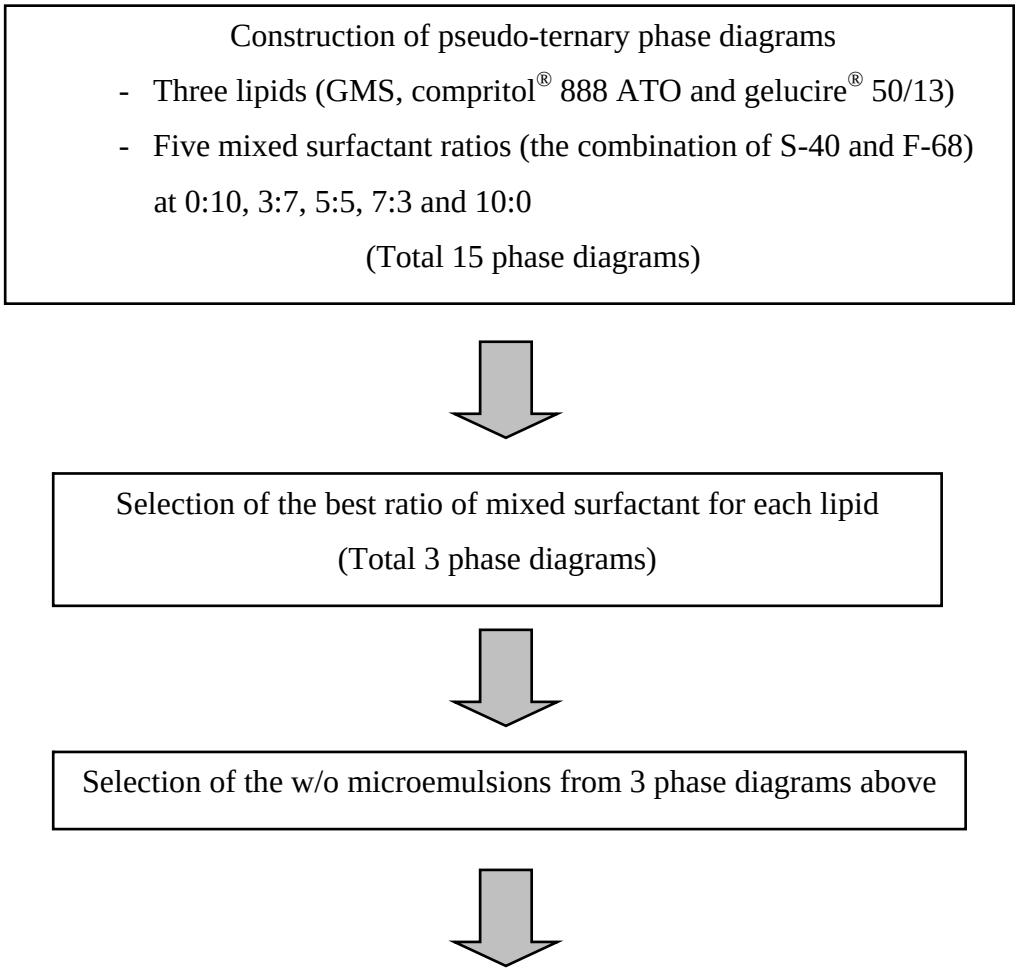


Figure 22 The scope of the study in the step of preparation of blank SLN by w/o/w double emulsion method (GMS = glyceryl monostearate, S-40 = polyoxyethylene 40 stearate, F-68 = poloxamer 188)

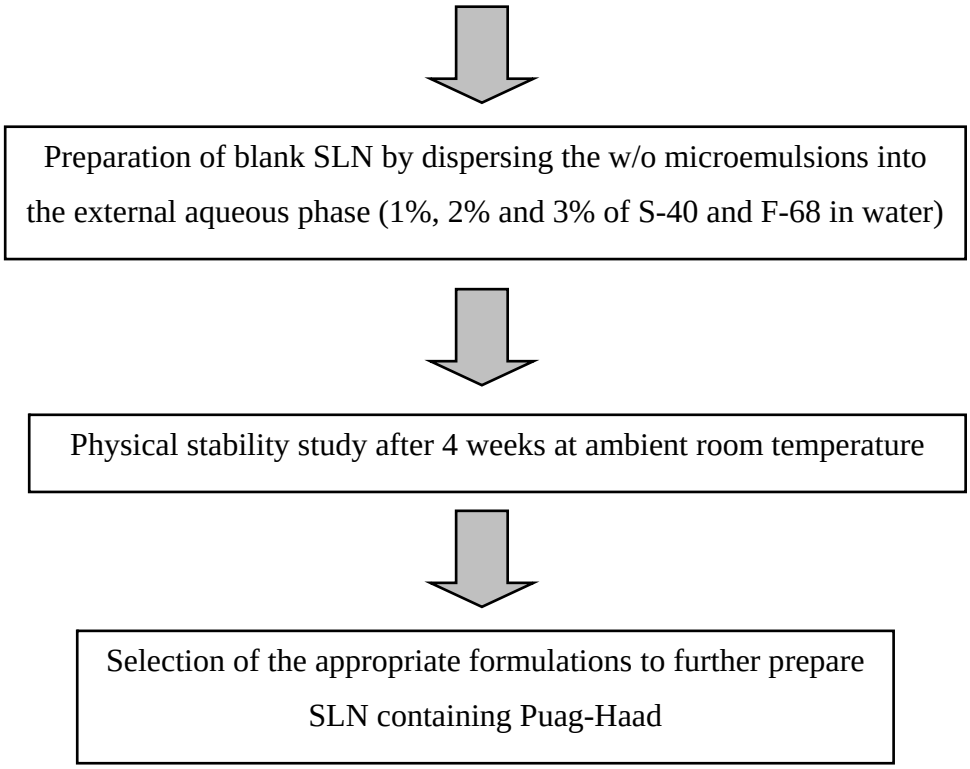


Figure 22 (Cont.) The scope of this study in the step of preparation of blank SLN by w/o/w double emulsion method (GMS = glyceryl monostearate, S-40 = polyoxyethylene 40 stearate, F-68 = poloxamer 188)

1.1.1 Construction of pseudo-ternary phase diagrams

The pseudo-ternary phase diagrams were constructed using lipids (glyceryl monostearate, compritol® 888 ATO or gelucire® 50/13), mixed surfactants (polyoxyethylene 40 stearate and poloxamer 188) and water at about 10°C above melting temperature of each lipid by water titration method (Gattefossé, 1994 cited in Djordjevic et al., 2004; Prapaporn Boonme et al., 2006). The ratio of the mixed surfactants (polyoxyethylene 40 stearate : poloxamer 188) was varied at 0:10, 3:7, 5:5, 7:3 and 10:0 for each phase diagram (modified from Ma et al., 2007). Briefly, the lipid and mixed surfactants were mixed at the weight ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 and then, melted at about 10°C above melting temperature of each lipid. These mixtures were added dropwish with water, under continuous stirring at the same temperature until the mixtures

became turbid liquid or gel formation. The samples were classified as microemulsions when they appeared as clear liquids. To construct phase diagram, all data were analysed by using CHEMIX School Program Version 3.50 (CHEMIX School & Lab, 2007). The best ratio that gave the highest area of microemulsion formation was chosen for each lipid.

1.1.2 Confirmation and type-identification of microemulsions

After the region of microemulsion and the boundary line had been identified from 1.1.1, a new set of lipid, mixed surfactants and water ratios was specified within the microemulsion region of each phase diagram. Microemulsions were again prepared at these ratios and at the same temperature as in 1.1.1 to confirm the formation of microemulsion as well as to identify the type of microemulsion so obtained. The desired microemulsion was chosen by judging from its clarity and flowablility.

To further identify a type of microemulsions, they were dispersed into water or their respective lipids at the same temperature. Only, the w/o microemulsions were selected for the next step.

1.1.3 Preparation of blank SLN

Using the appropriate ratios of lipid, mixed surfactants and water, the w/o microemulsions from 1.1.2 were prepared and dispersed into aqueous surfactant solution (1%, 2% and 3% w/v of polyoxyethylene 40 stearate or poloxamer 188 in water) at the same temperature with the ratio of microemulsion : surfactant solution of 1 g : 10 ml and then suddenly cooled in ice bath. Lipid was immediately precipitated to form SLN. The obtained SLN preparations were kept at 4 – 8°C for 24 hours to ensure complete SLN formation.

1.2 Preparation of blank SLN by cold homogenization method (modified from Almeida, Runge, and Müller, 1997)

The formula was as in the following:

Rx.			
Lipid (5%w/v)		5	g
(glyceryl monostearate, compritol 888 ATO or gelucire 50/13)			
Aqueous surfactant solution	qs. to	100	ml
(1%, 2% and 3% w/v of polyoxyethylene 40 stearate or poloxamer 188 in water)			

In this study, each of the lipid was first melted on water bath. The melted lipid was transferred to a mortar and cooled in freezer (-20°C). Then, the solid lipid was ground into lipid powder before being dispersed in a cold (4 – 8°C) aqueous surfactant solution using a high speed stirrer (WiggenHouser, Germany) at 10,000 rpm for 3 minutes yielding a pre-suspension. The pre-suspension was homogenized at ambient room temperature using a high pressure homogenizer (EmulsiFlex C5®, Avestin, Canada) at 800 bar 3 cycles. The prepared SLN were kept at 4 – 8°C for 24 hours to ensure complete SLN formation.

2. Selection of the appropriate formulations of blank SLN

All blank SLN formulations prepared by both methods were kept at ambient room temperature for 4 weeks to evaluate their initial particle size and size distribution as well as their physical stability so as to select appropriate formulations for subsequent preparation of SLN containing Puag-Haad. The desirable formulations needed to maintain a good appearance throughout the storage period. They must not be phase separation or gel formation. Moreover, they must become homogenous when redispersed.

3. Preparation of SLN containing Puag-Haad

Preparation of SLN containing Puag-Haad by w/o/w double emulsion method

After selection of appropriate blank SLN formulas, Puag-Haad was dissolved in mixture of lipid, mixed surfactant and aqueous internal phase (water or citrate buffer pH 5.5) to obtain w/o microemulsion and further processed as in 1.1.3 (using the same vehicle for aqueous internal and external phase) to obtain SLN. Citrate buffer pH 5.5 was used because there was an evident that citrate buffer pH 4.0-5.5 showed a good physical and chemical stability for Puag-Haad (Manatchaya Wanawatanakun, 2006). The final concentration of Puag-Haad in SLN dispersions were about 0.25% and 0.50% w/v. The formulas of SLN containing Puag-Haad by w/o/w double emulsion method show in table 2.

Table 2 The formulas of SLN containing Puag-Haad by w/o/w double emulsion method

Formulation	w/o microemulsion (g)	Puag-Haad (g)	Aqueous external phase (ml)
SLN containing Puag-Haad			
0.25% w/v	2	0.05	20
0.50% w/v	2	0.10	20

Preparation of SLN containing Puag-Haad by cold homogenization method

To prepare SLN containing Puag-Haad, the stock solution of 1%w/v Puag-Haad in methanol was prepared. This solution (12.5 or 25 ml) was added into melted lipid (2.5 g) under continuously stirring on boiling water bath. At a high temperature on water bath, methanol was evaporated, resulting in the dispersion of Puag-Haad in the melted lipid. The resultant melted lipid containing Puag-

Haad was then processed into 50 ml of SLN using the same procedure as in 1.2. The final concentrations of Puag-Haad in SLN dispersion were 0.25% and 0.50% w/v or 50 and 100 mg of Puag-Haad in lipid 1 g, respectively. The formulas of SLN containing Puag-Haad by cold homogenization method show in table 3.

Table 3 The formulas of SLN containing Puag-Haad by cold homogenization method

Formulation	1% w/v Puag-Haad stock solution (ml)	Lipid (g)	Total volume (ml)
SLN containing Puag-Haad			
0.25% w/v	12.5	2.5	50
0.50% w/v	25	2.5	50

Part C. Characterization of SLN containing Puag-Haad

The characteristics of freshly prepared SLN containing Puag-Haad such as physical appearances, morphology, size and size distribution, Zeta potential and the percentage of entrapment efficiency of selected formulations were investigated.

1. Physical Appearances

The physical appearances such as color, coalescence, gel formation and phase separation were visually observed**.**

2. Morphology

The morphological feature was investigated using transmission electron microscope, TEM (JEM-1230, Jeol, Japan). The samples were placed on a specimen mesh coated with collodian film, being stained by uranyl acetate (for SLN in water) or phosphotungstic acid (for SLN in citrate buffer pH 5.5) and dried under room temperature.

3. Size, size distribution and Zeta potential

The mean particle size (hydrodynamic diameter), particle size distribution (polydispersity index, PI) and zeta potential were measured by photon correlation spectroscopy, PCS (Brookhaven 90 Plus Particle size and Zeta Potential Analyzer, UK). A sample was dispersed and diluted in appropriate vehicle (water or citrate buffer pH 5.5) to obtain weak opalescence before use. The SLN dispersion was put in a quartz cuvette and placed inside the instrument chamber. The obtained value from each formulation was the average of 3 measurements.

4. The percentage of entrapment efficiency

1 g of SLN containing Puag-Haad were purified by ultrafiltration method (Schwarz and Mehnert, 1999; Hsu et al., 2003) using centrifugal filter device (Centriprep® with cellulose membrane MWCO = 50 K, equivalent to 15 – 30 nm, only free drug and micelle could pass this membrane) as shown in figure 23, at 2,000

rpm, 25°C for 30 minutes per cycle followed by washing with water 3 times (5 ml for each) to separate free Puag-Haad from entrapped Puag-Haad. The filtrates (free drug) were collected and the precipitated pellets containing Puag-Haad (entrapped drug) were lyzed by adding appropriate solvent. Both of the obtained solutions were analyzed for content of oxyresveratrol (as marker) using high performance liquid chromatography, HPLC (detail in part A). The entrapment efficiency (the percentage of entrapped drug), the percentage of free drug and recovery were calculated by equations below:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of drug entrapped}}{\text{Amount of initial drug added}} \times 100$$

$$\text{Free drug (\%)} = \frac{\text{Amount of free drug}}{\text{Amount of initial drug added}} \times 100$$

$$\text{Recovery (\%)} = \frac{\text{Amount of drug entrapped + Amount of free drug}}{\text{Amount of initial drug added}} \times 100$$

Figure 23 Centrifugal filter device (Centriprep® with MWCO = 50 K)

5. Thermal analysis by differential scanning calorimetric method

The differential scanning calorimetric (DSC) thermogram was determined by using differential scanning calorimeter (DSC822, Mettler Toledo, Switzerland). A

highly sensitive ceramic sensor in the DSC instrument was used to measure the difference between the heat flows to the sample and reference crucibles. The samples (bulk lipid and freeze dried SLN) (3-5 mg) were accurately weighed into standard aluminum pans (40 µl) and then sealed. The DSC runs were conducted over a temperature range 15-115 ºC at rate of 10ºC/min. All tests were performed under a nitrogen atmosphere of 2 ml/min. The recrystallization index (RI) was calculated as follows:

$$\text{Recrystallization index (\%)} = \frac{\text{Enthalpy}_{\text{SLN}}\ (\text{J/g})}{\text{Enthalpy}_{\text{bulk material}}\ (\text{J/g}) \times \text{Concentration of lipid}} \times 100$$

6. In vitro release property of SLN containing Puag-Haad

The in vitro release study was performed by using vertical Franz diffusion cell, which consisted of donor and receiver compartments. The dialysis membrane was placed between two compartments of Franz diffusion cell. The type of membrane was regenerated cellulose acetate membrane with molecular weight cutoff 12,000-14,000 (CelluSep®T4, Canada) (only free drug could pass this membrane). The membrane was soaked in distilled water for 24 hours, then washed by hot distilled water and soaked in receiver medium for an hour before use. The receiving compartment contained either 14 ml of water or citrate buffer pH 5.5 (depending on the external phase of SLN formulations) which was maintained at 37±0.5°C by a circulating water jacket. After equilibration, the membrane (diameter 1.8 cm) and the receptor compartment were carefully fitted with the donor compartment and 1 g of SLN formulation was dropped into the donor compartment, then covered with parafilm to prevent evaporation. The aqueous solution with the same concentration of Puag-Haad (in 20%w/v propylene glycol in water or citrate buffer pH 5.5) was used as control. The receptor fluid was continuously mixed by magnetic stirring bar throughout the time of study. A volume of 3 ml were taken from the receiver medium at certain time intervals of 0.25, 0.5, 1, 3, 5, 8, 12, 18 and 24 hours. The receptor compartment was replaced with the same volume of receptor solution to keep the constant volume during the experiment and to maintain sink conditions. The samples were analyzed for amount of oxyresveratrol released from the SLN by HPLC method. The amount of

drug released was calculated by multiplying the drug concentration with the medium volume. The percentage of drug release was calculated by the following equation:

Cumulative amount of drug release (%) = $(A_t / A_0) \times 100$

Where A_t is the cumulative amount of drug released at a particular time (μg)

A_0 is the initial amount of drug loaded to the donor compartment (μg)

Part D. Physical and chemical stability study of SLN containing Puag-Haad formulations in comparison with aqueous solutions of Puag-Haad.

1. Stability study of SLN containing Puag-Haad

The suspensions of SLN containing Puag-Haad were divided and kept in tightly closed amber glass bottles. The samples were kept at room temperature (30±2°C) for 4 months. The samples were withdrawn and investigated on month 0, 1, 2, 3 and 4 (detail in table 4) in three batches (n=3). Physical and chemical stability of the samples was evaluated as follows:

Physical appearance

The physical appearances such as color, coalescence, gel formation and phase separation were visually observed using the same procedure as in topic C1 in comparison with their respective freshly prepared formulations.

pH

The pH of the formulations was measured by pH meter (Orion model 420A, USA).

Size and size distribution

The particle size and size distribution of the formulations was measured using the same procedure as in topic C3.

Drug remaining

The total amount of oxyresveratrol remained in the formulations was determined by HPLC method as described under topic A4.

The percentage of entrapment efficiency

The percentage of entrapment efficiency was determined using the same procedure as in topic C4.

Thermal analysis by differential scanning calorimetric method

The DSC thermogram was determined using the same procedure as in topic C5.

2. Stability study of aqueous solution of Puag-Haad

The control solutions in this study contained the same concentration of Puag-Haad dissolved in aqueous solution of 3% w/v polyoxyethylene 40 stearate and 20% v/v propylene glycol.

They were divided and kept in tightly closed amber glass bottles. The solutions were kept at room temperature (30±2°C) for 4 months and investigated on month 0, 1, 2, 3 and 4 (detail in table 5) in three batches (n=3). Physical and chemical stability of the solutions was evaluated as follows:

Physical appearance

The physical appearances such as color and color changes were visually observed in comparison with their respective freshly prepared formulations.

pH

The pH of the solutions was measured by pH meter (Orion model 420A, USA).

Drug remaining

The total amount of oxyresveratrol remained in the solutions was determined by HPLC method as described under Topic A6.

Table 4 Physical and chemical stability of SLN containing Puag-Haad at 30±2°C protected from light

Month / Topics	0	1	2	3	4
1. Physical appearance	/	/	/	/	/
2. pH	/	/	/	/	/
3. Size and size distribution	/	/	/	/	/
4. Drug remaining	/	/	/	/	/
5. The percentage of entrapment efficiency	/	/	/	/	/
6. Thermal analysis by DSC	/	-	/	-	/

Table 5 Physical and chemical stability of aqueous solution of Puag-Haad at 30±2°C protected from light

Month / Topics	0	1	2	3	4
1. Physical appearance	/	/	/	/	/
2. pH	/	/	/	/	/
3. Drug remaining	/	/	/	/	/

Part E. Evaluation of whitening and anti-wrinkle efficacy of SLN containing Puag-Haad in human volunteers

The purpose of this part was to demonstrate the *in vivo* whitening and anti-wrinkle efficacy of SLN containing Puag-Haad in healthy volunteers in comparison with micellar solution of Puag-Haad. The optimum Puag-Haad concentration of 0.25% w/v had been previously found to that provide both the whitening effect (Parkpoom Tengamnuay et al., 2006) and the anti-wrinkle effect (Manatchaya Wanawatanakun, 2006) was selected to prepare SLN and micellar solution in this study.

1. Study design and subject selection

Twenty six healthy woman volunteers with age from 30-65 years participated in this single-blind study. The protocol was approved by an independent Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University as shown in appendix B. All of them had given written informed consents prior to enrollment. They were allowed to drop out from the study at any time and were closely monitored for any unwanted side effects such as skin rash or other inflammatory responses throughout the study period. A questionnaire was also given to each volunteer to fill out personal statistics and relevant information as shown in Appendix.

Subject selection

Inclusion criteria

- Healthy woman volunteers
- Age 30-65 years old
- Permission to write her informed consent before participating in the study
- Passed the patch test procedure for irritation risk

Exclusion criteria

- Allergic and/or hypersensitivity to cosmetics
- Allergic to the test sample

- History of skin disease such as eczema and psoriasis on their forearm
- Receiving systemic or topical drug which could interfere with the evaluation

All of them stopped using any cosmetics on the application areas for at least two weeks before the start of the experiments. The application areas were the inner area of both the upper left and right forearms. The melanin index, skin roughness, skin hydration, elasticity and sebum values were monitored for the start of the experiment to observe for baseline.

2. Patch test for irritation risk evaluation

The patch test consisted of 2 cycles

2.1 Cycle 1 (for acute hypersensitivity reaction)

0.3 ml of samples (SLN containing Puag-Haad or micellar solution of Puag-Haad) and purified water (using as control) were dropped on the (2.5 x 2.5 cm) gauze pad and secured to the inner side of the upper forearm with cloth tape. The right side was SLN and the left side was micellar solution. The control patch (water) was also placed along side of each SLN or micellar solution patch on both forearms. The patches remained in place for 24 hours (if any of the patches caused severe itching or pain, it was immediately removed). Then the patches were removed and the skin was flooded with water to remove any residual test substances, then dried with cotton. Visual reading was taken in 30 minutes and 3 days later. The tested area was inspected visually according to the following grading:

0	No erythema
+1	Mild erythema
+2	Severe erythema
+3	Erythema and papules
+4	Erythema, papules and vesiculation

2.2 Cycle 2 (for delayed hypersensitivity reaction) (after 7 – 10 days later from cycle 1)

The subjects that passed from cycle 1 (no erythema) were subsequently selected in this cycle. The procedure was the same as in cycle 1.

The subjects that passed both cycles were included in the next study.

3. Preparation of the test samples

The freshly prepared micellar solution of Puag-Haad (reference sample) and SLN containing Puag-Haad (test sample) at 0.25% w/v were prepared and assigned to volunteers for every two weeks. One forearm was the micellar solution and the other forearm was SLN containing Puag-Haad.

4. Application of the test and reference samples on the volunteers' skin

Each subject was randomly assigned to the samples. Half of the subjects were assigned such that the right forearm was SLN and the left forearm was solution whereas the other half was in the opposite, i.e., the right forearm was solution and the left forearm was SLN. The application amount was always 0.3 ml for both samples.

After the baseline measurements (no treatment), each volunteer would self-apply the samples twice daily, in the morning and at night time for 8 consecutive weeks. They were monitored for any changes in the melanin index, skin roughness, skin elasticity, skin hydration and sebum values at a two-week interval. Each subject was supplied with a pair of similar plastic bottles filled with samples. The content of each bottle was about 30 ml, which was sufficient for daily application up to two weeks before the next visit. They were all blind regarding which bottle was SLN or reference solution. The label on each bottle merely stated the coded letter and number of subject and whether the individual bottle was intended to use on which forearm. For example, at each visit subject no.1 received two bottles, one with a label "1 left" and the other with a label "1 right". Only the investigator who prepared all the samples knew the exact content within each bottle. All the samples were freshly prepared every two weeks. Upon the next visit, the individual subjects were again measured for the melanin index, skin roughness, skin hydration, elasticity and sebum values and received another pair of bottles for further application.

To achieve uniform application procedures, each subject was instructed to dispense 3 drops (equivalent to total of 0.3 ml) of the sample on the hand before gently apply with whirling motion on the forearm.

5. Measurement of the melanin index, skin roughness, skin elasticity, skin hydration and sebum

Melanin index is measured by Derma-Spectrometer®

Skin roughness is measured by Visioscan® VC98

Elasticity is measured by Dermalab®

Skin hydration is measured by Skin diagnostic® SD27

Sebum is measured by Skin diagnostic® SD27

The probe of each instrument was placed at the marked areas of both the left and right forearms. The melanin index, roughness and hydration parameters were read four times at 4 adjacent spots. The sebum and elasticity parameters were read twice at 2 adjacent spots. The average value was calculated for each parameter and further used in statistical analysis.

6. Statistical analysis

Paired student's t-test was also applied to test for any differences in values between the left and the right forearms (treatment difference) at every period of the study as well as values between starting time (week 0) and week 2, 4, 6 or 8 after application (time effect within each treatment). Comparison was made at 5% significance level (α).

In addition, Repeated measures ANOVA was also applied on each parameter to compare the overall efficacy between the test (SLN) and reference (micellar solution) samples at the same significance level.

CHAPTER IV

RESULTS AND DISCUSSION

Part A. Development and validation of UV-Visible Spectrophotometric and HPLC method for determination of Puag-Haad and oxyresveratrol content

1. Validation of UV-Visible Spectrophotometric method

The developed UV-Visible Spectrophotometric system was applied to analyze the Puag-Haad content in SLN containing Puag-Haad during the initial development phase. The method validation was performed in the topic below.

1.1 Specificity

Under the conditions used, the absorbance of Puag-Haad must not be interfered by the absorbance of other components in the sample. The amount of Puag-Haad was determined from its maximum absorption at 327 nm Methanol was used as solvent in this system. The typical spectra of Puag-Haad standard solution, blank SLN and other spectra are shown in Figures 24 – 28. All spectra are shown under the same scale.

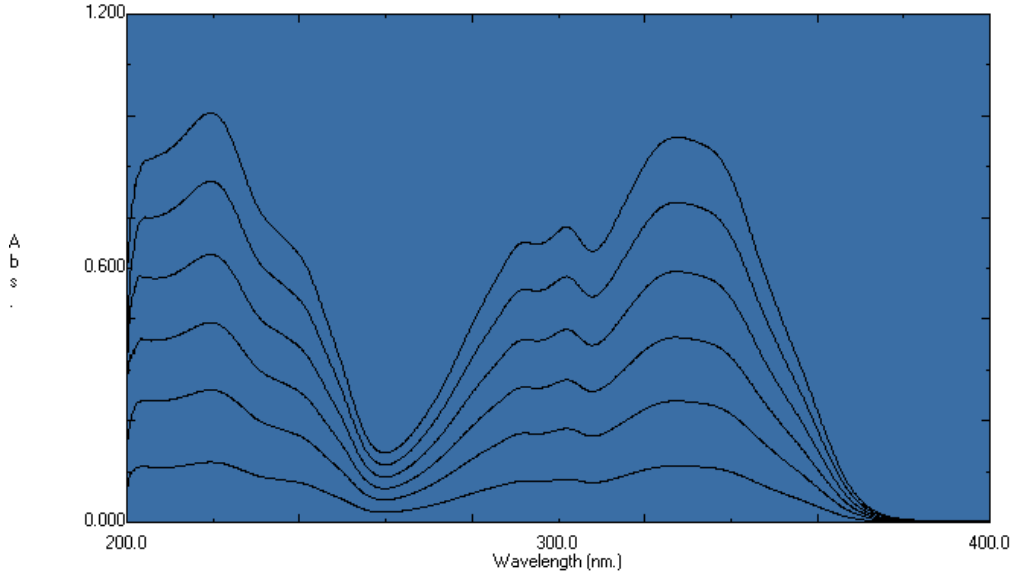


Figure 24 Chromatogram of Puag-Haad standard solution

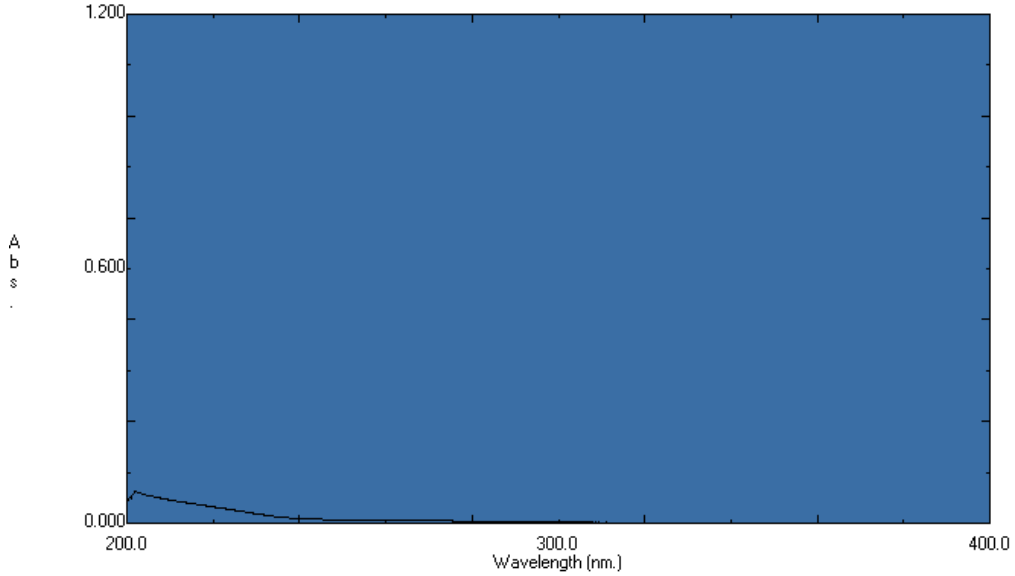


Figure 25 Chromatogram of blank SLN in water

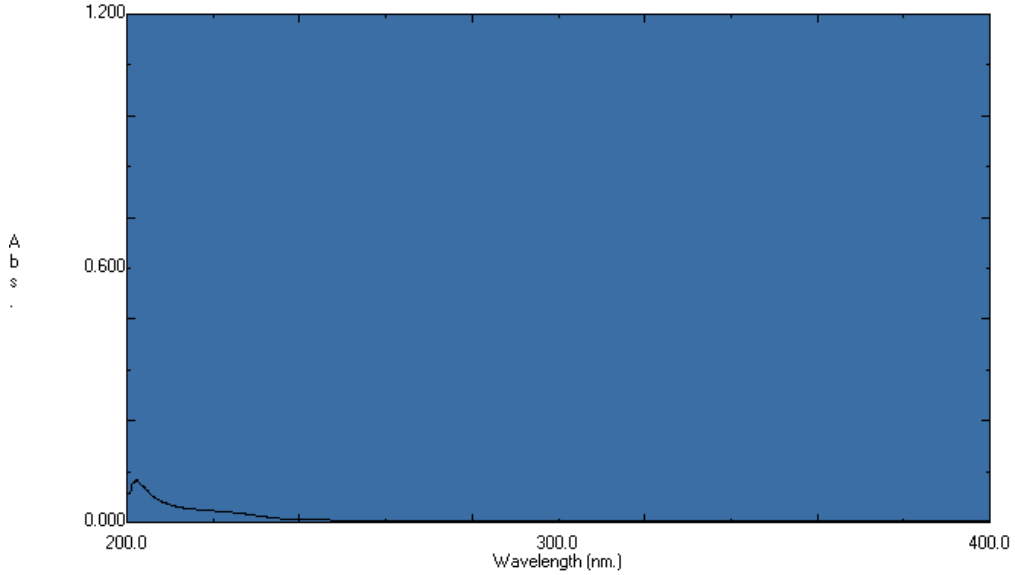


Figure 26 Chromatogram of blank SLN in citrate buffer pH 5.5

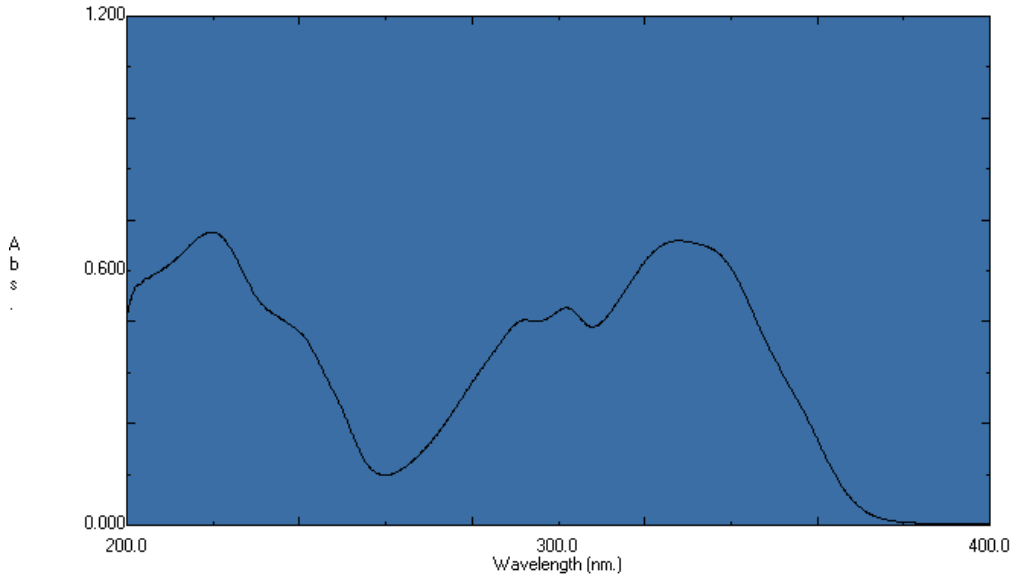


Figure 27 Chromatogram of SLN containing Puag-Haad in water

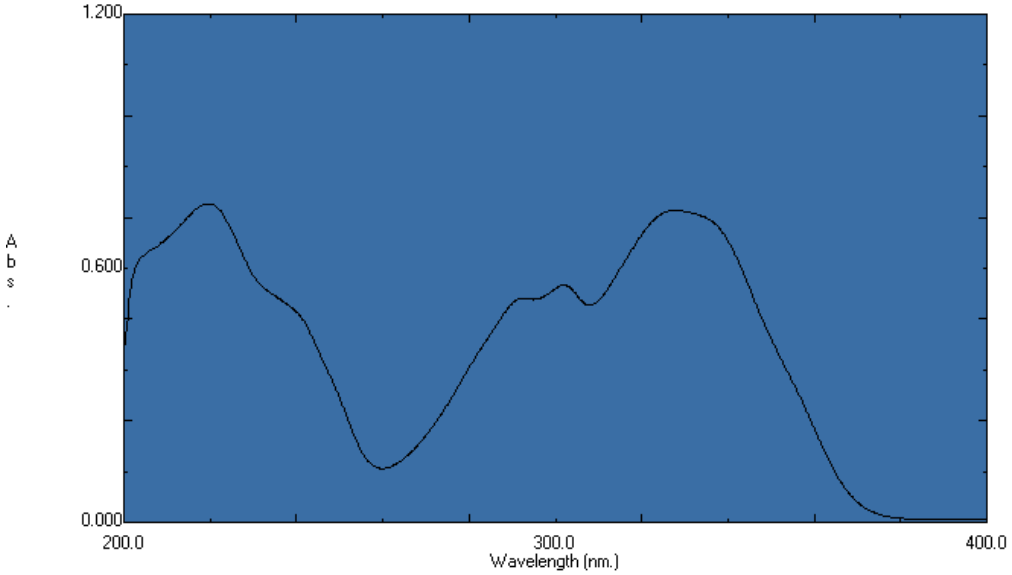


Figure 28 Chromatogram of SLN containing Puag-Haad in citrate buffer pH 5.5

From Figures 24 – 28, it was found that there was no interference from other SLN components in the spectra at 327 nm. So, the UV-Visible Spectrophotometric method was acceptable for specificity.

1.2 Linearity

The representative calibration curve data of Puag-Haad are shown in Table 6. The plot of standard Puag-Haad concentrations mixed with blank SLN versus absorbance are shown in Figure 29. It displayed the linear correlation in the concentration range studied of 2-12 μg/ml. The coefficient of determination (R^2) of this line was 0.9999. These results indicated that the UV-Visible Spectrophotometric method was acceptable for quantitative analysis of the crude extract (Puag-Haad) in the range studied.

Table 6 Data for calibration curve of Puag-Haad by UV-Visible Spectrophotometric method

Concentration of Puag-Haad (μg/ml)	Absorbance			Mean	SD
	Set 1	Set 2	Set 3		
2	0.123	0.133	0.120	0.125	0.007
4	0.276	0.282	0.290	0.283	0.007
6	0.415	0.435	0.440	0.430	0.013
8	0.574	0.583	0.595	0.584	0.011
10	0.731	0.741	0.754	0.742	0.012
12	0.883	0.898	0.898	0.893	0.009

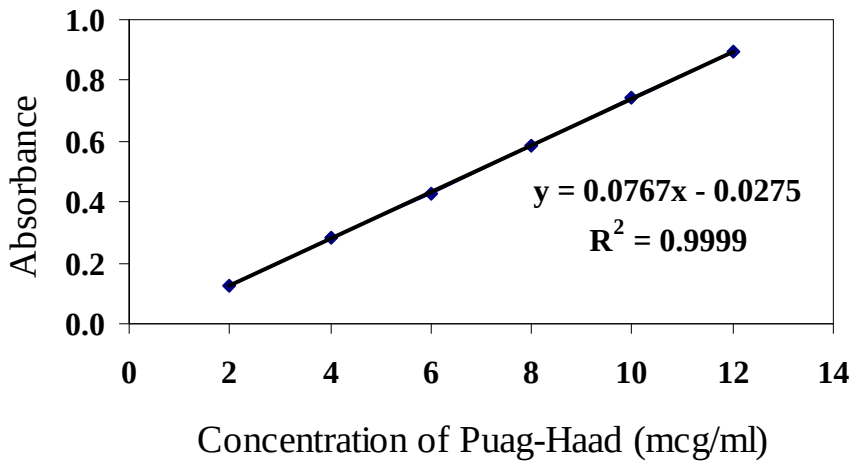


Figure 29 Calibration curve of Puag-Haad by UV-Visible Spectrophotometric method

1.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. The determination of accuracy was performed by analyzing five sets of three concentrations (3, 7 and 11 μg/ml). The inversely estimated concentrations and percentages of analytical recovery at each drug concentration are shown in Table 7 and Table 8, respectively. All percentages of

analytical recovery were in the range of 98.55-101.49%, which indicated that this method could be used for the analysis of Puag-Haad crude extract in all concentrations studied with high accuracy.

Table 7 The inversely estimated concentrations of Puag-Haad by Spectrophotometric method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean (SD)
	Set 1	Set 2	Set 3	Set 4	Set 5	
3	3.1300	3.1170	3.0650	3.1560	3.1300	3.1196 (0.0336)
7	7.2003	7.2653	7.1222	7.1743	7.3953	7.2315 (0.1051)
11	11.2835	11.3875	11.2575	11.4655	11.4395	11.3667 (0.0927)

Table 8 The percentage of analytical recovery of Puag-Haad by Spectrophotometric method

Concentration (μg/ml)	%Analytical recovery					Mean (SD)
	Set 1	Set 2	Set 3	Set 4	Set 5	
3	101.49	99.14	100.16	101.16	99.56	100.30 (1.01)
7	100.06	99.04	99.75	98.55	100.81	99.64 (0.88)
11	99.78	98.78	100.33	100.22	99.23	99.67 (0.66)

1.4 Precision

The precision of Puag-Haad analyzed by UV-Visible Spectrophotometric method were determined both within-run precision and between-run precision as illustrated in Tables 9 and 10. All coefficients of variation values were small, 0.82 – 1.45% and 1.37 – 1.84%, respectively. The coefficient of variation of an analytical

method should generally be less than 2%. Therefore, the UV-Visible Spectrophotometric method was precise for quantitative analysis of Puag-Haad in the range studied.

Table 9 Data of within-run precision by UV-Visible Spectrophotometric method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean (SD)	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5		
3	3.1300	3.1170	3.0650	3.1560	3.1300	3.1196 (0.0336)	1.0787
7	7.2003	7.2653	7.1222	7.1743	7.3953	7.2315 (0.1051)	1.4530
11	11.2835	11.3875	11.2575	11.4655	11.4395	11.3667 (0.0927)	0.8152

Table 10 Data of between-run precision by UV-Visible Spectrophotometric method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean (SD)	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5		
3	3.1300	3.0234	3.0344	3.0728	3.0529	3.0627 (0.0420)	1.3730
7	7.2003	6.9584	7.0344	7.1656	7.2114	7.1140 (0.1120)	1.5739
11	11.2835	10.8416	10.9682	10.9934	11.3004	11.0774 (0.2042)	1.8434

In conclusion, the analysis of Puag-Haad crude extract content in SLN containing Puag-Haad by UV-Visible Spectrophotometric method developed in this study showed good specificity, linearity, accuracy and precision. The LOQ or limit of quantitation from the validation results was 3 μg/ml. Thus, this method was used for determination of the content of Puag-Haad in SLN to evaluate its characteristics.

2. Validation of HPLC method

The developed HPLC system was applied to analyze the oxyresveratrol content in SLN containing Puag-Haad. The method validation was performed in the topic below.

Validation of the analytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. For HPLC assay validation, these include specificity, linearity, accuracy and precision.

2.1 Specificity

The specificity of an analytical method is its ability to measure the analyte accurately and with specificity in the presence of other components in the sample.

The internal standard technique was performed by determinating the peak area ratio of oxyresveratrol to furazolidone (internal standard) to give the complete separation, appropriate resolution and sharp peaks of all components. The methanol-water mixture (40:60%v/v) was used as the mobile phase. The typical chromatograms of oxyresveratrol standard solution, resveratrol standard solution (related substance), furazolidone internal standard solution, Puag-Haad solution, blank SLN and other chromatograms are shown in Figures 30 – 41. All chromatograms are shown under the same attenuation (9) and scale. The solvent used to prepare the final solution before HPLC injection was mobile phase (methanol and water with the ratio of 40:60 % v/v).

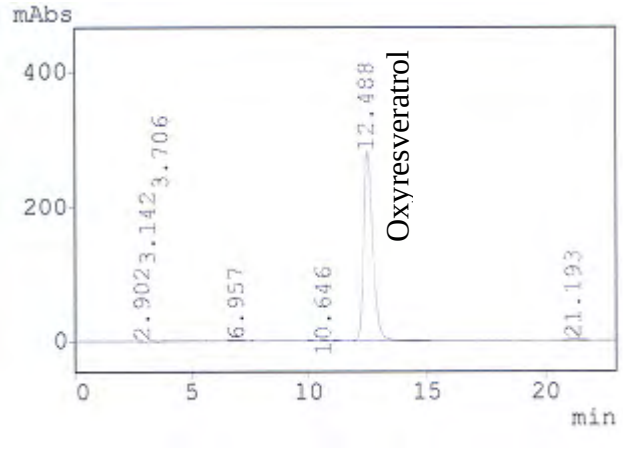


Figure 30 HPLC chromatogram of oxyresveratrol standard solution (final concentration of oxyresveratrol = 20 μg/ml)

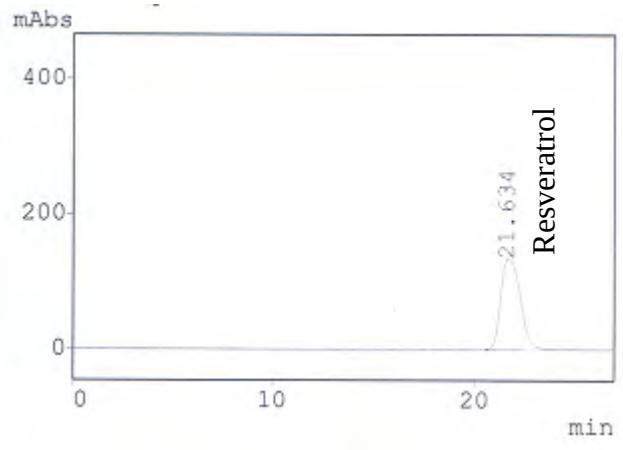


Figure 31 HPLC chromatogram of resveratrol standard solution (final concentration of resveratrol = 20 μg/ml)

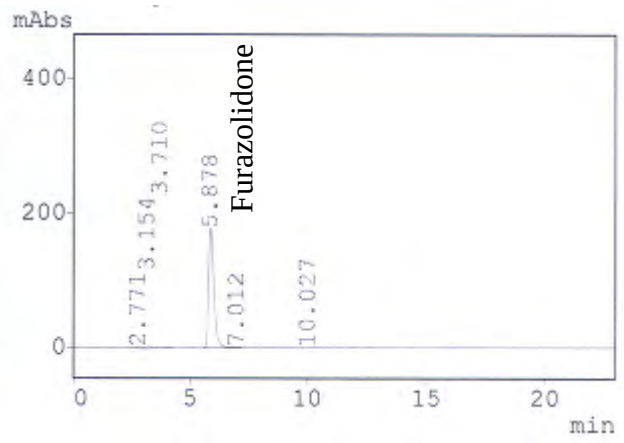


Figure 32 HPLC chromatogram of furazolidone internal standard solution (final concentration of furazolidone = 15 μg/ml)

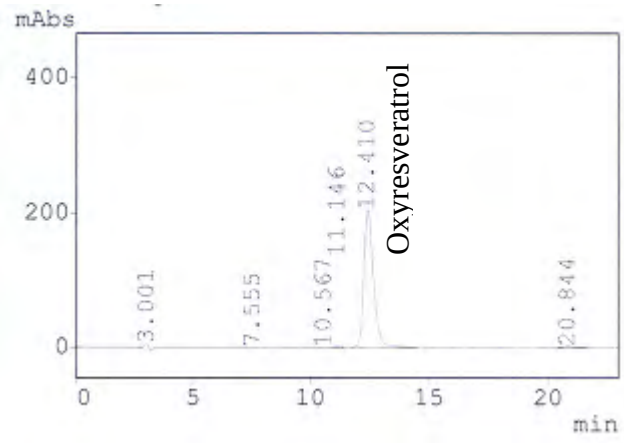


Figure 33 HPLC chromatogram of Puag-Haad solution (final concentration of Puag-Haad = 20 μg/ml)

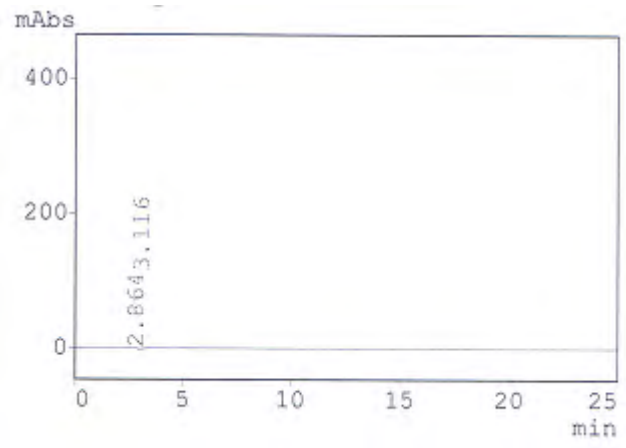


Figure 34 HPLC chromatogram of blank SLN in water
(final concentration of lipid = 100 μg/ml)

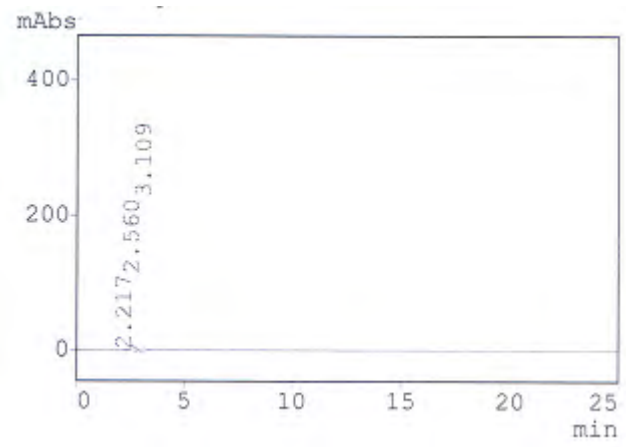


Figure 35 HPLC chromatogram of blank SLN in citrate buffer pH 5.5
(final concentration of lipid = 100 μg/ml)

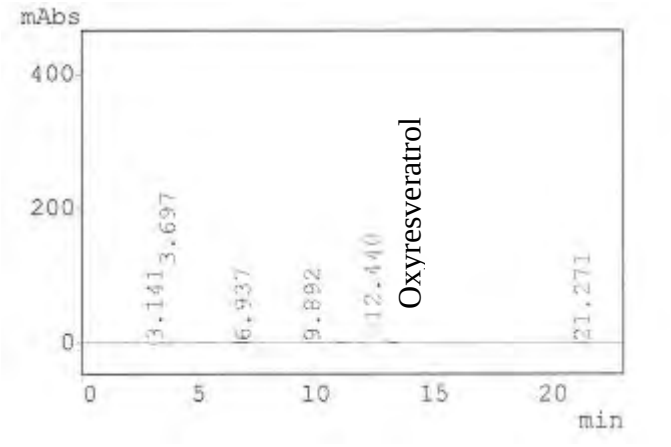


Figure 36 HPLC chromatogram of SLN containing Puag-Haad in water (final concentration of Puag-Haad = 5 μg/ml)

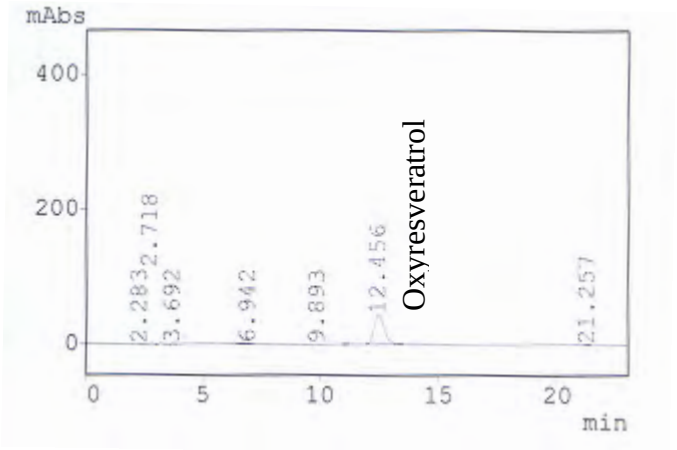


Figure 37 HPLC chromatogram of SLN containing Puag-Haad in citrate buffer pH 5.5 (final concentration of Puag-Haad = 5 μg/ml)

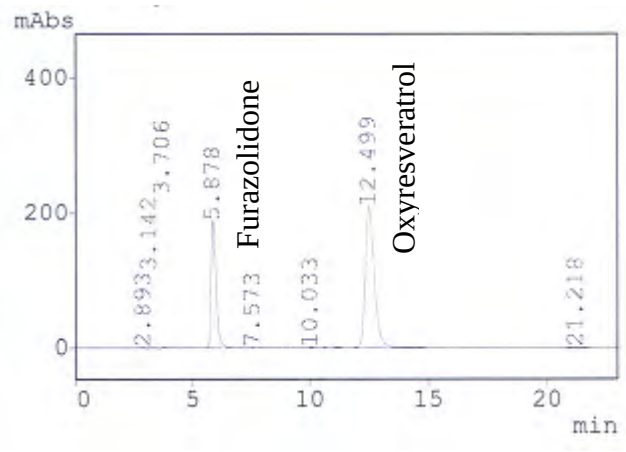


Figure 38 HPLC chromatogram of mixture of oxyresveratrol (final concentration of oxyresveratrol = 20 μg/ml) and internal standard (final concentration of furazolidone = 15 μg/ml)

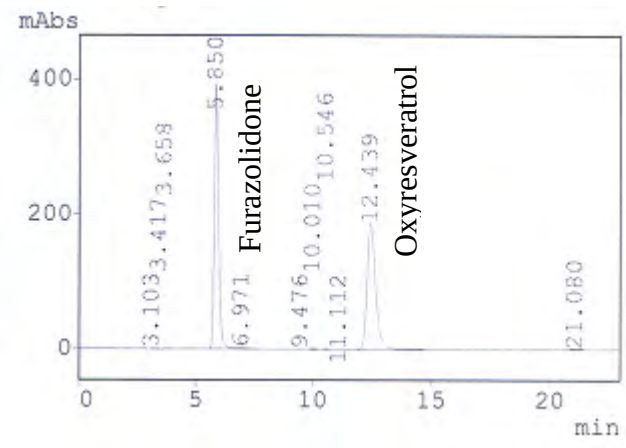


Figure 39 HPLC chromatogram of mixture of Puag-Haad (final concentration of Puag-Haad = 20 μg/ml) and internal standard (final concentration of furazolidone = 30 μg/ml)

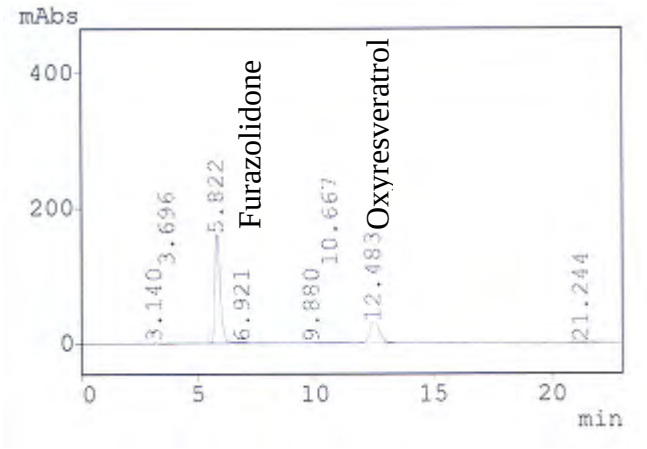


Figure 40 HPLC chromatogram of mixture of SLN containing Puag-Haad (final concentration of Puag-Haad = 5 μg/ml) in water and internal standard (final concentration of furazolidone = 15 μg/ml)

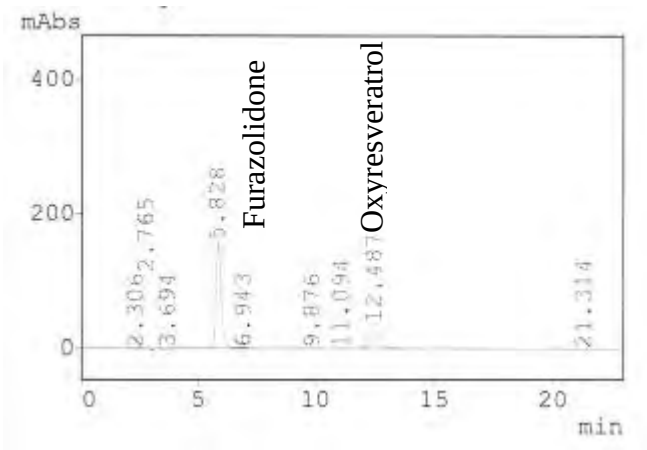


Figure 41 HPLC chromatogram of mixture of SLN containing Puag-Haad (final concentration of Puag-Haad = 5 μg/ml) in citrate buffer pH 5.5 and internal standard (final concentration of furazolidone = 15 μg/ml)

From Figures 30 – 41, it was found that there was no interference from other components in the chromatogram including the related substance (resveratrol), lipids and surfactants. So, the HPLC method was acceptable for specificity.

2.2 Linearity

The linearity of an analytical method is the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in the samples within a given range. The linearity is usually expressed in the terms of variance around the slope of the regression line calculated according to an established mathematical relationship from the test results obtained by the analysis of samples with varying concentration of analyte. The representative calibration curve data of oxyresveratrol standard solutions mixed with blank SLN are shown in Table 11. The plot of oxyresveratrol concentrations versus the peak area ratios of oxyresveratrol to its internal standard furazolidone illustrated the linear correlation in the concentration range of 0.5-25 μg/ml (Figure 42). The coefficient of determination (R^2) of this line was 0.9999. These results indicated that the HPLC method was acceptable for quantitative analysis of oxyresveratrol in the range studied.

Table 11 Data for calibration curve of oxyresveratrol by HPLC method

Concentration of oxyresveratrol (μg/ml)	Peak area ratio (oxyresveratrol to furazolidone)			Mean	SD
	Set 1	Set 2	Set 3		
0.5	0.0577	0.0562	0.0503	0.0547	0.0039
5	0.6925	0.6786	0.6249	0.6653	0.0357
10	1.3744	1.3506	1.2424	1.3225	0.0704
15	2.0660	2.0085	1.9733	2.0159	0.0468
20	2.7694	2.7359	2.6691	2.7248	0.0511
25	3.4572	3.4293	3.3649	3.4171	0.0473

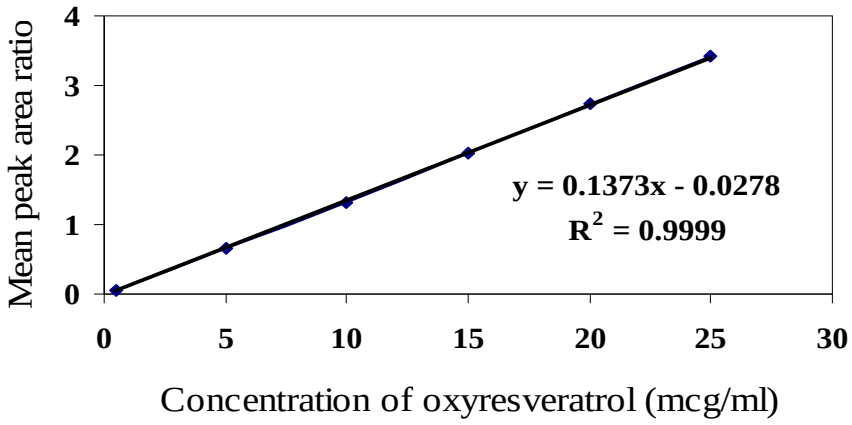


Figure 42 Calibration curve of oxyresveratrol by HPLC method

2.3 Accuracy

The determination of accuracy was performed by analyzing fives sets of three concentrations (1, 12 and 24 μg/ml) of oxyresveratrol in SLN medium. The inversely estimated concentrations and percentage of analytical recovery for each concentration are shown in Table 12 and Table 13, respectively. The percentages of analytical recovery were in the range of 98.47-101.46%, which indicated that this method could be used for analysis of oxyresveratrol at all concentrations studied with high accuracy.

Table 12 The inversely estimated concentrations of oxyresveratrol by HPLC method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean (SD)
	Set 1	Set 2	Set 3	Set 4	Set 5	
1	0.9938	0.9986	1.0033	1.0073	1.0033	1.0013 (0.0052)
12	11.7609	11.7367	11.7635	11.7668	11.7309	11.7518 (0.0167)
24	23.7292	24.0435	23.6607	23.9121	23.7989	23.8289 (0.1517)

Table 13 The percentage of analytical recovery of oxyresveratrol by HPLC method

Concentration (μg/ml)	%Analytical recovery					Mean (SD)
	Set 1	Set 2	Set 3	Set 4	Set 5	
1	100.10	100.59	101.06	101.46	101.06	100.85 (0.52)
12	98.72	98.51	98.74	98.77	98.47	98.64 (0.14)
24	99.59	100.91	99.30	100.36	99.88	100.01 (0.64)

2.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation, %CV) of a series of measurements.

Tables 14 and 15 illustrate the data of within-run precision and between-run precision, respectively, of oxyresveratrol in SLN medium. All coefficient of variation values were small, 0.14 – 0.64% and 0.47 – 0.99%, respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the HPLC method was precise for the quantitative analysis of oxyresveratrol in the range studied.

Table 14 Data of within-run precision by HPLC method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean (SD)	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5		
1	0.9938	0.9986	1.0033	1.0073	1.0033	1.0013 (0.0052)	0.5175
12	11.7609	11.7367	11.7635	11.7668	11.7309	11.7518 (0.0167)	0.1420
24	23.7292	24.0435	23.6607	23.9121	23.7989	23.8289 (0.1517)	0.6367

Table 15 Data of between-run precision by HPLC method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean (SD)	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5		
1	0.9938	0.9876	0.9828	1.0016	1.0068	0.9945 (0.0098)	0.9902
12	11.7609	11.8903	11.7853	11.8716	11.8164	11.8249 (0.0552)	0.4671
24	23.7292	23.5496	23.9740	23.7614	23.7412	23.7511 (0.1508)	0.6350

In conclusion, the analysis of oxyresveratrol content in SLN containing Puag-Haad by HPLC method developed in this study showed good specificity, linearity, accuracy and precision. The LOQ or limit of quantitation, or the lowest concentration at which oxyresveratrol can be measured with acceptable accuracy and precision, was 1 μg/ml. Thus, this method was used for determination of the content of oxyresveratrol in SLN containing Puag-Haad to evaluate its stability.

Part B. Development of SLN containing Puag-Haad

1. Preparation of blank SLN

1.1 Preparation of blank SLN by w/o/w double emulsion method

1.1.1 Construction of pseudo-ternary phase diagrams

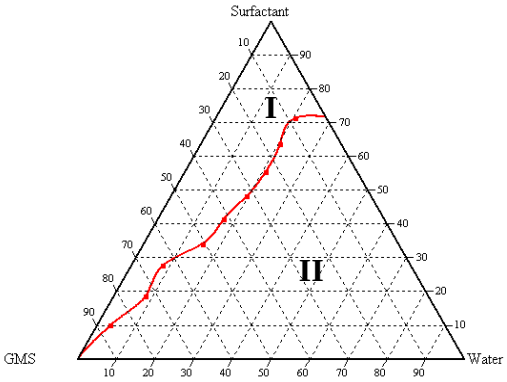
The pseudo-ternary phase diagrams were constructed using three different types of lipids, i.e., glyceryl monostearate (GMS), glyceryl behenate (Compritol® 888 ATO), or macrogol glyceryl stearate (Gelucire® 50/13), by water titration method at about 10°C above melting temperature of each lipid. Water was added into the mixtures of lipid and mixed surfactants (combination of polyoxyethylene 40 stearate (Myrj® 52 or S-40) and poloxamer 188 (Lutrol® F-68) at various ratios) under continuously stirring. To find the boundary line of microemulsion region, the ratios of lipid, mixed surfactants and water were calculated. Detail of titration results and amount of water added to form microemulsion boundary within each phase diagram is provided in appendix A. The data were plotted and the phase diagrams were constructed as shown in Figures 43 – 45.

From Figure 43, for GMS as lipid, the best ratio of mixed surfactants (S-40:F-68) that gave the highest area of microemulsion was 7:3. This result agreed with the previous study of Ma et al. (2007). Their phase diagram of the mixed surfactants (S-40:F-68) at ratio 7:3 (60°C) was chosen to prepare SLN. Although slight difference existed in the area of microemulsion region between the current and Ma's studies, this could be due to the differences in temperature, apparatus and end point evaluation. However, the best ratio of the mixed surfactants was still 7:3.

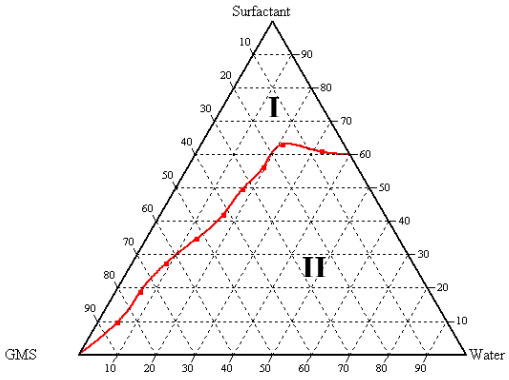
From Figure 44, for compritol as lipid, the best ratio of mixed surfactants (S-40:F-68) that gave the highest area of microemulsion was 10:0. This ratio was the same as in Figure 45 when gelucire was used as lipid.

So, the best ratios of S-40:F-68 for GMS, compritol and gelucire to generate microemulsion were 7:3, 10:0 and 10:0, respectively.

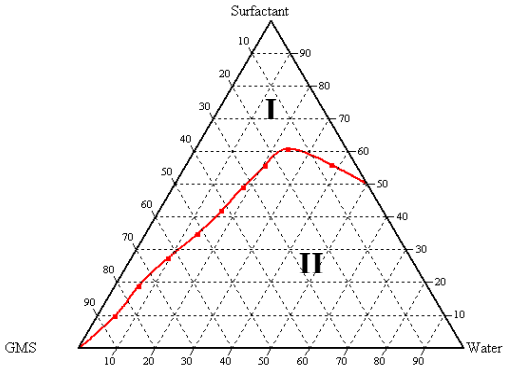
Surfactant = S-40:F-68 (0:10), at 70°C



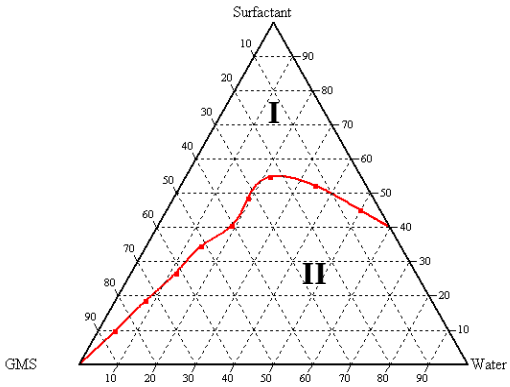
Surfactant = S-40:F-68 (3:7), at 70°C



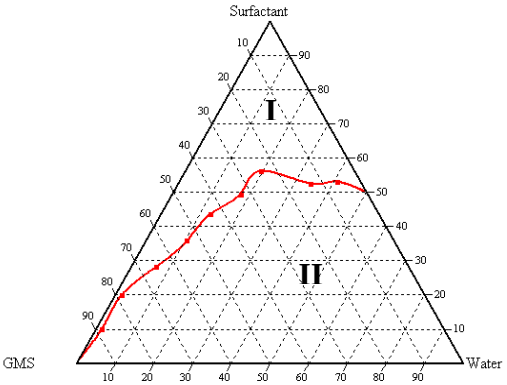
Surfactant = S-40:F-68 (5:5), at 70°C



Surfactant = S-40:F-68 (7:3), at 70°C



Surfactant = S-40:F-68 (10:0), at 70°C



GMS = glyceryl monostearate

S-40 = polyoxyethylene 40 stearate (Myrj® 52)

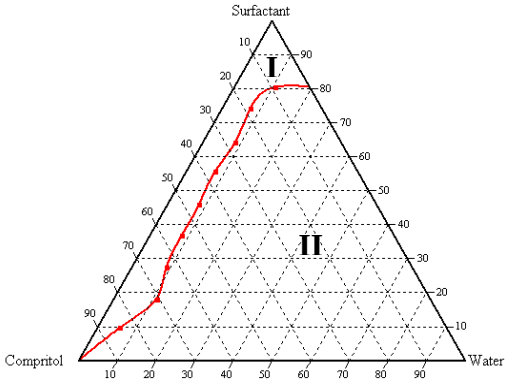
F-68 = poloxamer 188 (Lutrol® F-68)

I: flowable microemulsion region (liquid clear zone)

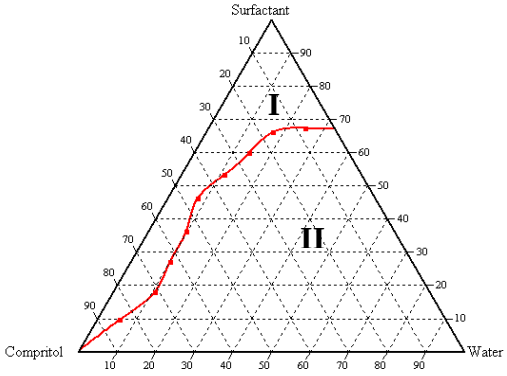
II: viscous or non-microemulsion region

Figure 43 The pseudo-ternary phase diagrams of lipid (GMS), mixed surfactants (S-40:F-68) and water at 70°C

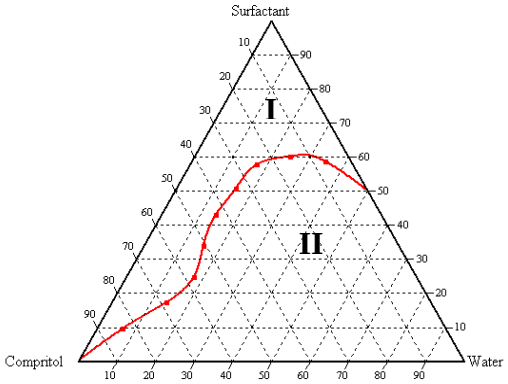
Surfactant = S-40:F-68 (0:10), at 85°C



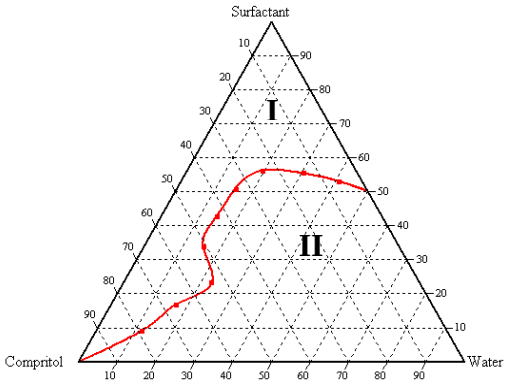
Surfactant = S-40:F-68 (3:7), at 85°C



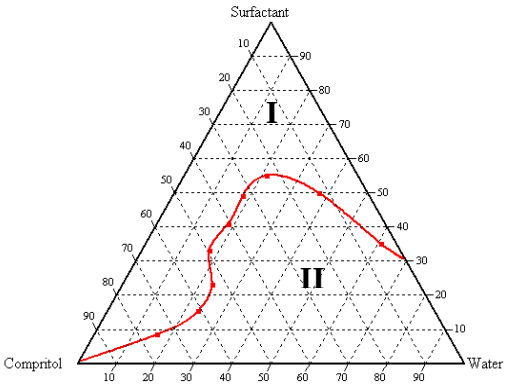
Surfactant = S-40:F-68 (5:5), at 85°C



Surfactant = S-40:F-68 (7:3), at 85°C



Surfactant = S-40:F-68 (10:0), at 85°C



Compritol = glyceryl behenate (Compritol® 888 ATO)

S-40 = polyoxyethylene 40 stearate (Myrj® 52)

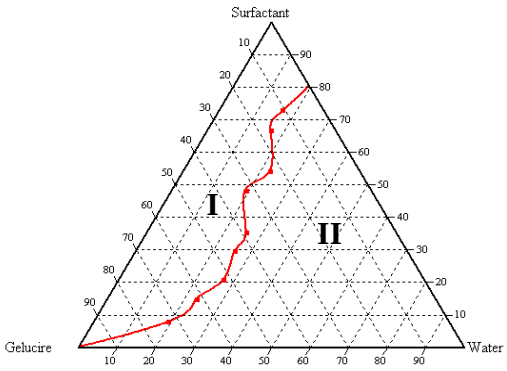
F-68 = poloxamer 188 (Lutrol® F-68)

I: flowable microemulsion region (liquid clear zone)

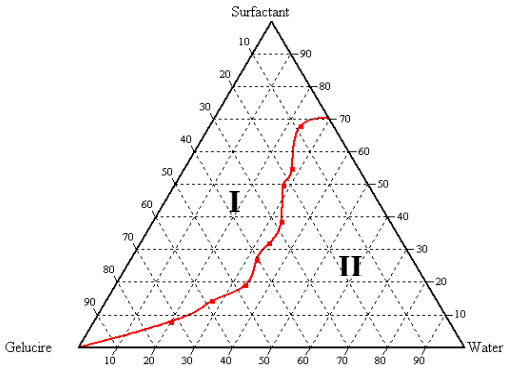
II: viscous or non-microemulsion region

Figure 44 The pseudo-ternary phase diagrams of lipid (compritol 888 ATO), mixed surfactants (S-40:F-68) and water at 85°C

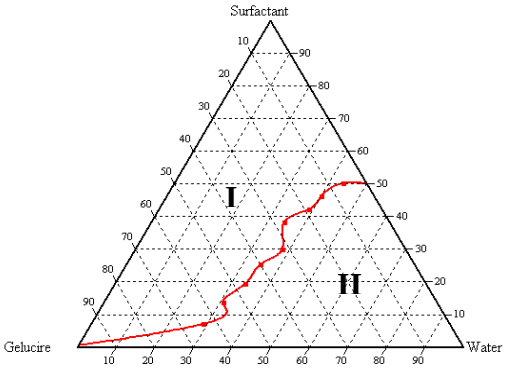
Surfactant = S-40:F-68 (0:10), at 60°C



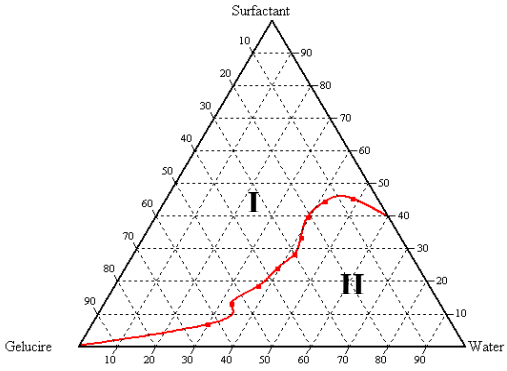
Surfactant = S-40:F-68 (3:7), at 60°C



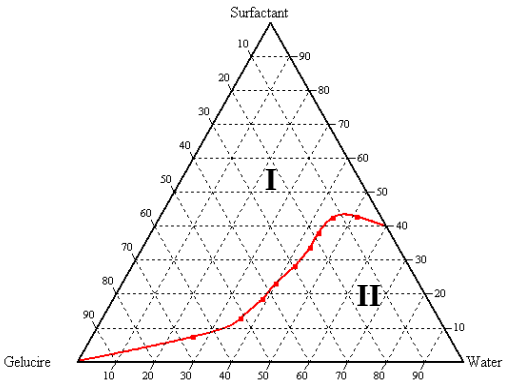
Surfactant = S-40:F-68 (5:5), at 60°C



Surfactant = S-40:F-68 (7:3), at 60°C



Surfactant = S-40:F-68 (10:0), at 60°C



Gelucire = macrogol glyceryl stearate (Gelucire® 50/13)

S-40 = polyoxyethylene 40 stearate (Myrj® 52)

F-68 = poloxamer 188 (Lutrol® F-68)

I: flowable microemulsion region (liquid clear zone)

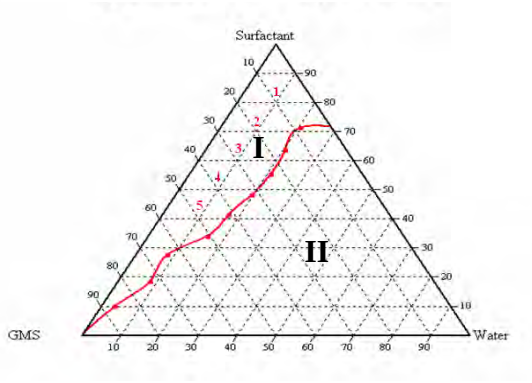
II: viscous or non-microemulsion region

Figure 45 The pseudo-ternary phase diagrams of lipid (Gelucire® 50/13), mixed surfactants (S-40:F-68) and water at 60°C

Confirmation and identification of microemulsion type

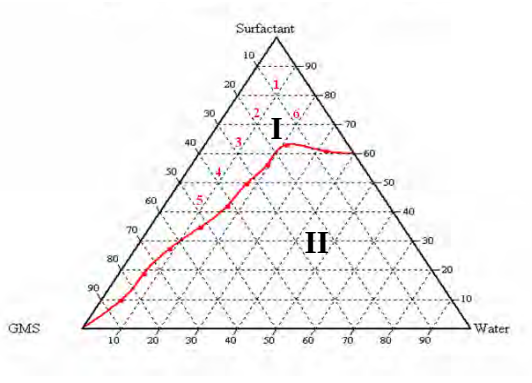
Various ratios of lipid, mixed surfactants and water were specified and prepared again within the area of microemulsion formation of each phase diagram in Figures 43 – 45 in order to confirm the formation and identify the type of microemulsions obtained. The samples were prepared and identified for w/o microemulsion as shown in Figures 46 – 48.

Surfactant = S-40:F-68 (0:10), at 70°C



At the ratio of S-40:F-68 = 0:10, The samples that were identified as w/o microemulsion were number 2, 3, 4 and 5 (4 samples from a total of 5 samples prepared).

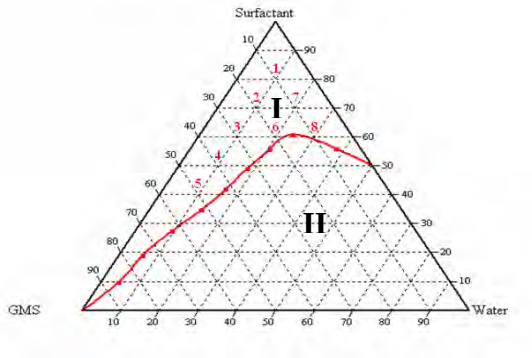
Surfactant = S-40:F-68 (3:7), at 70°C



At the ratio of S-40:F-68 = 3:7, The samples that were identified as w/o microemulsion were number 2, 3, 4 and 5 (4 samples from a total of 6 samples prepared).

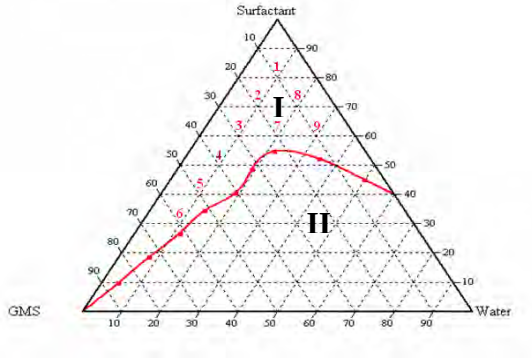
Figure 46 Confirmation and identification of samples in the microemulsion region of pseudo-ternary phase diagrams containing GMS as lipid at 70°C (I = flowable microemulsion region (liquid clear zone), II = viscous or non-microemulsion region).

Surfactant = S-40:F-68 (5:5), at 70°C



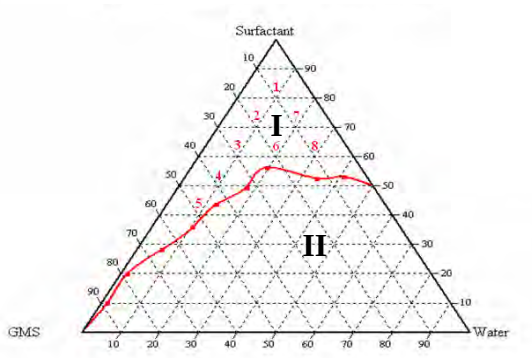
At the ratio of S-40:F-68 = 5:5,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5 and 6 (5 samples from a total of 8 samples prepared).

Surfactant = S-40:F-68 (7:3), at 70°C



At the ratio of S-40:F-68 = 7:3,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5, 6 and 7 (6 samples from a total of 9 samples prepared).

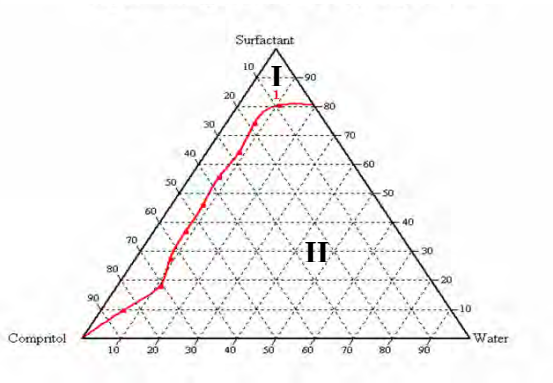
Surfactant = S-40:F-68 (10:0), at 70°C



At the ratio of S-40:F-68 = 10:0,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5 and 6 (5 samples from a total of 8 samples prepared).

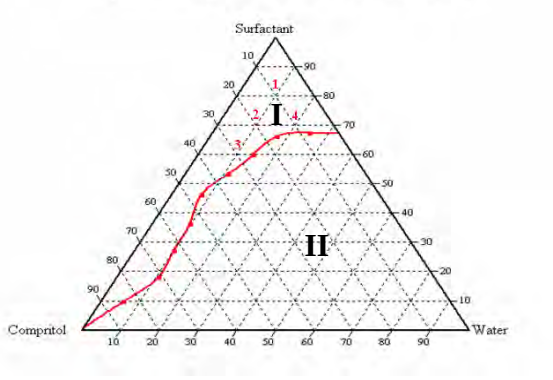
Figure 46 (Cont.) Confirmation and identification of samples in the microemulsion region of pseudo-ternary phase diagrams containing GMS as lipid at 70°C (I = flowable microemulsion region (liquid clear zone), II = viscous or non-microemulsion region).

Surfactant = S-40:F-68 (0:10), at 85°C



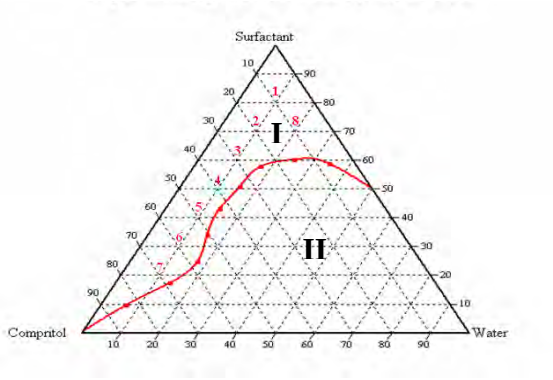
At the ratio of S-40:F-68 = 0:10,
The sample that was identified as w/o microemulsion was number 1 (only one sample prepared).

Surfactant = S-40:F-68 (3:7), at 85°C



At the ratio of S-40:F-68 = 3:7,
The samples that were identified as w/o microemulsion were number 1, 2 and 3 (3 samples from a total of 4 samples prepared).

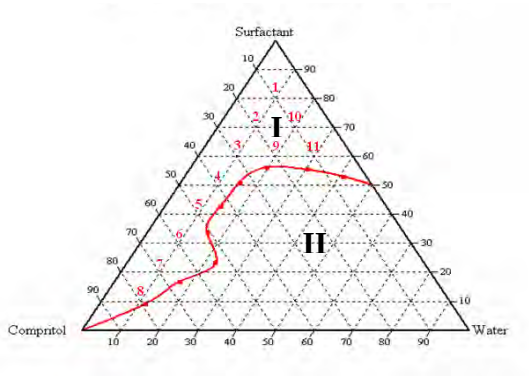
Surfactant = S-40:F-68 (5:5), at 85°C



At the ratio of S-40:F-68 = 5:5,
The samples that were identified as w/o microemulsion were number 1, 2, 3, 4, 5, 6, 7 and 8 (8 samples from a total of 8 samples prepared).

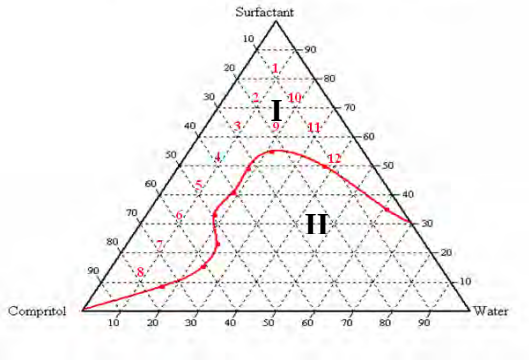
Figure 47 Confirmation and identification of samples in the microemulsion region of pseudo-ternary phase diagrams containing compritol® 888 ATO as lipid at 85°C (I = flowable microemulsion region (liquid clear zone), II = viscous or non-microemulsion region).

Surfactant = S-40:F-68 (7:3), at 85°C



At the ratio of S-40:F-68 = 7:3,
The samples that were identified as w/o microemulsion were number 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (10 samples from a total of 11 samples prepared).

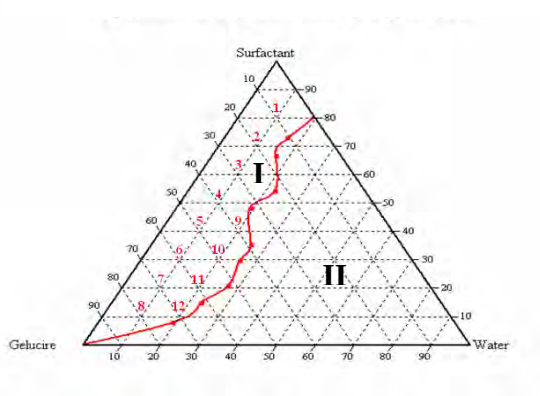
Surfactant = S-40:F-68 (10:0), at 85°C



At the ratio of S-40:F-68 = 10:0,
The samples that were identified as w/o microemulsion were number 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (10 samples from a total of 12 samples prepared).

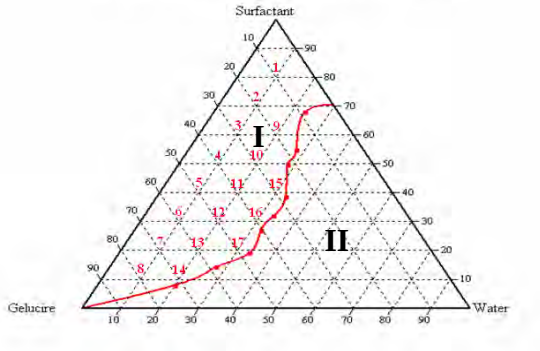
Figure 47 (Cont.) Confirmation and identification of samples in the microemulsion region of pseudo-ternary phase diagrams containing compritol® 888 ATO as lipid at 85°C (I = flowable microemulsion region (liquid clear zone), II = viscous or non-microemulsion region).

Surfactant = S-40:F-68 (0:10), at 60°C



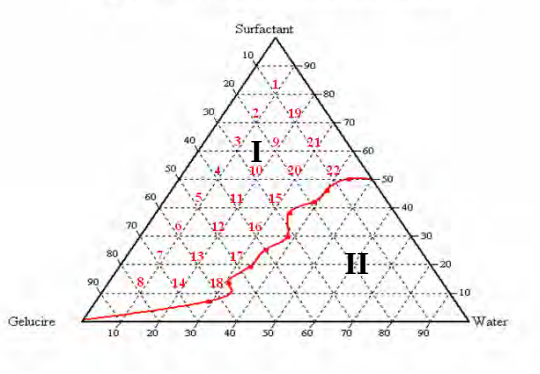
At the ratio of S-40:F-68 = 0:10,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 (11 samples from a total of 12 samples prepared).

Surfactant = S-40:F-68 (3:7), at 60°C



At the ratio of S-40:F-68 = 3:7,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 16 and 17
(14 samples from a total of 17 samples prepared).

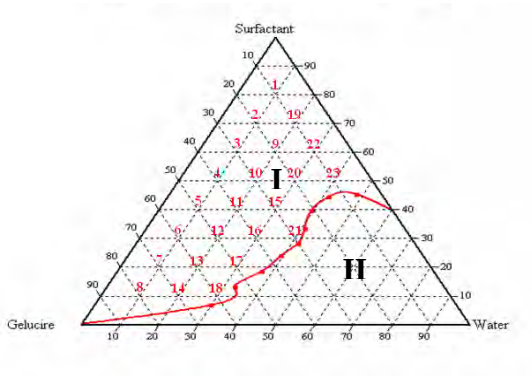
Surfactant = S-40:F-68 (5:5), at 60°C



At the ratio of S-40:F-68 = 5:5,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17 and 18
(15 samples from a total of 22 samples prepared).

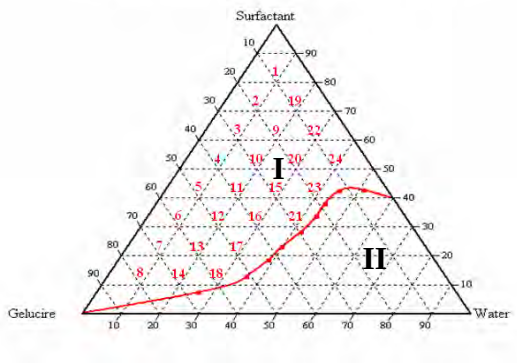
Figure 48 Confirmation and identification of samples in the microemulsion region of pseudo-ternary phase diagrams containing gelucire® 50/13 as lipid at 60°C
(I = flowable microemulsion region (liquid clear zone), II = viscous or non-microemulsion region).

Surfactant = S-40:F-68 (7:3), at 60°C



At the ratio of S-40:F-68 = 7:3,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17 and 18 (15 samples from a total of 23 samples prepared).

Surfactant = S-40:F-68 (10:0), at 60°C



At the ratio of S-40:F-68 = 10:0,
The samples that were identified as w/o microemulsion were number 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17 and 18 (15 samples from a total of 24 samples prepared).

Figure 48 (Cont.) Confirmation and identification of samples in the microemulsion region of pseudo-ternary phase diagrams containing gelucire® 50/13 as lipid at 60°C (I = flowable microemulsion region (liquid clear zone), II = viscous or non-microemulsion region).

Considering Figures 46 – 48, it was found that the w/o microemulsion region appeared to increase when the ratio of S-40 in the mixed surfactants was increased for all lipids. This could be due to the lower HLB value of S-40 (HLB = 16.9) than F-68 (HLB = 29) (Rowe, Sheskey, and Owen, 2006). S-40 may facilitate the formation of w/o microemulsion better than F-68. Therefore, the best surfactant ratio (S-40:F-68) that generated the largest region of w/o microemulsion was 7:3, 10:0 and 10:0 for lipid GMS, compritol and gelucire , respectively.

The formulas of lipid, surfactant and water that formed w/o microemulsion are summarized in Table 16 for each lipid at its respective optimum surfactant ratio.

Table 16 The formulas of lipid, surfactant and water that formed w/o microemulsion for each lipid at its respective optimum surfactant ratio

Type of lipid	Formula No.	Surfactant (g)		Water (g)	Lipid (g)	Total (g)
		S-40	F-68			
GMS (7:3)	2	0.49	0.21	0.1	0.2	1
	3	0.42	0.18	0.1	0.3	1
	4	0.35	0.15	0.1	0.4	1
	5	0.28	0.12	0.1	0.5	1
	6	0.21	0.09	0.1	0.6	1
	7	0.42	0.18	0.2	0.2	1
Compritol (10:0)	1	0.8	-	0.1	0.1	1
	2	0.7	-	0.1	0.2	1
	3	0.6	-	0.1	0.3	1
	4	0.5	-	0.1	0.4	1
	5	0.4	-	0.1	0.5	1
	6	0.3	-	0.1	0.6	1
	7	0.2	-	0.1	0.7	1
	8	0.1	-	0.1	0.8	1
	9	0.6	-	0.2	0.2	1
	10	0.7	-	0.2	0.1	1

Table 16 (Cont.) The formulas of lipid, surfactant and water that formed w/o microemulsion for each lipid at its respective optimum surfactant ratio

Type of lipid	Formula No.	Surfactant (g)		Water (g)	Lipid (g)	Total (g)
		S-40	F-68			
Gelucire (10:0)	2	0.7	-	0.1	0.2	1
	3	0.6	-	0.1	0.3	1
	4	0.5	-	0.1	0.4	1
	5	0.4	-	0.1	0.5	1
	6	0.3	-	0.1	0.6	1
	7	0.2	-	0.1	0.7	1
	8	0.1	-	0.1	0.8	1
	10	0.5	-	0.2	0.3	1
	11	0.4	-	0.2	0.4	1
	12	0.3	-	0.2	0.5	1
	13	0.2	-	0.2	0.6	1
	14	0.1	-	0.2	0.7	1
	16	0.3	-	0.3	0.4	1
	17	0.2	-	0.3	0.5	1
	18	0.1	-	0.3	0.6	1

Preparation of blank SLN and physical stability after 4 weeks at ambient room temperature

The w/o microemulsions were again prepared according to Table 16 and transformed into SLN by dispersing them into 1%, 2% and 3% w/v aqueous surfactant solutions (S-40 or F-68). Then, they were kept for 4 weeks at ambient room temperature to select formulations that showed a good physical stability.

To understand easily, the identification codes were created as follows:

“type of lipid / ratio of S-40:F-68 / number in the microemulsion region / % and type of external surfactant”.

For example, “GMS/7:3/2/1S” means that the formulation was prepared by using GMS as lipid, the ratio of mixed surfactants (S-40:F-68) = 7:3, number in the microemulsion region = 2 and it was dispersed into 1% w/v S-40 external surfactant solution.

To study physical stability of various blank SLN formulations, the following definitions were used:

Stable (S) : Good stability, no sedimentation and no gelation

Gelation, flowable (GF) : Increase in viscosity, no sediment occurred and the SLN was still able to flow. Partial gelation may also be observed but the SLN was still flowable

Gelation, not flowable (GN) : Extensive gelation with SLN unable to flow

Phase separation (PS) : Sedimentation with clear supernatant. Sediment was easily redispersible

Volume of sedimentation (F) : The number in (_) was the volume of sedimentation that could be calculated from the equation below:

$$\text{Volume of sedimentation (F)} = \frac{\text{Height of sediment (cm)}}{\text{Total height (cm)}}$$

Caking (C) : Sedimentation with milky supernatant. Sediment consisted of hard aggregates, which were difficult to redisperse

Degree of caking (+1 to +5) : Extent of caking
+1 = slight caking (≤ 1% total height)
+5 = extensive caking (≥ 5% total height)

Tables 17 – 21 show the physical stability of blank SLN formulations by w/o/w double emulsion method when stored at ambient room temperature for 4 weeks.

Table 17 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using GMS as lipid and S-40 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
2	GMS/7:3/2/1S	20	70	10	95.0	0.119	S	PS (0.95)	-
3	GMS/7:3/3/1S	30	60	10	116.1	0.141	S	S	GF
4	GMS/7:3/4/1S	40	50	10	119.4	0.091	S	S	S
5	GMS/7:3/5/1S	50	40	10	175.3	0.194	S	S	S
6	GMS/7:3/6/1S	60	30	10	200.6	0.247	S	S	S
7	GMS/7:3/7/1S	20	60	20	99.9	0.131	S	PS (0.95)	-
2	GMS/7:3/2/2S	20	70	10	82.8	0.181	S	PS (0.97)	-
3	GMS/7:3/3/2S	30	60	10	96.4	0.154	S	S	PS (0.97)
4	GMS/7:3/4/2S	40	50	10	107.7	0.166	S	S	S
5	GMS/7:3/5/2S	50	40	10	140.8	0.196	S	S	S
6	GMS/7:3/6/2S	60	30	10	146.3	0.264	S	S	GF
7	GMS/7:3/7/2S	20	60	20	92.3	0.157	S	PS (0.98)	-
2	GMS/7:3/2/3S	20	70	10	70.4	0.161	S	S	PS (0.92)
3	GMS/7:3/3/3S	30	60	10	90.5	0.122	S	S	PS (0.94)
4	GMS/7:3/4/3S	40	50	10	97.4	0.132	S	S	PS (0.96)
5	GMS/7:3/5/3S	50	40	10	124.6	0.162	S	S	S
6	GMS/7:3/6/3S	60	30	10	133.2	0.275	S	S	GF
7	GMS/7:3/7/3S	20	60	20	87.3	0.128	S	S	S

Note : PI = polydispersity index

Size and PI were determined only at week 0.

1S, 2S and 3S = 1%, 2% and 3% S-40 external surfactant, respectively

Table 18 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using GMS as lipid and F-68 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
2	GMS/7:3/2/1F	20	70	10	108.9	0.147	S	PS (0.94)	-
3	GMS/7:3/3/1F	30	60	10	126.7	0.276	S	S	PS (0.94)
4	GMS/7:3/4/1F	40	50	10	154.6	0.106	S	S	GF
5	GMS/7:3/5/1F	50	40	10	277.1	0.292	S	S	S
6	GMS/7:3/6/1F	60	30	10	N/A	N/A	GN	-	-
7	GMS/7:3/7/1F	20	60	20	113.9	0.159	S	PS (0.92)	-
2	GMS/7:3/2/2F	20	70	10	92.4	0.135	S	PS (0.86)	-
3	GMS/7:3/3/2F	30	60	10	121.8	0.124	S	S	PS (0.93)
4	GMS/7:3/4/2F	40	50	10	139.6	0.153	S	S	S
5	GMS/7:3/5/2F	50	40	10	232.7	0.267	S	S	S
6	GMS/7:3/6/2F	60	30	10	N/A	N/A	GN	-	-
7	GMS/7:3/7/2F	20	60	20	98.0	0.165	S	PS (0.66)	-
2	GMS/7:3/2/3F	20	70	10	86.6	0.191	S	PS (0.76)	-
3	GMS/7:3/3/3F	30	60	10	104.8	0.156	S	S	PS (0.97)
4	GMS/7:3/4/3F	40	50	10	133.5	0.150	S	GF	-
5	GMS/7:3/5/3F	50	40	10	226.2	0.278	S	GN	-
6	GMS/7:3/6/3F	60	30	10	N/A	N/A	GN	-	-
7	GMS/7:3/7/3F	20	60	20	93.4	0.076	S	PS (0.41)	-

Note : PI = polydispersity index

Size and PI were determined only at week 0.

1F, 2F and 3F = 1%, 2% and 3% F-68 external surfactant, respectively

Considering the effect of lipid and mixed surfactants ratios on the mean particle size, it was found that the mean particle size generally increased when increasing the percentage of lipid. On the contrary, increasing the percentage of surfactant, either internal (during w/o microemulsion formation) or external (during dispersion to form w/o/w double emulsion) resulted in the decrease in mean particle size especially for GMS and compritol. The higher amount of surfactants could emulsify lipid to smaller droplets in the step of double emulsion formation due to their surface active properties. From this study, when the percentage of external surfactant was increased from 1% to 3%, the mean particle sizes decreased for both S-40 and F-68 in case of GMS and compritol. High concentrations of surfactant could reduce the surface tension and facilitate the particle breakage during the size reduction process resulting in smaller particle size. Both the contents of lipid and surfactants in the SLN formulations thus influenced the mean particle sizes of the SLN and showed the same general trend for all lipids and surfactants used in this study.

To select the formulations that showed appropriate appearance, particle size and good physical stability, all formulations were visually observed immediately after preparation and during storage at ambient room temperature for 4 weeks. For GMS as lipid, all formulations had a good appearance when freshly prepared and had a small particle size (not more than 300 nm) and narrow size distribution (not more than 0.3). Considering the type of external surfactant, S-40 or F-68, it was found that S-40 generated smaller particle size than F-68 at every concentration for GMS (Tables 17 and 18). This could be due to interplay of several factors such as HLB, size and geometry of each surfactant. Furthermore, the physical stability of SLN formulations when using S-40 as surfactant showed better physical stability than using F-68 due to smaller particle size. Therefore, S-40 was selected to prepare SLN for GMS. For lipid concentration at 50% (in w/o microemulsion), they showed a good appearance when freshly prepared, had an appropriate particle size (and size distribution) and showed a good physical stability. Moreover, the appropriate concentration of lipid at 5% in SLN formulations (after dispersion in external surfactant solution) might be enough to load and protect entrapped Puag-Haad. Thus, formulas GMS/7:3/5/1S and GMS/7:3/5/3S were selected as the SLN base to entrap Puag-Haad in the next step.

Table 19 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using compritol as lipid and S-40 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
1	COM/10:0/1/1S	10	80	10	187.6	0.177	S	S	S
2	COM/10:0/2/1S	20	70	10	201.3	0.433	S	S	GF
3	COM/10:0/3/1S	30	60	10	225.4	0.031	S	S	PS (0.49)
4	COM/10:0/4/1S	40	50	10	326.7	0.048	S	S	S
5	COM/10:0/5/1S	50	40	10	357.6	0.015	S	S	S
6	COM/10:0/6/1S	60	30	10	648.3	0.359	S	S	S
7	COM/10:0/7/1S	70	20	10	766.3	0.251	S	C (+2)	C (+3)
8	COM/10:0/8/1S	80	10	10	869.1	0.120	S	C (+3)	C (+3)
9	COM/10:0/9/1S	20	60	20	211.4	0.278	S	S	GF
10	COM/10:0/10/1S	10	70	20	199.1	0.163	S	S	GF
1	COM/10:0/1/2S	10	80	10	177.1	0.111	S	S	GF
2	COM/10:0/2/2S	20	70	10	184.3	0.141	S	S	GF
3	COM/10:0/3/2S	30	60	10	192.6	0.088	S	S	PS (0.41)
4	COM/10:0/4/2S	40	50	10	315.5	0.005	S	S	S
5	COM/10:0/5/2S	50	40	10	342.3	0.005	S	S	S
6	COM/10:0/6/2S	60	30	10	568.5	0.364	S	S	S
7	COM/10:0/7/2S	70	20	10	618.4	0.091	S	C (+1)	C (+2)
8	COM/10:0/8/2S	80	10	10	809.7	0.054	S	C (+2)	C (+2)
9	COM/10:0/9/2S	20	60	20	191.3	0.274	S	S	C (+1)
10	COM/10:0/10/2S	10	70	20	171.7	0.232	S	S	GF

Note : COM = Compritol® 888 ATO, PI = polydispersity index

Size and PI were determined only at week 0.

1S, 2S and 3S = 1%, 2% and 3% S-40 external surfactant, respectively

Table 19 (Cont.) Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using compritol as lipid and S-40 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
1	COM/10:0/1/3S	10	80	10	152.2	0.285	S	S	S
2	COM/10:0/2/3S	20	70	10	159.5	0.178	S	GF	GF
3	COM/10:0/3/3S	30	60	10	174.1	0.221	S	S	PS (0.40)
4	COM/10:0/4/3S	40	50	10	300.8	0.005	S	S	S
5	COM/10:0/5/3S	50	40	10	329.0	0.005	S	S	S
6	COM/10:0/6/3S	60	30	10	498.5	0.365	S	S	S
7	COM/10:0/7/3S	70	20	10	565.4	0.005	S	C (+1)	C (+2)
8	COM/10:0/8/3S	80	10	10	711.0	0.029	S	C (+1)	C (+2)
9	COM/10:0/9/3S	20	60	20	168.2	0.228	S	S	GF
10	COM/10:0/10/3S	10	70	20	157.5	0.132	S	S	S

Note : COM = Compritol® 888 ATO, PI = polydispersity index

Size and PI were determined only at week 0.

1S, 2S and 3S = 1%, 2% and 3% S-40 external surfactant, respectively

Table 20 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using compritol as lipid and F-68 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
1	COM/10:0/1/1F	10	80	10	186.6	0.005	S	S	S
2	COM/10:0/2/1F	20	70	10	200.7	0.423	S	S	S
3	COM/10:0/3/1F	30	60	10	225.5	0.005	S	S	S
4	COM/10:0/4/1F	40	50	10	289.9	0.005	S	S	S
5	COM/10:0/5/1F	50	40	10	341.6	0.005	S	S	S
6	COM/10:0/6/1F	60	30	10	360.4	0.005	S	S	S
7	COM/10:0/7/1F	70	20	10	429.6	0.005	S	C (+1)	C (+1)
8	COM/10:0/8/1F	80	10	10	542.5	0.005	S	C (+2)	C (+3)
9	COM/10:0/9/1F	20	60	20	219.4	0.005	S	S	C (+1)
10	COM/10:0/10/1F	10	70	20	181.1	0.395	S	C (+1)	GF
1	COM/10:0/1/2F	10	80	10	172.9	0.005	S	S	S
2	COM/10:0/2/2F	20	70	10	193.2	0.363	S	S	S
3	COM/10:0/3/2F	30	60	10	212.7	0.005	S	S	S
4	COM/10:0/4/2F	40	50	10	264.5	0.005	S	S	S
5	COM/10:0/5/2F	50	40	10	289.6	0.005	S	S	S
6	COM/10:0/6/2F	60	30	10	343.0	0.005	S	S	S
7	COM/10:0/7/2F	70	20	10	402.7	0.005	S	C (+1)	C (+1)
8	COM/10:0/8/2F	80	10	10	483.4	0.005	S	C (+2)	C (+3)
9	COM/10:0/9/2F	20	60	20	208.4	0.005	S	S	S
10	COM/10:0/10/2F	10	70	20	171.9	0.089	S	C (+1)	GF

Note : COM = Compritol® 888 ATO, PI = polydispersity index

Size and PI were determined only at week 0.

1F, 2F and 3F = 1%, 2% and 3% F-68 external surfactant, respectively

Table 20 (Cont.) Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using compritol as lipid and F-68 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
1	COM/10:0/1/3F	10	80	10	152.3	0.005	S	S	S
2	COM/10:0/2/3F	20	70	10	184.8	0.043	S	GF	GF
3	COM/10:0/3/3F	30	60	10	191.7	0.057	S	S	S
4	COM/10:0/4/3F	40	50	10	237.8	0.005	S	S	S
5	COM/10:0/5/3F	50	40	10	280.4	0.005	S	S	S
6	COM/10:0/6/3F	60	30	10	322.1	0.005	S	S	S
7	COM/10:0/7/3F	70	20	10	375.0	0.005	S	C (+1)	C (+1)
8	COM/10:0/8/3F	80	10	10	407.6	0.005	S	C (+2)	C (+3)
9	COM/10:0/9/3F	20	60	20	188.4	0.005	S	S	S
10	COM/10:0/10/3F	10	70	20	155.5	0.005	S	C (+1)	GF

Note : COM = Compritol® 888 ATO, PI = polydispersity index

Size and PI were determined only at week 0.

1F, 2F and 3F = 1%, 2% and 3% F-68 external surfactant, respectively

For compritol as lipid, SLN formulations generally gave larger particle sizes than GMS. It might be due to higher melting point and lower HLB of compritol. Considering the type of external surfactant, S-40 could generate the particle sizes not different from F-68 when using low concentration of lipid. But, at high concentration of lipid, F-68 could generate smaller particle sizes than S-40 (Tables 19 and 20). Compritol was a very lipophilic lipid (soluble in chloroform), so the surfactant with high HLB like F-68 is necessary to emulsify this lipid in water to small droplets. It is possible that the effect of surfactant HLB may be more pronounced in the case of compritol. Furthermore, the physical stability of SLN formulations when using F-68 as surfactant showed better physical stability than using S-40. Therefore, F-68 was selected to prepare SLN for compritol. For lipid concentration at 50% (in w/o microemulsion), they also showed a good appearance when freshly prepared, had an appropriate particle size and good physical stability. Similarly, the appropriate concentration of lipid at 5% in SLN formulations (after dispersion) might be enough to load and protect entrapped Puag-Haad. Thus, formulas COM/10:0/5/1F and COM/10:0/5/3F were the selected formulations to entrap Puag-Haad in the next step.

For gelucire as lipid and S-40 as external surfactant, no SLN were formed in this study. The formulations were clear and their appearances were like solution. No particles were detected by visual observation. It thus appeared that w/o/w double emulsion could not be formed after addition of S-40 to microemulsion of gelucire.

For gelucire as lipid and F-68 as external surfactant, w/o/w double emulsion and subsequent SLN could be formed. This was probably due to the more appropriate HLB value of F-68 which facilitated emulsification of oil droplets in water. However, only 5 formulations of SLN could be obtained with F-68. These formulations did not show desirable milky appearances and had large particle sizes (Table 21). Therefore, gelucire was not selected to prepare SLN containing Puag-Haad in the next step.

Table 21 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by w/o/w double emulsion method using gelucire as lipid and F-68 as external surfactant

No.	Code	Lipid (%)	Mixed surfactants (%)	Water (%)	Freshly prepared SLN		Appearance		
							Week		
					Size (nm)	PI	0	2	4
8	GE/10:0/8/2F	80	10	10	353.8	0.274	S	S	S
14	GE/10:0/14/2F	70	10	20	249.9	0.332	S	S	S
8	GE/10:0/8/3F	80	10	10	626.7	0.315	S	S	S
14	GE/10:0/14/3F	70	10	20	490.1	0.268	S	S	S
18	GE/10:0/18/3F	60	10	30	301.1	0.366	S	S	GF

Note : GE = Gelucire® 50/13, PI = polydispersity index
Size and PI were determined only at week 0.
1F, 2F and 3F = 1%, 2% and 3% F-68 external surfactant, respectively

Figures 49 – 51 are photographs of representative SLN formulations prepared by w/o/w double emulsion method using 3 different lipids.

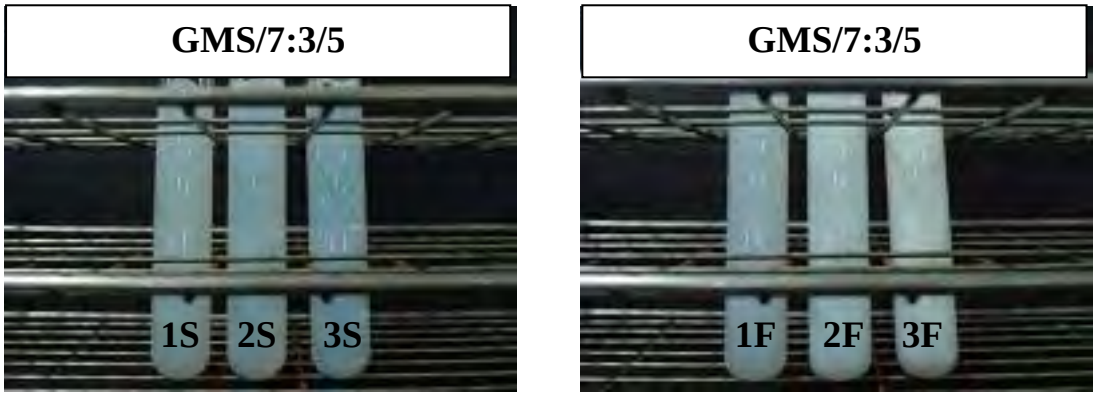


Figure 49 Freshly prepared blank SLN by w/o/w double emulsion method using GMS as lipid and S-40:F-68 mixed surfactant ratio of 7:3 (formula no. 5)

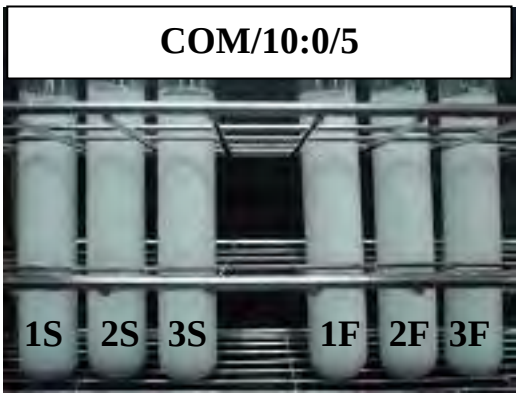


Figure 50 Freshly prepared blank SLN by w/o/w double emulsion method using compritol® 888 ATO as lipid and S-40:F-68 mixed surfactant ratio of 10:0 (formula no. 5)

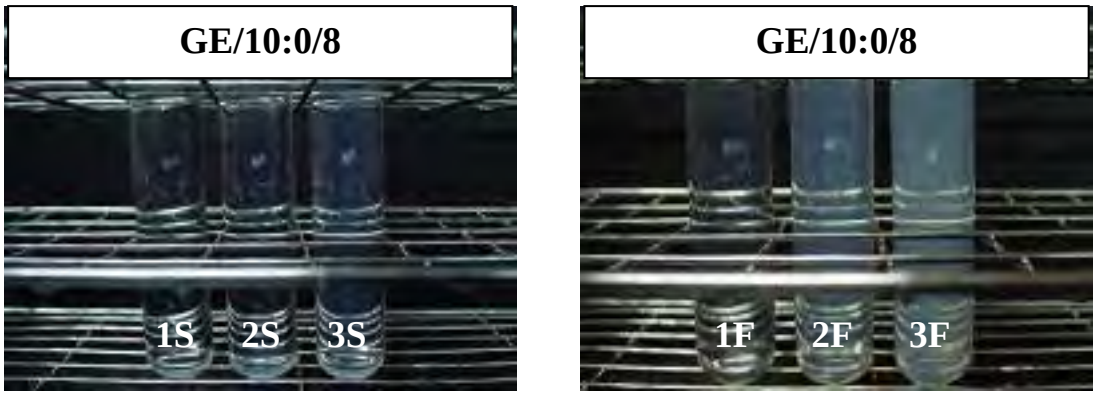


Figure 51 Freshly prepared blank SLN by w/o/w double emulsion method using gelucire® 50/13 as lipid and S-40:F-68 mixed surfactant ratio of 10:0 (formula no. 8)

1.2 Preparation of blank SLN by cold homogenization method

1.2.1 Preparation of blank SLN and physical stability after 4 weeks at ambient room temperature

The solid lipid was ground and dispersed into 1%, 2% and 3% w/v aqueous surfactant solutions using a high speed stirrer at 10,000 rpm for 3 minutes. Then, they were homogenized at ambient room temperature using a

high pressure homogenizer at 800 bar 3 cycles. The concentration of lipid in the formulations was 5% w/v. Then, they were kept for 4 weeks at ambient room temperature to select formulations that gave the best physical stability.

To understand easily, the codes were designated as follows:

"type of lipid / % and type of external surfactant".

For example, "GMS/1S" means that the formulation was prepared using GMS as lipid and dispersed into 1% w/v S-40.

Tables 22 – 23 show the physical stability during 4-week storage at ambient room temperature of blank SLN formulations prepared by cold homogenization method.

Table 22 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by cold homogenization method using GMS as lipid

Sample	Lipid (%)	Surfactant (Type and %)	Freshly prepared SLN		Appearance		
					Week		
			Size (nm)	PI	0	2	4
GMS/1S	5%	1% S-40	322.7	0.232	S	S	S
GMS/2S	5%	2% S-40	313.4	0.236	S	S	S
GMS/3S	5%	3% S-40	290.5	0.256	S	S	S
GMS/1F	5%	1% F-68	399.5	0.306	S	S	PS(0.89)
GMS/2F	5%	2% F-68	326.0	0.272	S	S	PS(0.88)
GMS/3F	5%	3% F-68	318.8	0.224	S	S	PS(0.92)

For GMS, the particle size of SLN formulations by this method were about 290 – 400 nm depending on formulations. Similar to w/o/w double emulsion method, the percentage of surfactant influenced the particle size of SLN formulations. Increasing surfactant concentration resulted in smaller particle size. S-40 also generated smaller particle size and better physical stability than F-68 at all concentrations. Therefore,

formulas GMS/1S and GMS/3S were selected to prepare SLN containing Puag-Haad in the next step.

For compritol as lipid, no SLN formulations were prepared by cold homogenization method because they were difficult to grind up to small particles. When these formulations were passed through the high pressure homogenizer, the pressure was raised over the optimal limit of instrument. Then, the instrument stopped working. In general, lipids that have high melting point are appropriate to produce SLN by cold homogenization method because the difference between the melting point of lipid and homogenization temperature is large enough to avoid melting of lipid during homogenization process. For compritol, its melting point was 65 – 77°C which was higher than GMS (55 – 60°C) (Rowe, Sheskey, and Owen, 2006) and gelucire (about 50°C). It was thus regrettable that no SLN formulations could be prepared by this method when using compritol as lipid. However, provided that a more sophisticated instrument or an improved process is available, it may be possible to produce SLN formulations from compritol by this method.

For gelucire as lipid, the particle size of SLN formulations were about 400 – 520 nm depending on formulations. Similar to other lipids, the percentage of surfactant influenced the particle size of SLN formulations. F-68 generated smaller particle size than S-40. Both S-40 and F-68 showed good physical stability of SLN formulations. Unfortunately for gelucire, freshly prepared SLN formulations did not give good appearances. These formulations were translucent and not milky regardless of surfactants. They could not hide the yellow color of Puag-Haad to improve product appearance. Therefore, gelucire was not selected to prepare SLN containing Puag-Haad in the next step.

Table 23 Size, polydispersity index (PI) and physical stability during storage at ambient room temperature of various blank SLN formulations prepared by cold homogenization method using gelucire as lipid

Sample	Lipid (%)	Surfactant (Type and %)	Freshly prepared SLN		Appearance		
					Week		
			Size (nm)	PI	0	2	4
GE/1S	5%	1% S-40	513.3	0.293	S	S	S
GE/2S	5%	2% S-40	462.1	0.232	S	S	S
GE/3S	5%	3% S-40	423.0	0.352	S	S	S
GE/1F	5%	1% F-68	508.4	0.244	S	S	S
GE/2F	5%	2% F-68	434.6	0.207	S	S	S
GE/3F	5%	3% F-68	407.5	0.112	S	S	S

Figures 52 – 53 show the samples of SLN formulations obtained by cold homogenization method when using GMS and gelucire (compritol could not prepare SLN by this method).

Figure 52 Freshly prepared blank SLN by cold homogenization method using GMS as lipid

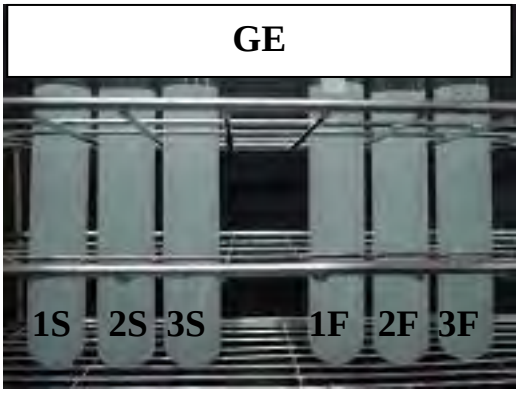


Figure 53 Freshly prepared blank SLN by cold homogenization method using gelucire® 50/13 as lipid

2. Preparation of SLN containing Puag-Haad

To study the effects of Puag-Haad loading and surfactant concentration on particle size and entrapment efficiency, the blank SLN formulations from 1.1.3 and 1.2.1 were selected to entrap Puag-Haad. These were formulas GMS/7:3/5/1S; GMS/7:3/5/3S; COM/10:0/5/1F; COM/10:0/5/3F; GMS/1S and GMS/3S. SLN containing Puag-Haad were prepared to final concentration of Puag-Haad about 0.25% and 0.50% w/v. The concentrations of external surfactant were 1% and 3% w/v using water and citrate buffer pH 5.5 as vehicle. In addition, the theoretical final lipid concentration in all formulations was 5% w/v. All formulations were prepared in 3 batches. Details of each formulas are given in Table 24.

Table 24 The selected formulations to prepare SLN containing Puag-Haad

Code	Lipid	Method	Type and % surfactant	Vehicle	% Puag-Haad loading
GMS/7:3/5/1S	GMS	w/o/w double emulsion	1% S-40	Water or citrate buffer pH 5.5	0.25% or 0.50%
GMS/7:3/5/3S	GMS	w/o/w double emulsion	3% S-40	Water or citrate buffer pH 5.5	0.25% or 0.50%
COM/10:0/5/1F	Compritol	w/o/w double emulsion	1% F-68	Water or citrate buffer pH 5.5	0.25% or 0.50%
COM/10:0/5/3F	Compritol	w/o/w double emulsion	3% F-68	Water or citrate buffer pH 5.5	0.25% or 0.50%
GMS/1S	GMS	Cold homogenization	1% S-40	Water or citrate buffer pH 5.5	0.25% or 0.50%
GMS/3S	GMS	Cold homogenization	3% S-40	Water or citrate buffer pH 5.5	0.25% or 0.50%

2.1 Preparation of SLN containing Puag-Haad by w/o/w double emulsion method

2.1.1 Effect on particle size

The different SLN formulations were respectively measured by PCS. All data are shown as mean ± SD (n = 3).

Tables 25 – 26 and Figures 54 – 55 show the particle size and PI of freshly prepared SLN formulations by w/o/w double emulsion method using GMS as lipid.

The particle size of all formulations were about 100 – 280 nm in water, about 110 – 700 nm in citrate buffer pH 5.5 and were different from each other depending on formulations.

Table 25 Particle size and size distribution of freshly prepared SLN formulations by w/o/w double emulsion method when using water as vehicle (for GMS)

Sample	% Puag-Haad loading	Size (nm)±SD	PI±SD
GMS/7:3/5/1S	Blank	186.4±1.72	0.261±0.007
GMS/7:3/5/1S	0.25%	206.6±4.23	0.263±0.038
GMS/7:3/5/1S	0.50%	271.0±5.51	0.297±0.010
GMS/7:3/5/3S	Blank	107.6±2.17	0.195±0.035
GMS/7:3/5/3S	0.25%	140.4±2.88	0.212±0.008
GMS/7:3/5/3S	0.50%	171.1±7.05	0.259±0.022

Table 26 Particle size and size distribution of freshly prepared SLN formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS)

Sample	% Puag-Haad loading	Size (nm)±SD	PI±SD
GMS/7:3/5/1S	Blank	186.8±3.97	0.272±0.002
GMS/7:3/5/1S	0.25%	350.7±5.27	0.351±0.017
GMS/7:3/5/1S	0.50%	673.6±27.02	0.346±0.010
GMS/7:3/5/3S	Blank	112.2±2.83	0.180±0.034
GMS/7:3/5/3S	0.25%	167.3±4.84	0.269±0.019
GMS/7:3/5/3S	0.50%	246.9±5.77	0.298±0.031

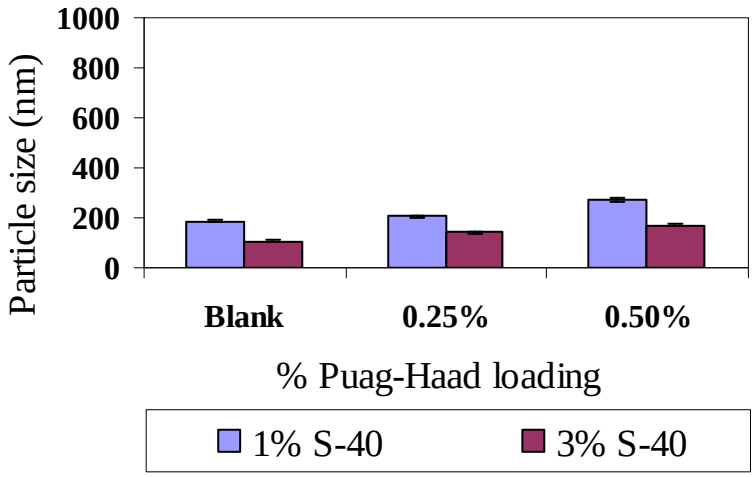


Figure 54 Effect of Puag-Haad loading and percentage external surfactant (S-40) on particle size of freshly prepared SLN by w/o/w double emulsion method when using water as vehicle (for GMS)

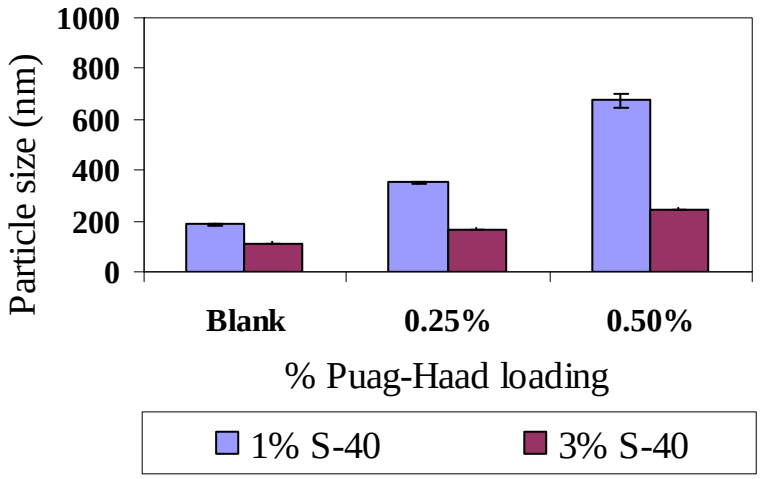


Figure 55 Effect of Puag-Haad loading and percentage external surfactant (S-40) on particle size of freshly prepared SLN by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS)

After statistical analysis by one-way ANOVA and multiple comparison (LSD), it was found that they were significantly different in particle size of formulations depending on the concentration of Puag-Haad loading and external surfactant. The particle size of SLN containing Puag-Haad were significantly different from blank SLN ($P < 0.05$). The incorporation of Puag-Haad into SLN showed the increase in the particle size. This result agreed with the previous study of Ma et al. (2007) who developed SLN containing tea polyphenols.

Moreover, the concentration of surfactant in the aqueous external phase could influence the particle size. The particle size and PI were significantly decreased with the increase in surfactant concentration ($P < 0.05$). High concentrations of surfactant could reduce the surface tension and facilitate the particle partition during the size reduction process resulting in smaller particle size. On the contrary, Waree Tiyaboonchai et al. (2007), who formulated SLN containing curcuminoids by microemulsion method using stearic acid, F-68 and dioctyl sodium sulfosuccinate, reported that when the amounts of surfactant and co-surfactant in SLN were increased, the mean particle size and PI also increased. They explained that the observed aggregation may involve an intrinsic thermodynamic instability of the nanoparticle system with dispersed molecules of the surfactant and co-surfactant in the lipid matrix but finally resulting in an adsorption of the emulsifier to the particle surface. At very low concentration, the emulsifier is adsorbed directly onto the surface of the particles. However, at high concentration of the emulsifier, compression of the emulsifier molecules at the particles surface with formation of loops and tails became prominent and finally leading to the bridging between the primary nanoparticles and size increase (Waree Tiyaboonchai et al., 2007; Freitas and Müller, 1999; Goppert and Müller, 2005).

The same trend of Puag-Haad loading and surfactant concentration on particle size was also observed when using citrate buffer pH 5.5 as vehicle.

Comparing the effect of vehicle on particle size of SLN formulations, it was found that the particle size of blank SLN formulations in citrate buffer pH 5.5 were not significantly different from blank SLN formulation in water system. However, the significant difference occurred between SLN containing Puag-Haad in water and in citrate buffer pH 5.5. SLN containing Puag-Haad in citrate buffer always gave larger

particles than their corresponding water system. Currently, there was no clear explanation about this observation. The presence of citrate ions might interact with Puag-Haad components, lipid and surfactant in some way that resulted in formation of larger particles or aggregations.

2.1.2 Effect on zeta potential

The different SLN formulations were respectively measured for zeta potential by PCS. All data are shown as mean ± SD (n = 3).

Tables 27 – 28 show the zeta potential of freshly prepared SLN formulations by w/o/w double emulsion method using GMS as lipid.

The zeta potentials of all SLN formulations in water system were negative (Table 27). The incorporation of Puag-Haad into SLN showed a significant increase in positive charge on the zeta potentials of nanoparticles ($P < 0.05$).

In contrast to the water system, the zeta potentials of all SLN formulations in citrate buffer pH 5.5 system were positive (Table 28). However, incorporation of Puag-Haad into SLN caused a significant increase in positive charge on the zeta potentials of nanoparticles similar to the water system. As with the vehicle effect on particle size, no clear explanation could be made regarding the vehicle effect on the zeta potential. In water, the SLN had negative zeta potential values indicating that the net charge on the surface of the tightly bound layer surrounding the particles was negative. When citrate buffer was used as vehicle, it is possible that sodium ions from sodium citrate may have acted as counter ion and reversed the zeta potential from negative to positive values. Puag-Haad may also process some unknown cationic components that may act as counter ions and contribute to more positive zeta potential.

Table 27 Zeta potential of freshly prepared SLN formulations by w/o/w double emulsion method when using water as vehicle (for GMS)

Sample	% Puag-Haad loading	Zeta potential (mV)±SD
GMS/7:3/5/3S	Blank	-27.11±5.33
GMS/7:3/5/3S	0.25%	-19.70±0.85
GMS/7:3/5/3S	0.50%	-19.50±0.90

Table 28 Zeta potential of freshly prepared SLN formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS)

Sample	% Puag-Haad loading	Zeta potential (mV)±SD
GMS/7:3/5/3S	Blank	21.80±3.30
GMS/7:3/5/3S	0.25%	29.51±3.78
GMS/7:3/5/3S	0.50%	29.98±3.40

2.1.3 Effect on entrapment efficiency

The entrapment efficiency of Puag-Haad was determined using ultrafiltration method and UV-Visible Spectrophotometry. All data are shown as mean ± SD (n = 3).

Tables 29 – 30 and Figures 56 – 57 show the percentage of entrapment efficiency and recovery of freshly prepared SLN formulations by w/o/w double emulsion method using GMS as lipid.

All formulations had moderate to high entrapment efficiency with high recovery. The values were different depending on formulations. The entrapment efficiency of SLN formulations were about 60 – 85% with > 80% of recovery in both water and citrate buffer pH 5.5 systems. Entrapment efficiency appeared to decrease when citrate buffer was used as vehicle, particularly at low external surfactant concentration (1% S-40). This was mainly due to larger particle size of SLN in the citrate buffer.

Table 29 The percentage of entrapment efficiency and recovery of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using water as vehicle (for GMS) (The amount of Puag-Haad was analyzed by UV-Visible Spectrophotometric method)

Sample	% Puag-Haad loading	Entrapment efficiency (%)±SD	Recovery (%)±SD
GMS/7:3/5/1S	0.25%	75.29±0.17	99.85±0.49
GMS/7:3/5/1S	0.50%	64.50±1.08	87.21±1.92
GMS/7:3/5/3S	0.25%	85.68±0.68	100.73±1.17
GMS/7:3/5/3S	0.50%	79.00±1.86	92.17±1.75

Table 30 The percentage of entrapment efficiency and recovery of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS) (The amount of Puag-Haad was analyzed by UV-Visible Spectrophotometric method)

Sample	% Puag-Haad loading	Entrapment efficiency (%)±SD	Recovery (%)±SD
GMS/7:3/5/1S	0.25%	67.90±1.83	87.19±2.66
GMS/7:3/5/1S	0.50%	59.96±0.58	81.61±0.44
GMS/7:3/5/3S	0.25%	84.36±1.87	97.61±1.40
GMS/7:3/5/3S	0.50%	77.33±1.19	91.56±1.94

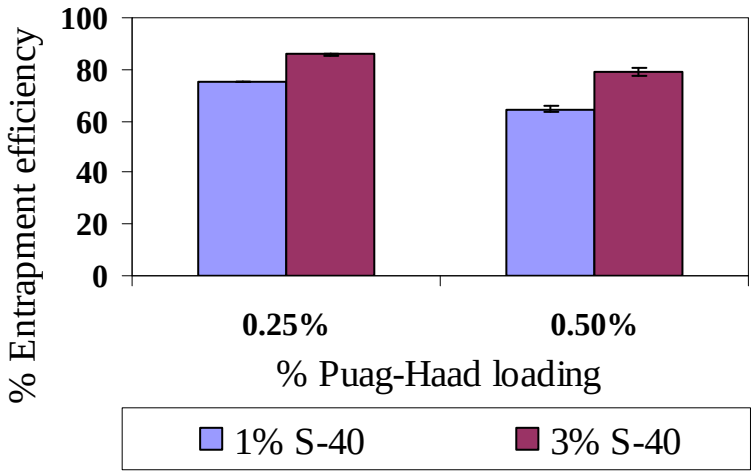


Figure 56 The percentage of entrapment efficiency of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using water as vehicle (for GMS)

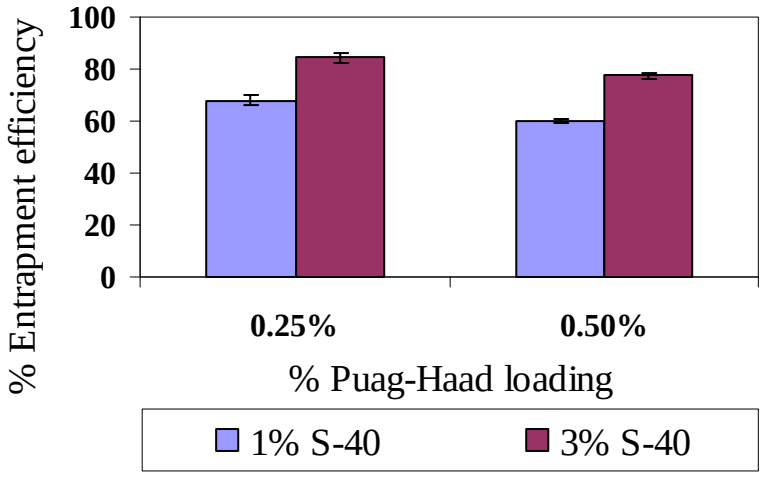


Figure 57 The percentage of entrapment efficiency of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS)

After statistical analysis by one-way ANOVA and multiple comparison (LSD), it was found that they were significant differences in the percentage of entrapment efficiency of formulations depending on the concentration of Puag-Haad loading and external surfactant. It was found that 0.25% Puag-Haad loading always showed higher entrapment efficiency than 0.50% regardless of external surfactant concentration and vehicle. This result agreed with the previous study of Ma et al. (2007) who reported that as the concentration of incorporated drug was increased, the entrapment efficiency decreased accordingly.

Furthermore, the concentration of surfactant in the aqueous external phase could influence the entrapment efficiency. 3% S-40 showed higher entrapment efficiency than 1% S-40 regardless of % Puag-Haad loading and type of vehicle. The increase in the surfactant concentration significantly increased in the entrapment efficiency ($P < 0.05$). The addition of surfactant in aqueous external phase might stabilize the lipid particles, reduce the diffusion speed of the drug and therefore increase the entrapment efficiency. On the other hand, 1% S-40 might not be sufficient to stabilize SLN containing Puag-Haad as compared to 3% S-40 resulting in lower entrapment efficiency. Another explanation for increased entrapment of Puag-Haad could be due to smaller particle size. From section 2.1.1, it was found that increasing surfactant concentration decreased the average particle size and thus the overall surface area was increased. It is possible that some Puag-Haad molecules might distribute to the surface of the particles and/or could be stabilized at the surfactant layer on the surface of SLN. Therefore, when the surface area increased, more Puag-Haad could be trapped at the nanoparticles surface.

From the finding of Waree Tiyaboonchai et al. (2007), the higher surfactant concentration resulted in bridging and forming of larger particle sizes. Since their drug (curcuminoids) was mainly incorporated in the surfactant layer at the surface of SLN, the entrapment efficiency was accordingly decreased when increasing the surfactant concentration due to smaller surface area of particles.

The same trend on the entrapment efficiency was observed when using citrate buffer pH 5.5 as vehicle.

2.1.4 Particle size-entrapment efficiency scattering plot

To define the model of drug incorporation in SLN, a particle size – entrapment efficiency scattering plot had been constructed. Data are shown in Tables 31 – 32 and the plots are shown in Figures 58 – 59.

Table 31 The correlation between particle size and entrapment efficiency of SLN containing Puag-Haad formulations by w/o/w double emulsion method when using water as vehicle (for GMS)

Sample	% Puag-Haad loading	Particle size (nm)	Entrapment efficiency (%)
GMS/7:3/5/1S	0.25%	203.1	75.45
GMS/7:3/5/1S	0.25%	205.4	75.31
GMS/7:3/5/1S	0.25%	211.3	75.11
GMS/7:3/5/1S	0.50%	270.0	65.65
GMS/7:3/5/1S	0.50%	266.0	64.34
GMS/7:3/5/1S	0.50%	276.9	63.52
GMS/7:3/5/3S	0.25%	142.4	84.94
GMS/7:3/5/3S	0.25%	137.1	85.82
GMS/7:3/5/3S	0.25%	141.7	86.27
GMS/7:3/5/3S	0.50%	176.4	79.30
GMS/7:3/5/3S	0.50%	173.8	80.69
GMS/7:3/5/3S	0.50%	163.1	77.00

Table 32 The correlation between particle size and entrapment efficiency of SLN containing Puag-Haad formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS)

Sample	% Puag-Haad loading	Particle size (nm)	Entrapment efficiency (%)
GMS/7:3/5/1S	0.25%	345.7	69.38
GMS/7:3/5/1S	0.25%	356.2	68.48
GMS/7:3/5/1S	0.25%	350.2	65.85
GMS/7:3/5/1S	0.50%	642.4	60.62
GMS/7:3/5/1S	0.50%	689.1	59.74
GMS/7:3/5/1S	0.50%	689.3	59.52
GMS/7:3/5/3S	0.25%	171.3	82.99
GMS/7:3/5/3S	0.25%	161.9	83.59
GMS/7:3/5/3S	0.25%	168.6	86.49
GMS/7:3/5/3S	0.50%	243.7	78.14
GMS/7:3/5/3S	0.50%	253.6	77.88
GMS/7:3/5/3S	0.50%	243.5	75.97

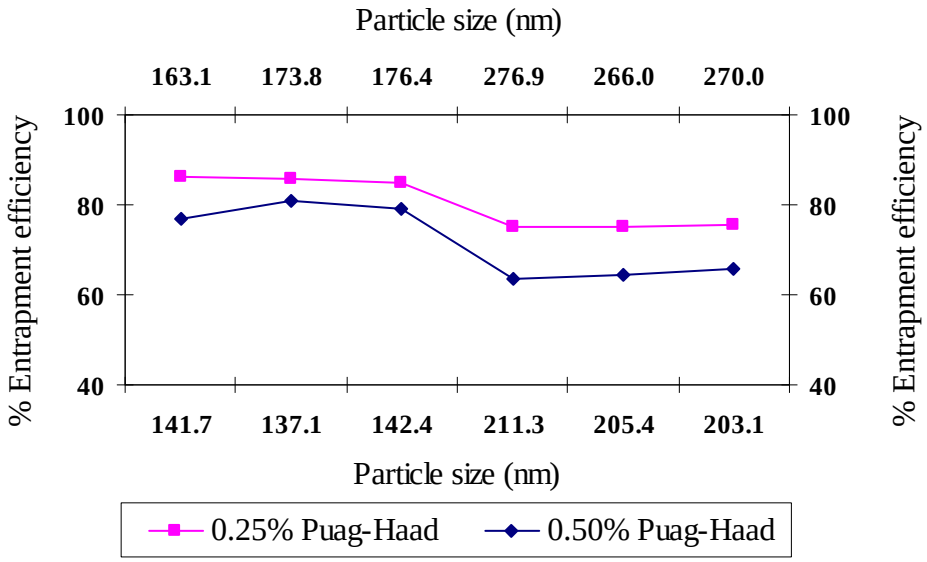


Figure 58 Particle size-entrapment efficiency scattering plot of SLN containing Puag-Haad by w/o/w double emulsion method when using water as vehicle (for GMS)

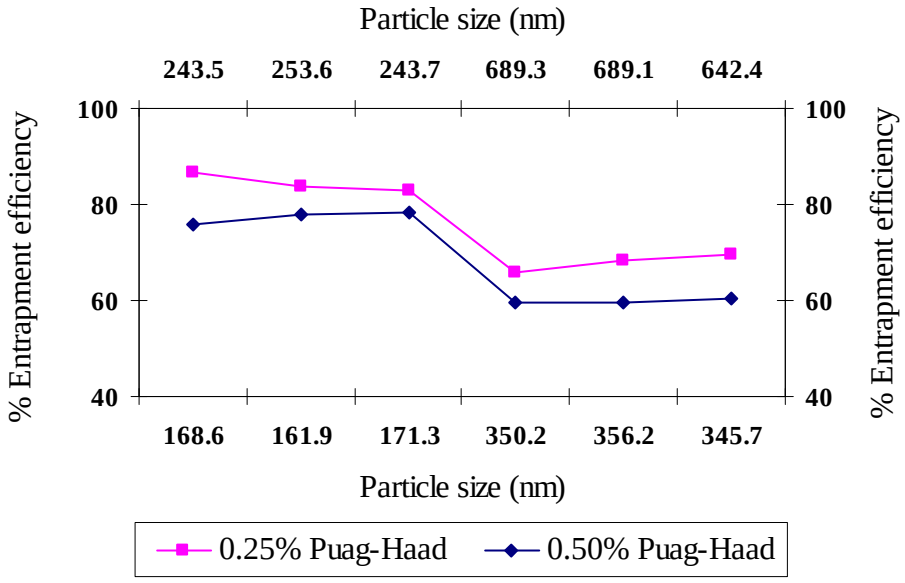


Figure 59 Particle size-entrapment efficiency scattering plot of SLN containing Puag-Haad by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for GMS)

From this result, it was found that the entrapment efficiency was decreased when the particle size was increased. The surface area of particles depended on the particle size in that the smaller particles had more surface area than larger particles. If Puag-Haad molecules had been stabilized at the surface of SLN, the smaller particles should have higher entrapment efficiency than larger particles. Therefore, the results of this study corresponded with the above assumption. However, this assumption should be further confirmed by release studies (Results and discussion in part C).

2.1.5 Effect of lipid

To study the effect of lipid on particle size and entrapment efficiency, SLN containing Puag-Haad were prepared using compritol as lipid in comparison with GMS.

Tables 33 – 34 and Figures 60 – 61 show the particle size and PI of freshly prepared SLN formulations by w/o/w double emulsion method using compritol as lipid and F-68 as external surfactant (Data = mean±SD, n = 3).

Table 33 Particle size and size distribution of freshly prepared SLN formulations by w/o/w double emulsion method when using water as vehicle (for compritol® 188 ATO)

Sample	% Puag-Haad loading	Size (nm)±SD	PI±SD
COM/10:0/5/1F	Blank	372.2±22.34	0.144±0.088
COM/10:0/5/1F	0.25%	459.5±19.86	0.171±0.051
COM/10:0/5/1F	0.50%	543.3±32.89	0.188±0.066
COM/10:0/5/3F	Blank	331.3±23.47	0.126±0.020
COM/10:0/5/3F	0.25%	373.8±18.57	0.005±0.000
COM/10:0/5/3F	0.50%	464.7±38.15	0.005±0.000

Table 34 Particle size and size distribution of freshly prepared SLN formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for compritol® 188 ATO)

Sample	% Puag-Haad loading	Size (nm)±SD	PI±SD
COM/10:0/5/1F	Blank	435.4±35.42	0.209±0.087
COM/10:0/5/1F	0.25%	491.2±35.50	0.212±0.074
COM/10:0/5/1F	0.50%	553.3±14.47	0.245±0.154
COM/10:0/5/3F	Blank	372.3±21.10	0.201±0.065
COM/10:0/5/3F	0.25%	457.7±27.06	0.193±0.095
COM/10:0/5/3F	0.50%	516.6±9.47	0.232±0.178

The particle size of all formulations were about 330 – 550 nm in water, about 370 – 560 nm in citrate buffer pH 5.5 and differed from each other depending on formulations. All SLN formulations when using compritol as lipid showed larger particle size than GMS.

For compritol as lipid, the effects of drug loading and surfactant concentration on particle size were the same as using GMS as described above. Briefly, the drug loading and surfactant concentration influenced the particle size of formulations. The increase in drug loading resulted in increasing in particle size. In contrast, when the surfactant concentration was increased, the particle size decreased. The trend on particle size was similar between using water and citrate buffer pH 5.5 as vehicle. Citrate buffer also gave larger particle size than their corresponding water systems.

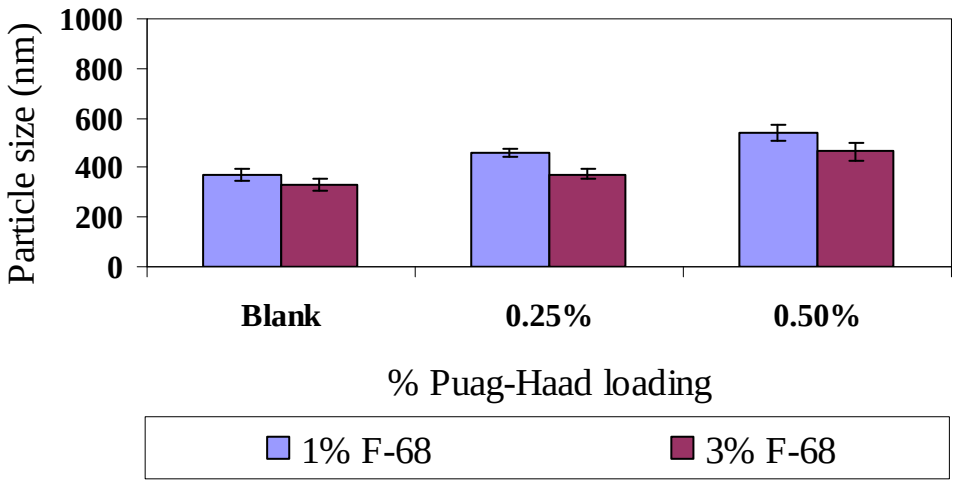


Figure 60 Particle size of freshly prepared SLN formulations by w/o/w double emulsion method when using water as vehicle (for compritol® 188 ATO)

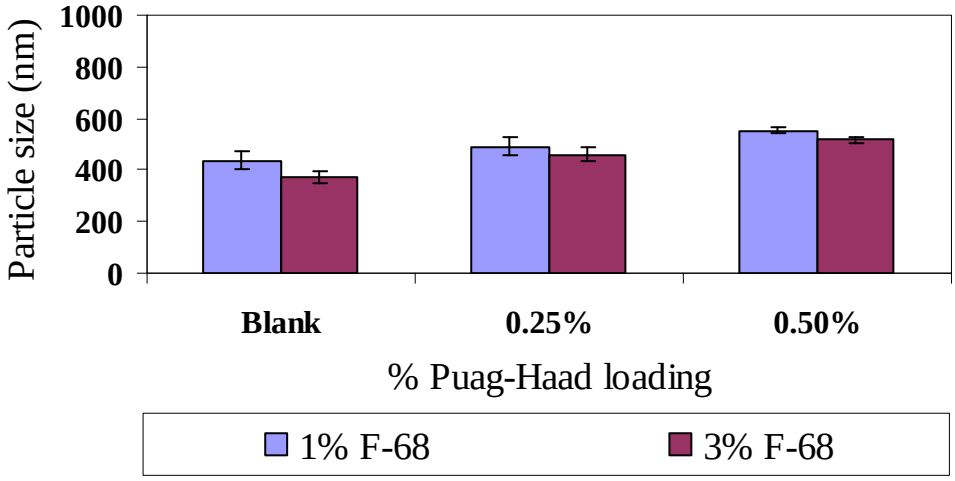


Figure 61 Particle size of freshly prepared SLN formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for compritol® 188 ATO)

Tables 35 – 36 and Figures 62 – 63 show the percentage of entrapment efficiency and recovery of freshly prepared SLN formulations by w/o/w double emulsion method using compritol as lipid and F-68 as external surfactant (Data = mean±SD, n = 3).

All formulations had high entrapment efficiency with high recovery. The values were different depending on formulations. The entrapment efficiency of SLN formulations were about 76 – 88% with > 90% of recovery in both water and citrate buffer pH 5.5 systems.

Table 35 The percentage of entrapment efficiency and recovery of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using water as vehicle (for compritol® 188 ATO) (The amount of Puag-Haad was analyzed by UV-Visible Spectrophotometric method)

Sample	% Puag-Haad loading	Entrapment efficiency (%)±SD	Recovery (%)±SD
COM/10:0/5/1F	0.25%	85.78±0.56	98.24±0.61
COM/10:0/5/1F	0.50%	84.37±1.45	99.51±1.97
COM/10:0/5/3F	0.25%	86.59±0.62	101.56±1.26
COM/10:0/5/3F	0.50%	82.16±1.53	97.18±1.93

Table 36 The percentage of entrapment efficiency and recovery of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for compritol® 188 ATO) (The amount of Puag-Haad was analyzed by UV-Visible Spectrophotometric method)

Sample	% Puag-Haad loading	Entrapment efficiency (%)±SD	Recovery (%)±SD
COM/10:0/5/1F	0.25%	85.58±1.03	100.12±1.41
COM/10:0/5/1F	0.50%	79.26±0.56	96.71±1.12
COM/10:0/5/3F	0.25%	87.86±2.00	101.80±1.56
COM/10:0/5/3F	0.50%	76.27±1.10	94.46±1.61

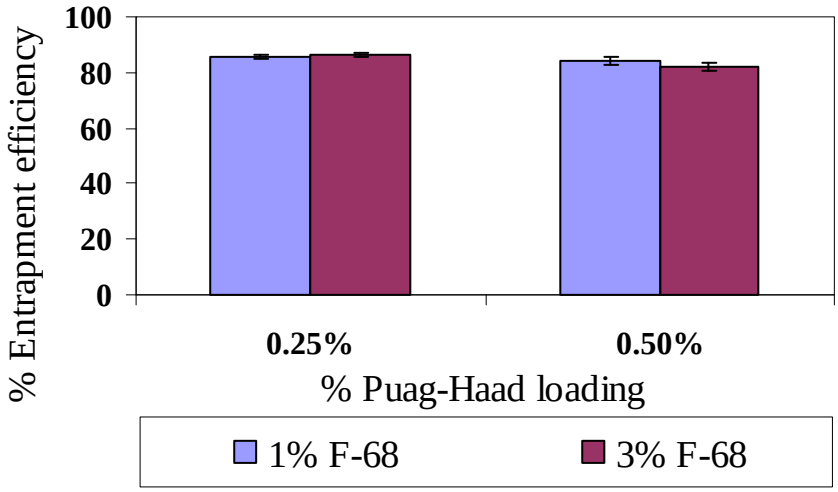


Figure 62 The percentage of entrapment efficiency of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using water as vehicle (for compritol® 188 ATO)

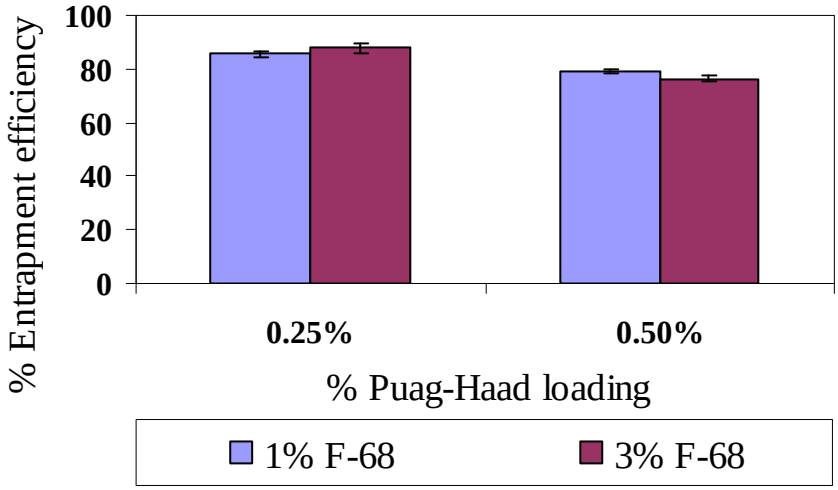


Figure 63 The percentage of entrapment efficiency of freshly prepared SLN containing Puag-Haad formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for compritol® 188 ATO)

The entrapment efficiency of all formulations when using compritol as lipid were higher than using GMS. Compritol 888 ATO or glyceryl behenate was the mixture of diacylglycerols, mainly dibehenylglycerol, together with variable

quantities of mono- and triacylglycerols, obtained by esterification of glycerol with behenic acid. It was composed of about 13 – 21% monoacylglycerols, 40 – 60% diacylglycerols and 21 – 35% triacylglycerols (BP 2004). The chemical nature of the lipid was also important because lipids which form highly crystalline particles with a perfect lattice (e.g. monostearin or GMS) led to drug expulsion. More complex lipids being mixtures of mono-, di- and triglycerides (e.g. glyceryl behenate or compritol) formed less perfect crystals with many imperfections offering space to accommodate the drugs (Müller, Mäder, and Gohla, 2000). For this reason, SLN containing Puag-Haad with compritol as lipid showed higher entrapment efficiency than using GMS.

There were both similarity and difference on the entrapment efficiency of the formulations occurred when using compritol as lipid. The similarity was that when increasing in drug loading, the entrapment efficiency was decreased, especially in citrate buffer. However, increasing the surfactant concentration (F-68) did not appear to affect the entrapment efficiency. The values of percentage entrapment remained relatively unchanged at the same drug loading and same vehicle type. Regarding the effect of vehicle, changing from water to citrate buffer pH 5.5 resulted in an increase in particle size in all cases. But there was a corresponding decrease in %entrapment observed only in the case of high Puag-Haad loading (0.5% Puag-Haad). At low Puag-Haad loading of 0.25%, the citrate buffer did not appear to adversely affect %entrapment. Thus, the decrease in percentage entrapment of Puag-Haad in SLN was mainly due to the drug loading. Citrate buffer affected %entrapment only at high drug loading whereas surfactant concentration did not seem to influence %entrapment. It is possible that most of the entrapped drug resided mainly inside the compritol matrix, thereby making the extent of entrapment less dependent on the particle size and surface area of the SLN. Tables 37 – 38 and Figures 64 – 65 show the correlation between particle size and entrapment efficiency for compritol. It was found that the particle size did not appear to correlate with the entrapment efficiency of formulations. It is possible that Puag-Haad molecules that were stabilized by surfactant at the surface of compritol-based SLN particles were not as much as GMS-based SLN.

Table 37 The correlation between particle size and entrapment efficiency of SLN containing Puag-Haad formulations by w/o/w double emulsion method when using water as vehicle (for compritol)

Sample	% Puag-Haad loading	Particle size (nm)	Entrapment efficiency (%)
COM/10:0/5/1F	0.25%	439.3	86.15
COM/10:0/5/1F	0.25%	460.1	86.05
COM/10:0/5/1F	0.25%	479.0	85.14
COM/10:0/5/1F	0.50%	549.5	83.56
COM/10:0/5/1F	0.50%	572.7	86.04
COM/10:0/5/1F	0.50%	507.8	83.51
COM/10:0/5/3F	0.25%	391.0	85.89
COM/10:0/5/3F	0.25%	376.2	87.09
COM/10:0/5/3F	0.25%	354.1	86.78
COM/10:0/5/3F	0.50%	502.7	83.91
COM/10:0/5/3F	0.50%	464.9	81.40
COM/10:0/5/3F	0.50%	426.4	81.16

Table 38 The correlation between particle size and entrapment efficiency of SLN containing Puag-Haad formulations by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for compritol)

Sample	% Puag-Haad loading	Particle size (nm)	Entrapment efficiency (%)
COM/10:0/5/1F	0.25%	490.5	84.77
COM/10:0/5/1F	0.25%	527.0	86.75
COM/10:0/5/1F	0.25%	456.0	85.23
COM/10:0/5/1F	0.50%	552.5	78.82
COM/10:0/5/1F	0.50%	539.3	79.90
COM/10:0/5/1F	0.50%	568.2	79.07
COM/10:0/5/3F	0.25%	458.7	85.56
COM/10:0/5/3F	0.25%	430.1	89.18
COM/10:0/5/3F	0.25%	484.2	88.84
COM/10:0/5/3F	0.50%	508.5	75.11
COM/10:0/5/3F	0.50%	527.0	76.37
COM/10:0/5/3F	0.50%	514.2	77.32

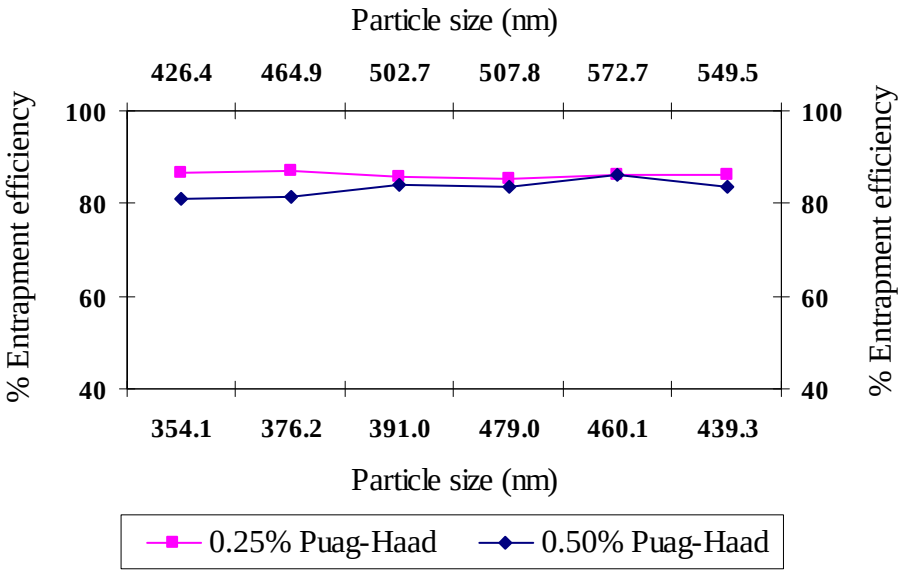


Figure 64 Particle size-entrapment efficiency scattering plot of SLN containing Puag-Haad by w/o/w double emulsion method when using water as vehicle (for compritol)

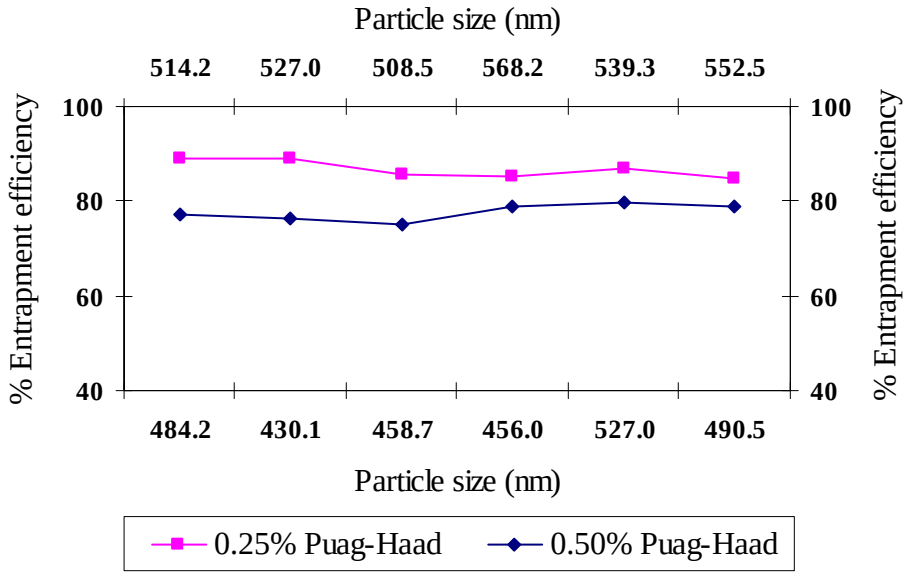


Figure 65 Particle size-entrapment efficiency scattering plot of SLN containing Puag-Haad by w/o/w double emulsion method when using citrate buffer pH 5.5 as vehicle (for compritol)

The explanation of the effect of drug loading and surfactant concentration on particle size and entrapment efficiency therefore could be different depending on many factors such as the nature of the incorporated drug, the type of lipid and surfactant used.

2.2 Preparation of SLN containing Puag-Haad by cold homogenization method

2.2.1 Effect on particle size

The different SLN formulations were respectively measured by PCS. All data are shown as mean ± SD (n = 3).

Tables 39 – 40 and Figures 66 – 67 show the particle size and PI of freshly prepared SLN formulations by cold homogenization method using GMS as lipid and S-40 as surfactant.

For 1% S-40, the SLN containing Puag-Haad could not be formed with acceptable physical appearance in both vehicles. The phase separation occurred quite rapidly when standing at room temperature after 30 minutes. The solid lipid particles were floating on the top and the clear solution was separated at the bottom of the beaker. This effect was obviously observed when using citrate buffer pH 5.5 as vehicle. At 1% S-40, the surfactant concentration might not be enough to stabilize and form SLN even with the blank formula. However, higher concentration of surfactant (3% S-40) was effective in stabilizing both the blank SLN and SLN containing Puag-Haad regardless of the vehicle type.

Table 39 Particle size and size distribution of freshly prepared SLN formulations by cold homogenization method when using water as vehicle (for GMS)

Sample	% Puag-Haad loading	Size (nm)±SD	PI±SD
GMS/1S	Blank	325.5±23.22	0.187±0.044
GMS/1S	0.25%	-	-
GMS/1S	0.50%	-	-
GMS/3S	Blank	287.0±10.16	0.190±0.032
GMS/3S	0.25%	289.3±8.75	0.138±0.020
GMS/3S	0.50%	290.6±18.77	0.176±0.052

Table 40 Particle size and size distribution of freshly prepared SLN formulations by cold homogenization method when using citrate buffer pH 5.5 as vehicle (for GMS)

Sample	% Puag-Haad loading	Size (nm)±SD	PI±SD
GMS/1S	Blank	-	-
GMS/1S	0.25%	-	-
GMS/1S	0.50%	-	-
GMS/3S	Blank	332.4±8.81	0.125±0.100
GMS/3S	0.25%	339.6±18.12	0.136±0.069
GMS/3S	0.50%	343.4±10.25	0.144±0.055

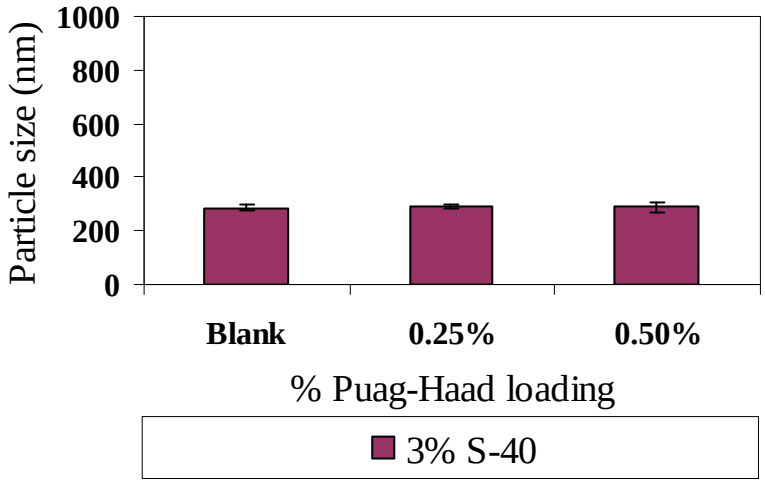


Figure 66 Particle size of freshly prepared SLN formulations by cold homogenization method when using water as vehicle (for GMS)

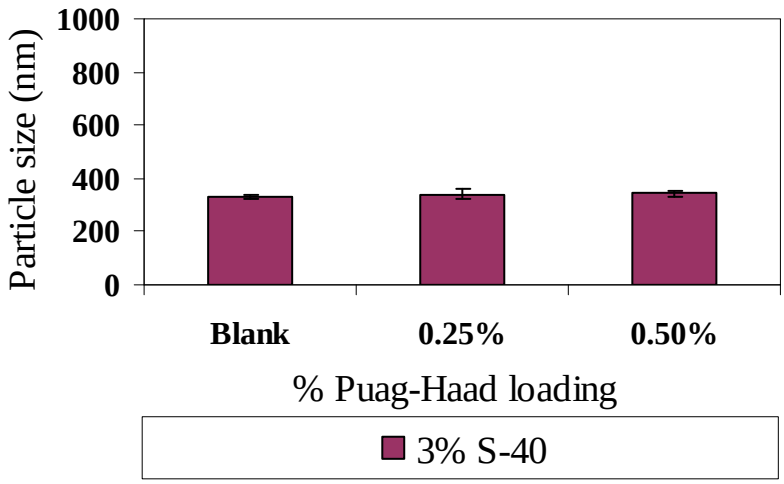


Figure 67 Particle size of freshly prepared SLN formulations by cold homogenization method when using citrate buffer pH 5.5 as vehicle (for GMS)

The particle sizes of all formulations were about 300 nm both in water and citrate buffer pH 5.5. The mean particle size by this method was typically large with broad size distribution because the dispersion of solid lipids required a high energy input to break the lipid microparticles directly to SLN.

After statistical analysis by one-way ANOVA and multiple comparison (LSD), it was found that there was no significant difference in the particle size of formulations due to Puag-Haad loading. For cold homogenization method, Puag-Haad might be more evenly dispersed throughout the lipid matrix. The Puag-Haad molecules were trapped inside the space of crystal lattice of the lipid. So, the drug loading may not significantly influence the particle size of the formulations. Similar to w/o/w double emulsion method, the particle size of SLN from the water systems was smaller than the citrate buffer counterparts.

2.2.2 Effect on zeta potential

The different SLN formulations were respectively measured for zeta potential by PCS. All data are means ± SD (n = 3).

Tables 41 – 42 show the zeta potential of freshly prepared SLN formulations by cold homogenization method using GMS as lipid and 3% S-40 as surfactant.

Table 41 Zeta potential of freshly prepared SLN formulations by cold homogenization method when using water as vehicle (for GMS)

Sample	% Puag-Haad loading	Zeta potential (mV)±SD
GMS/3S	Blank	-30.17±2.87
GMS/3S	0.25%	-25.13±1.68
GMS/3S	0.50%	-25.49±1.64

Table 42 Zeta potential of freshly prepared SLN formulations by cold homogenization method when using citrate buffer pH 5.5 as vehicle (for GMS)

Sample	% Puag-Haad loading	Zeta potential (mV)±SD
GMS/3S	Blank	12.39±2.78
GMS/3S	0.25%	18.15±1.86
GMS/3S	0.50%	18.46±2.38

Similar to SLN formulation by w/o/w double emulsion method, the zeta potentials of SLN obtained from cold homogenization in water were negative. The incorporation of Puag-Haad into SLN also showed a significant increase in positive charge on the zeta potentials of nanoparticles.

The zeta potentials of SLN formulations in citrate buffer pH 5.5 system were also positive as previously observed with the w/o/w double emulsion method. It was possible that the adsorption of buffer cationic species such as sodium ions onto the particles might occur. Similarly, the incorporation of Puag-Haad into the SLN showed a marked increase in positive charge on the zeta potentials of nanoparticles.

2.2.3 Effect on entrapment efficiency

Tables 43 – 44 and Figures 68 – 69 show the percentage of entrapment efficiency and recovery of freshly prepared SLN formulations by cold homogenization method using GMS as lipid and 3% S-40 as surfactant.

Table 43 The percentage of entrapment efficiency and recovery of freshly prepared SLN containing Puag-Haad by cold homogenization method when using water as vehicle (for GMS) (The amount of Puag-Haad was analyzed by UV-Visible Spectrophotometric method)

Sample	% Puag-Haad loading	Entrapment efficiency (%)±SD	Recovery (%)±SD
GMS/3S	0.25%	67.07±1.14	92.23±0.62
GMS/3S	0.50%	65.19±1.29	90.00±0.89

Table 44 The percentage of entrapment efficiency and recovery of freshly prepared SLN containing Puag-Haad by cold homogenization method when using citrate buffer pH 5.5 as vehicle (for GMS) (The amount of Puag-Haad was analyzed by UV-Visible Spectrophotometric method)

Sample	% Puag-Haad loading	Entrapment efficiency (%)±SD	Recovery (%)±SD
GMS/3S	0.25%	70.88±0.78	89.76±1.50
GMS/3S	0.50%	68.28±0.42	88.60±0.09

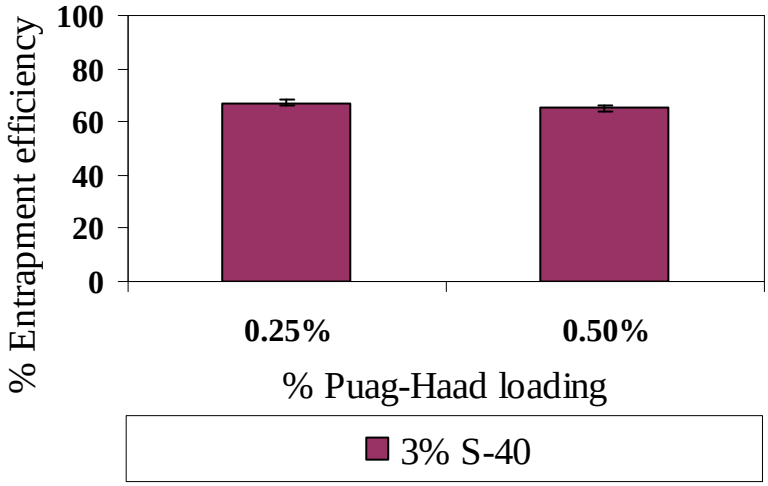


Figure 68 The percentage of entrapment efficiency of freshly prepared SLN containing Puag-Haad by cold homogenization method when using water as vehicle (for GMS)

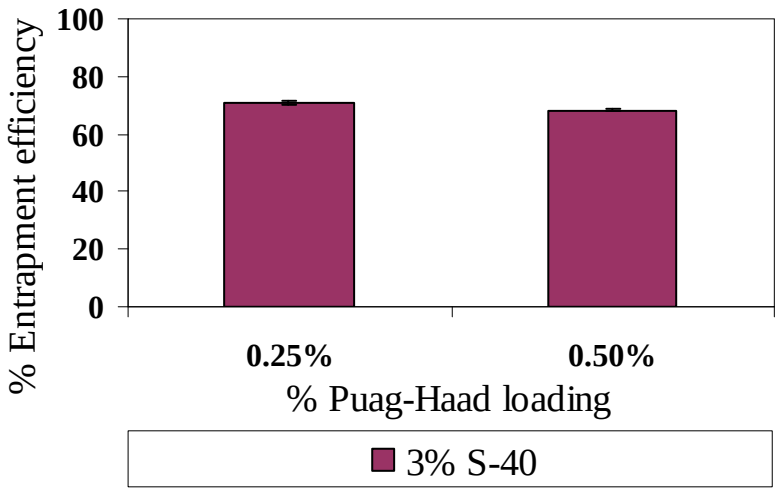


Figure 69 The percentage of entrapment efficiency of freshly prepared SLN containing Puag-Haad by cold homogenization method when using citrate buffer pH 5.5 as vehicle (for GMS)

All formulations had moderate entrapment efficiency with high recovery. The entrapment efficiency of SLN formulations were about 65 – 70% with > 85% recovery in both water and citrate buffer pH 5.5 systems.

After statistical analysis by one-way ANOVA and multiple comparison (LSD), it was found that there were no significant differences in the entrapment efficiency of formulations due to Puag-Haad loading or due to particle size difference ($P > 0.05$).

2.2.4 Particle size-entrapment efficiency scattering plot

Tables 45 – 46 and Figures 70 – 71 show the correlation between particle size and entrapment efficiency of SLN containing Puag-Haad formulations by cold homogenization method (for GMS).

It was found that the particle size did not have any influence on the entrapment efficiency of formulations regardless of the vehicle type. Although the type of vehicle seemed to affect the particle size of SLN obtained from this method in a similar way to w/o/w double emulsion method (citrate buffer yielded larger particles), the entrapment efficiency of the two vehicle systems was relatively the same or even slightly increased in the citrate buffer system.

Table 45 The correlation between particle size and entrapment efficiency of SLN containing Puag-Haad prepared by cold homogenization method using water as vehicle (for GMS)

Sample	% Puag-Haad loading	Particle size (nm)	Entrapment efficiency (%)
GMS/3S	0.25%	299.4	66.40
GMS/3S	0.25%	285.0	68.39
GMS/3S	0.25%	283.6	66.40
GMS/3S	0.50%	310.3	65.24
GMS/3S	0.50%	272.9	66.45
GMS/3S	0.50%	288.7	63.88

Table 46 The correlation between particle size and entrapment efficiency of SLN containing Puag-Haad prepared by cold homogenization method using citrate buffer pH 5.5 as vehicle (for GMS)

Sample	% Puag-Haad loading	Particle size (nm)	Entrapment efficiency (%)
GMS/3S	0.25%	319.7	69.99
GMS/3S	0.25%	355.2	71.45
GMS/3S	0.25%	343.8	71.20
GMS/3S	0.50%	332.6	68.47
GMS/3S	0.50%	353.0	68.58
GMS/3S	0.50%	344.6	67.81

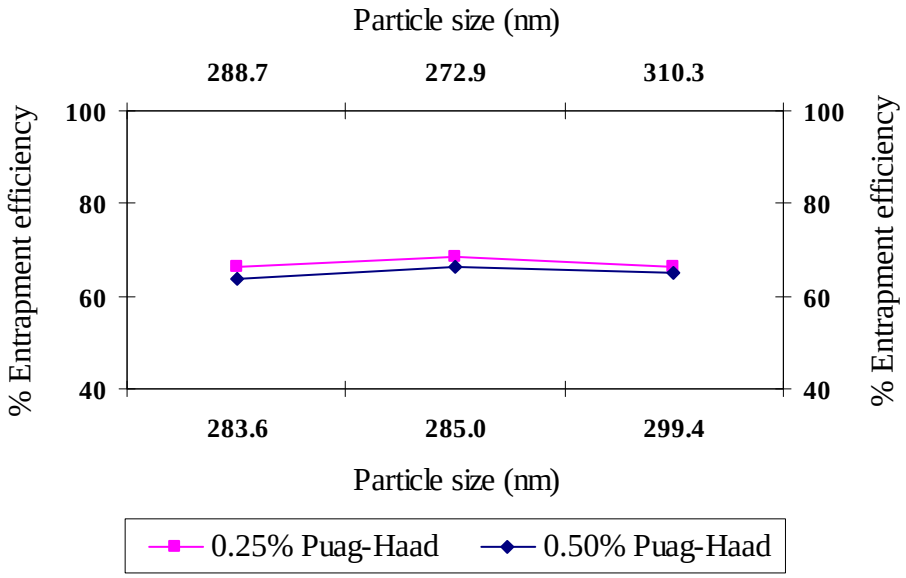


Figure 70 Particle size-entrapment efficiency scattering plot of SLN containing Puag-Haad by cold homogenization method using water as vehicle (for GMS)

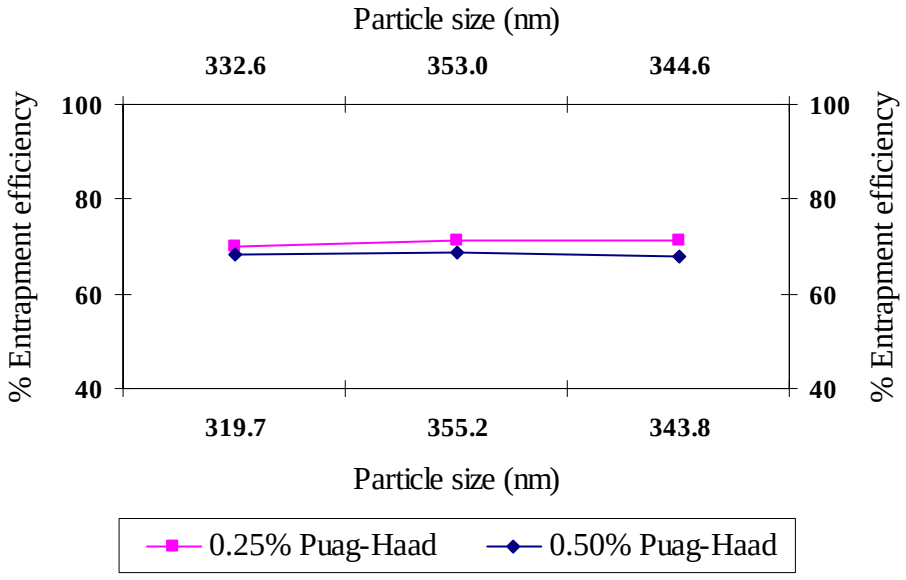


Figure 71 Particle size-entrapment efficiency scattering plot of SLN containing Puag-Haad by cold homogenization method using citrate buffer pH 5.5 as vehicle (for GMS)

Part C. Characterization of SLN containing Puag-Haad

The appropriate formulations of freshly prepared SLN containing Puag-Haad were selected from part B based on high entrapment efficiency and preferable particle size range. Four SLN formulations were prepared from GMS and S-40 using both methods and types of vehicles. They are shown in Table 47 and coded for convenience of identification.

Table 47 The selected formulations of SLN containing Puag-Haad

No.	Code	Lipid	%Loading	Method	Type and % of surfactant	Vehicle
1	MGW	GMS	0.25% w/v	w/o/w double emulsion	3% w/v S-40	Water
2	MGB	GMS	0.25% w/v	w/o/w double emulsion	3% w/v S-40	Citrate buffer pH 5.5
3	CGW	GMS	0.25% w/v	Cold homogenization	3% w/v S-40	Water
4	CGB	GMS	0.25% w/v	Cold homogenization	3% w/v S-40	Citrate buffer pH 5.5

To understand easily, the code will be created.

"1 2 3" The first letter represented production method (M = w/o/w double emulsion method, C = cold homogenization method).
The second letter represented type of lipid (G = GMS).
The third letter represented type of vehicle (W = water, B = citrate buffer pH 5.5).

All of the selected formulations in Table 47 were prepared in 3 batches and characterized as described in the following sections.

1. Physical appearances

The physical appearances such as color, coalescence, gel formation and phase separation were visually observed. The photos of freshly prepared SLN containing Puag-Haad are shown in Figures 72 – 73.

All SLN formulations showed good physical appearances. They were well dispersed with milky and pearl-like color. No coalescence, gel formation and phase separation occurred.

(a) (b)

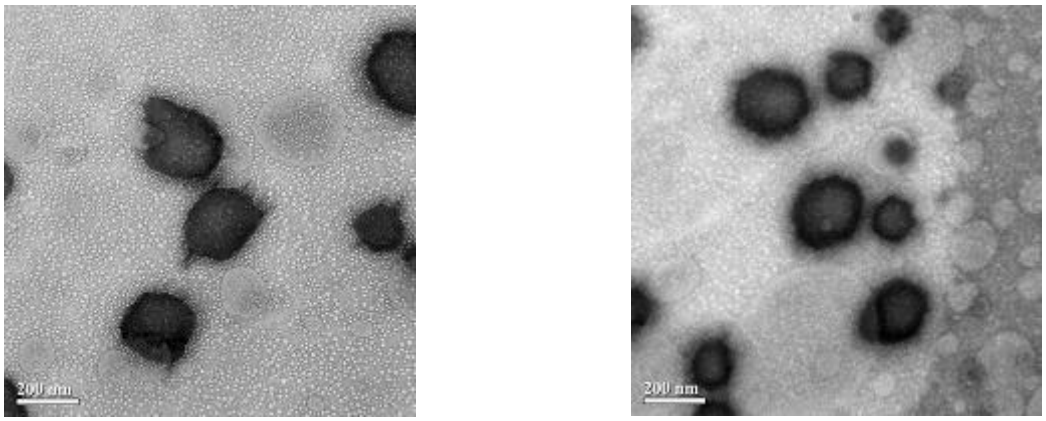
Figure 72 Freshly prepared SLN by w/o/w double emulsion method (a : using water as vehicle, b : using citrate buffer pH 5.5 as vehicle) (Left : Blank SLN, B and Right : SLN containing Puag-Haad, P)

(a) (b)

Figure 73 Freshly prepared SLN by cold homogenization method (a : using water as vehicle, b : using citrate buffer pH 5.5 as vehicle) (Left : Blank SLN, B and Right : SLN containing Puag-Haad, P)

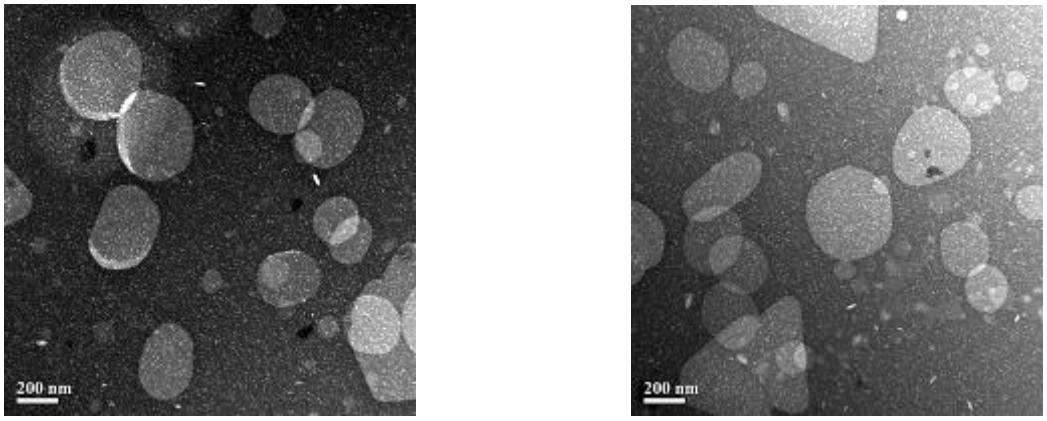
2. Morphology

The morphological feature of SLN containing Puag-Haad was investigated using transmission electron microscope (TEM). The observation of size and shape is shown in Figures 74 – 75. The particles of all formulations showed regular, spherical and uniform nanospheres. The nanoparticles exhibited spherical shape and were homogenous in size distribution. The particle size of the particles was approximately around 200 nm for SLN containing Puag-Haad by w/o/w double emulsion method and around 300 nm by cold homogenization method.



(a) (b)

Figure 74 TEM photomicrographs of freshly prepared SLN containing Puag-Haad by w/o/w double emulsion method (a : using water as vehicle, b : using citrate buffer pH 5.5 as vehicle)



(a) (b)

Figure 75 TEM photomicrographs of freshly prepared SLN containing Puag-Haad by cold homogenization method (a : using water as vehicle, b : using citrate buffer pH 5.5 as vehicle)

3. Size, size distribution and zeta potential

The mean particle size, particle size distribution and zeta potential of these SLN formulations were measured by PCS. The data are shown in Table 48 and expressed as mean±SD (n = 3).

Table 48 The mean particle size, particle size distribution and zeta potential of freshly prepared SLN containing Puag-Haad by PCS

No.	Code	Mean particle size (nm±SD)	Particle size distribution (PI±SD)	Zeta potential (mV±SD)
1	MGW	155.9±3.07	0.212±0.01	-19.70±0.85
2	MGB	186.9±5.74	0.259±0.02	29.51±3.78
3	CGW	253.3±18.52	0.174±0.07	-25.13±1.68
4	CGB	312.2±10.63	0.156±0.02	18.15±1.86

From Table 48, the mean diameters of all formulations were in nanometer range. It was found that the mean particle size of SLN containing Puag-Haad by w/o/w double emulsion method was 155.9±3.07 and 186.9±5.74 nm when using water and

citrate buffer pH 5.5 as vehicle, respectively. Furthermore, for cold homogenization method, it was 253.3±18.52 and 312.2±10.63 nm when using water and citrate buffer pH 5.5 as vehicle, respectively. The PI of all formulations was narrow which indicated that the formulations were of narrow size distribution.

The particle size determination by PCS agreed with the results from TEM studies. It was found that the particle size of SLN containing Puag-Haad by w/o/w double emulsion and cold homogenization method was around 200 and 300 nm, respectively.

The zeta potential of SLN containing Puag-Haad in water was negative charge for both formulations by w/o/w double emulsion and cold homogenization method. In contrast, the zeta potential of SLN containing Puag-Haad in citrate buffer pH 5.5 was positive charge for both methods. It was possible that the adsorption of cationic components of buffer onto the particles might occur.

4. The percentage of entrapment efficiency

The determination of the amount of entrapped oxyresveratrol in SLN containing Puag-Haad was carried out by ultrafiltration and analyzed by HPLC. The percentage of entrapment efficiency of SLN containing Puag-Haad is shown in Table 49. The values are mean±SD of three batches for each formulation. The results in Table 49 clearly show that all the formulations gave high entrapment efficiency about 68-86% depending on the formulations. Table 50 shows the content of unentrapped or free oxyresveratrol. Moreover, Table 51 shows the percentage of recovery of entrapment efficiency which included the combined content of entrapped and unentrapped oxyresveratrol. The high percentage of recovery was more than 85%. It indicated that only the small amount of oxyresveratrol was lost during preparation and ultrafiltration process.

Table 49 The percentage of entrapment efficiency of oxyresveratrol in freshly prepared SLN containing Puag-Haad by HPLC

No	Code	Entrapment efficiency (%±SD)
1	MGW	85.48±1.15
2	MGB	85.63±1.19
3	CGW	68.36±0.97
4	CGB	71.82±0.87

Table 50 The percentage of unentrapped oxyresveratrol in freshly prepared SLN containing Puag-Haad by HPLC

No	Code	Unentrapped oxyresveratrol (%±SD)
1	MGW	14.58±0.61
2	MGB	13.20±0.50
3	CGW	18.64±0.57
4	CGB	20.55±0.29

Table 51 The percentage of recovery of oxyresveratrol in freshly prepared SLN containing Puag-Haad by HPLC

No	Code	Recovery (%±SD)
1	MGW	100.06±1.57
2	MGB	98.82±0.77
3	CGW	87.00±1.13
4	CGB	92.37±1.17

5. Thermal analysis by differential scanning calorimetric method

Differential scanning calorimetric (DSC) analysis was performed for all selected formulations and the recorded DSC parameters are presented in Table 52. The DSC thermogram of bulk lipid, GMS, showed endothermic peak at 73.29°C. Vivek, Reddy,

and Murthy (2007) reported that peak maximum of GMS was at 68.53°C. Although both results had a little difference in peak maximum, the peak of GMS was still in the range of melting point in USPNF 20 (≥ 55°C). Normally, there is a difference in melting point of lipid depending on the batch.

Table 52 DSC results of bulk lipid, freshly prepared blank SLN and SLN containing Puag-Haad formulations

No	Code	Endotherm				RI (%)
		Enthalpy (J/g)	Peak (°C)	Onset (°C)	End set (°C)	
1	Bulk lipid (GMS)	194.27	73.29	69.67	75.68	-
2	MGW (blank)	43.71	64.89	58.45	67.44	37.12
	(Puag-Haad)	45.48	64.26	56.61	67.44	38.63
3	MGB (blank)	45.47	65.92	59.65	68.54	38.62
	(Puag-Haad)	46.97	65.68	60.58	67.69	39.89
4	CGW (blank)	84.85	67.04	58.58	69.65	72.07
	(Puag-Haad)	93.74	69.11	62.90	70.58	79.62
5	CGB (blank)	77.17	67.72	63.90	69.67	65.54
	(Puag-Haad)	87.02	68.20	64.26	70.98	73.91

Figures 76 – 77 show DSC thermograms of bulk lipid, freshly prepared blank SLN and SLN containing Puag-Haad formulations

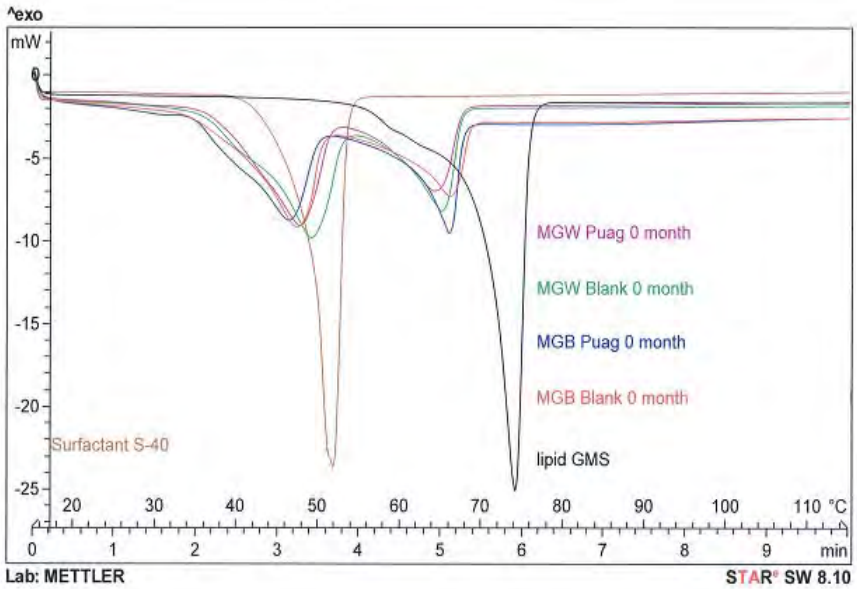


Figure 76 DSC thermograms of bulk lipid, freshly prepared blank SLN and SLN containing Puag-Haad formulations by w/o/w double emulsion method

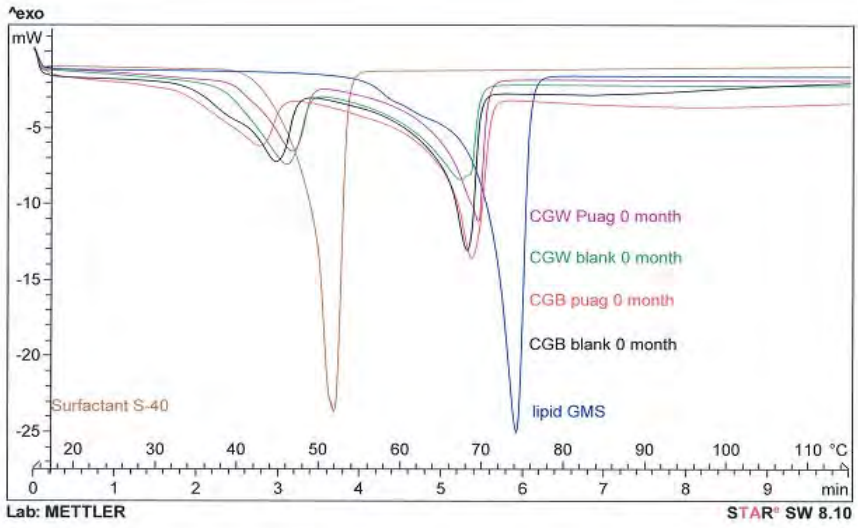


Figure 77 DSC thermograms of bulk lipid, freshly prepared blank SLN and SLN containing Puag-Haad formulations by cold homogenization method

From data obtained in Table 52 and Figures 76 – 77, reduction in the melting point and enthalpy endotherm was observed when formulated as SLN. This result could be correlated with the impurities or less ordered crystal lattice, so the substance required less energy than perfect crystalline material. Furthermore, small particle size of SLN leads to high surface energy, which creates an energetically suboptimal state causing a decrease in the melting point (Vivek, Reddy, and Murthy, 2007).

For SLN prepared by w/o/w double emulsion method, the decrease in enthalpy and melting point from bulk lipid was higher than SLN prepared by cold homogenization method due to the presence of surfactant molecule in the step of microemulsion formation. Furthermore, the enthalpies of all SLN containing Puag-Haad especially by cold homogenization method were much more than their respective blank SLN. The presence of the drug prevented formation of unstable modification or accelerated the transition to the more stable form. Thus, drug incorporation could accelerate the transformation to the more stable polymorph leading to higher enthalpy (and slightly higher melting point) (Mühlen, Schwarz, and Mehnert, 1998). However, this was observed mainly in cold homogenization method. It might be due to the incorporation of Puag-Haad in SLN by this method mainly entrapped into the lipid matrix rather than the surface of SLN. In case of w/o/w double emulsion method, the impurities lattices of SLN formulation in the presence of Puag-Haad and surfactant molecules comparing to bulk lipid may have resulted in similar enthalpy and melting points between the drug-loaded and blank SLN. The imperfection lattice due to surfactant might offer space to accommodate Puag-Haad for the w/o/w double emulsion method than the cold homogenization method. These results were corresponding to the high entrapment efficiency of Puag-Haad in lipid of SLN dispersion especially for the w/o/w double emulsion method.

6. In vitro release property of SLN containing Puag-Haad

To investigate the structure of SLN containing Puag-Haad, in vitro release property was studied. The SLN containing Puag-Haad formulations were evaluated for the release profiles by using Franz diffusion cell over 24 hours. The receiver fluids were either water or citrate buffer pH 5.5 depending on vehicle of SLN formulations. The sink condition was maintained over the period of study. The amount of

oxyresveratrol release was analyzed by using HPLC method. The experiment was conducted in five replicates, n=5. The aqueous solution of Puag-Haad which was dissolved in mixture of 20% v/v propylene glycol in water or 20% v/v propylene glycol in citrate buffer pH 5.5 was used as a control. The release profiles of control solution and SLN containing Puag-Haad formulations are shown in Figures 78 – 79.

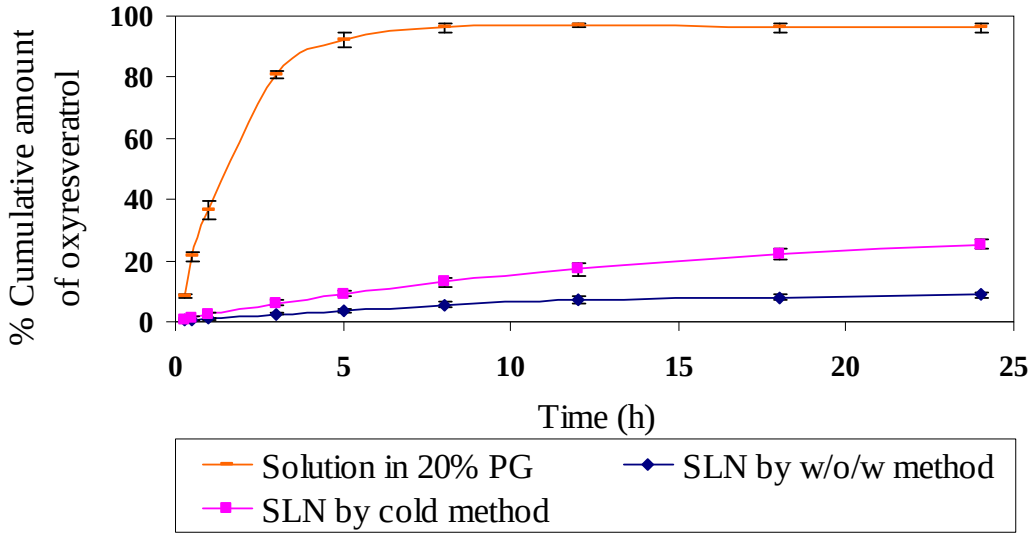


Figure 78 The release profiles of formulations using water as vehicle (medium also = water)

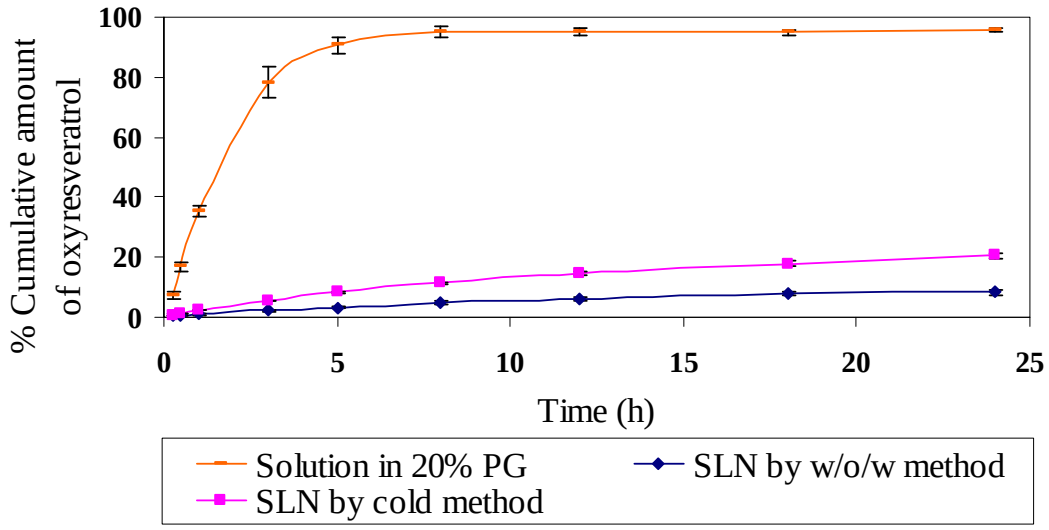


Figure 79 The release profiles of formulations using citrate buffer pH 5.5 as vehicle (medium also = citrate buffer pH 5.5)

The release of oxyresveratrol from the control solution was rapid and reached the plateau at about 8 hours with high recovery (99.30±1.71 and 98.53±0.78 for water and citrate buffer pH 5.5, respectively). All the SLN formulations showed sustained release profiles of oxyresveratrol regardless of the method and type of vehicle. The release data over the whole time period were explained according to the treatment proposed by Higuchi for oxyresveratrol from SLN formulations.

To quantify the release data for comparison, the release profiles were plotted according to Higuchi's equation.

$$Q_t = k_H t^{1/2}$$

where: Q_t is the amount of drug release at time t ($\mu g/cm^2$)

k_H is the release rate constants of Higuchi ($\mu gcm^{-2}h^{-1/2}$)

t is the release time (h)

Figures 80 – 81 show the Higuchi plots of oxyresveratrol released from SLN the formulations. The Figures were plotted between the cumulative amount (Q_t) of oxyresveratrol release versus the square root of time. The coefficient of determination (R^2) and the release rate constants of SLN formulations are presented in Table 53.

Table 53 The coefficient of determination (R^2) from Higuchi plots and the release rate constants of various SLN containing Puag-Haad

No	Code	R^2	Release rate constant ($\mu gcm^{-2}h^{-1/2}$)
1	MGW	0.9838	14.089
2	MGB	0.9914	16.412
3	CGW	0.9959	29.540
4	CGB	0.9972	30.908

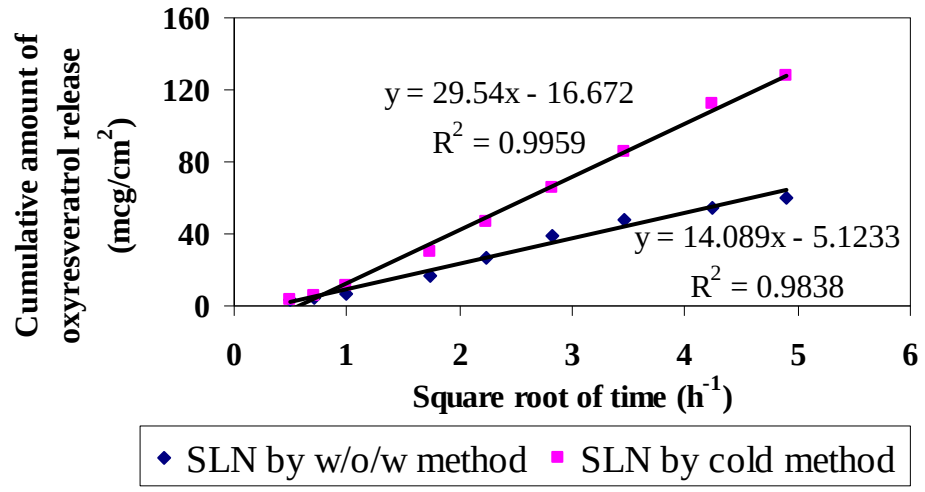


Figure 80 Higuchi plots of SLN containing Puag-Haad using water as vehicle

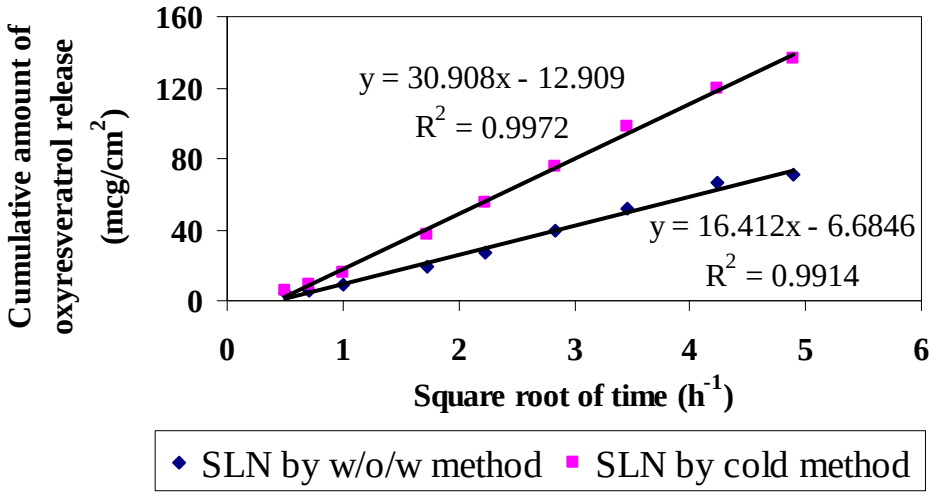
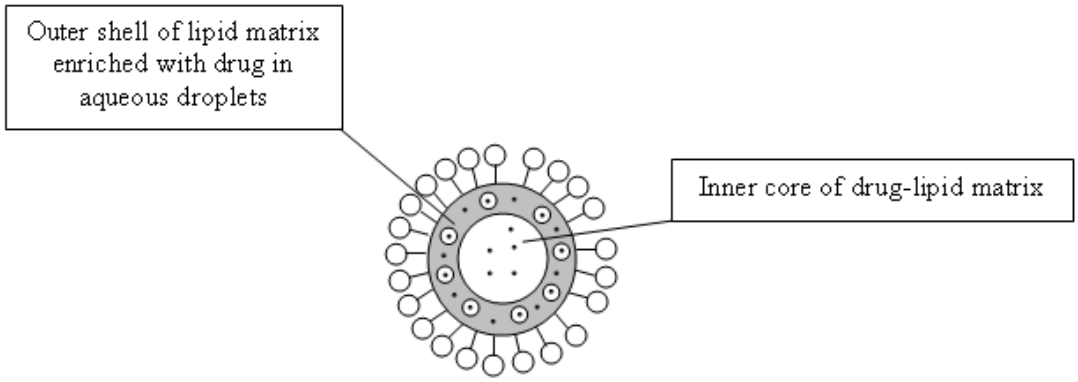


Figure 81 Higuchi plots of SLN containing Puag-Haad using citrate buffer pH 5.5 as vehicle

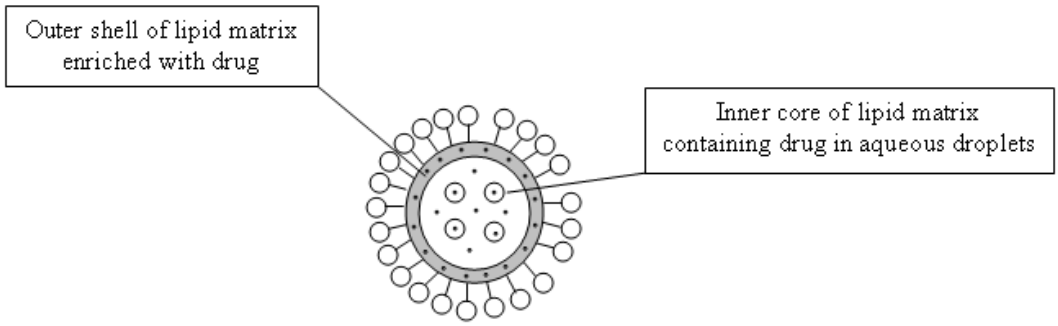
The release profiles of all SLN formulations well fitted the Higuchi equation with $R^2 > 0.98$. The Higuchi equation describes the diffusion of drug from matrix systems. The drug release from a matrix system is said to follow Higuchi's release kinetics if the amount of drug released is directly proportional to the square root of time. The slopes obtained from the above plots were proportional to an apparent diffusion coefficient. Linear fits were obtained indicating that the release was diffusional. The release profiles of all SLN formulations showed sustained release without any burst at all. The sustained release was due to the diffusion of drug from the lipid matrix.

The release profile data can be used to hypothesize the drug incorporation model. Based on the production method, the particle size – entrapment efficiency scattering plot (from part B) and the release profiles, it can be deduced as follows:

For w/o/w double emulsion method that used high temperature during the process, the particle size of GMS-based SLN formulations influenced their entrapment efficiency. The increase in particle size might decrease in entrapment efficiency due to lower surface area of the particles. Furthermore, the release profile showed sustained release without burst effect and fitted to Higuchi plot. It was possible that the drug incorporation model of SLN containing Puag-Haad by w/o/w double emulsion method may be more complex than the basic model of homogeneous dispersion in lipid. The possible models are shown in Figure 82.



(a)



(b)

Figure 82 The possible models of GMS-based SLN containing Puag-Haad by w/o/w double emulsion method

During the incorporation of Puag-Haad in SLN, Puag-Haad was dissolved in aqueous internal phase with melted surfactant mixtures at a temperature above the melting point of lipid to high amount loading of Puag-Haad. The addition of Puag-Haad with surfactant (and cosurfactant) might increase the solubility of Puag-Haad in internal water phase. But, when the lipid was added to form w/o microemulsion, the presence of surfactants might as well play a role in the solubilization of Puag-Haad in the lipid phase. The partition coefficient of oxyresveratrol (the ratio of oxyresveratrol concentration in octanol to water at equilibrium, K_D) was 48 (Nitaya Poopyruchpong et al., 1978). Some Puag-Haad molecules were partition from aqueous internal phase

to lipid phase during the process of microemulsion formation. An outer shell enriched with Puag-Haad might be formed when phase separation occurred during the cooling process from the liquid oil droplet to the formation of SLN. As the w/o/w double emulsion was formed and the oil droplet precipitated to become solid particles, some of the drug molecules (especially oxyresveratrol) might be expelled to the surface of the particles which were stabilized by the adsorbed surfactant molecules (the outer shell). However, most of the expelled drug did not diffuse into the aqueous external phase but remained attached (probably by surface adsorption) to the outer shell. Strong adhesion to this outer shell may explain the particle size-dependent entrapment efficiency of the drug as well as the observed sustained release profile without burst release effect. However, the model for incorporation of Puag-Haad in SLN by w/o/w double emulsion is still not fully understood and requires further investigation.

For cold homogenization method that avoided using high temperature during the process, the particle size of SLN formulations did not influence their entrapment efficiency as with the w/o/w double emulsion method. However, the release profile also showed sustained release without burst effect and fitted to Higuchi plot. It was possible that the drug incorporation of SLN containing Puag-Haad by cold homogenization method could be described by the homogeneous matrix model, one of the basic model for the incorporation of active ingredient. This model is shown in Figure 83.

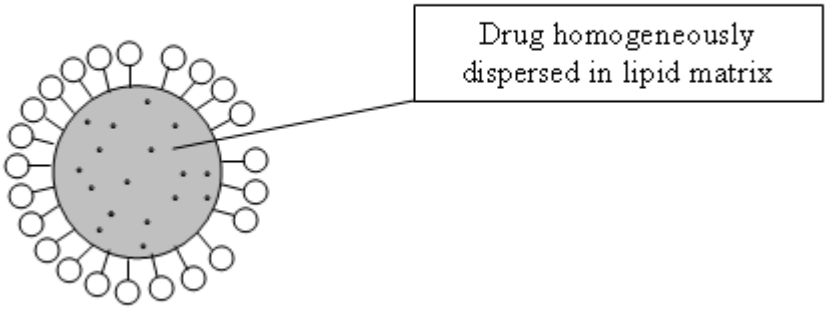


Figure 83 The possible model of GMS-based SLN containing Puag-Haad by cold homogenization method

A homogeneous matrix with molecularly dispersed drug or in amorphous clusters was thought to occur when applying the cold homogenization method. In the cold homogenization method, the bulk lipid contained the dissolved drug in molecularly dispersed form. Mechanical breaking by high pressure homogenization led to nanoparticles having the homogeneous matrix structure. As a result, it could give sustained release without burst effect (Müller, Radtke, and Wissing, 2002).

Part D. Physical and chemical stability study of SLN containing Puag-Haad formulations in comparison with aqueous solutions of Puag-Haad

One of the advantages of SLN was to protect incorporated drug from physical and chemical degradation. So, to prove this advantage, the physical and chemical stability of SLN containing Puag-Haad was evaluated in comparison with aqueous solutions which contained the same concentration of Puag-Haad (0.25% w/v) in 20%v/v propylene glycol and 3%w/v S-40. They were kept at room temperature (30±2°C) and protected from light. The samples were withdrawn and investigated on months 0, 1, 2, 3 and 4. The physical and chemical stability was evaluated as shown in the following topics.

1. Stability study of SLN containing Puag-Haad

Four SLN containing Puag-Haad formulations from part C were selected to evaluate their stability. Table 54 shows the SLN containing Puag-Haad formulations that were selected to study the physical and chemical stability at 30±2°C for 4 months.

Table 54 The selected SLN containing Puag-Haad formulations to study the physical and chemical stability

No.	Code	Lipid	%Loading	Method	Type and % of surfactant	Vehicle
1	MGW	GMS	0.25% w/v	w/o/w double emulsion	3% w/v S-40	Water
2	MGB	GMS	0.25% w/v	w/o/w double emulsion	3% w/v S-40	Citrate buffer pH 5.5
3	CGW	GMS	0.25% w/v	Cold homogenization	3% w/v S-40	Water
4	CGB	GMS	0.25% w/v	Cold homogenization	3% w/v S-40	Citrate buffer pH 5.5

1.1 Physical appearance

The physical appearances such as color, coalescence, gel formation and phase separation were visually observed. All formulations were freshly prepared to compare with their own samples every month. The physical stability study of SLN formulations at 30±2°C for 4 months is shown in Figures 84 – 87 and Table 55.

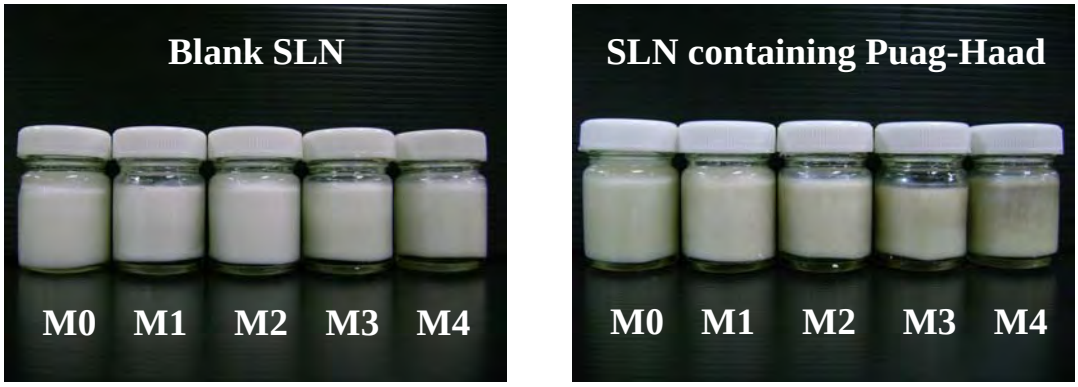


Figure 84 Physical stability of SLN formulations by w/o/w double emulsion method using water as vehicle at 30±2°C (MGW) (Left : Blank SLN, Right : SLN containing Puag-Haad) (M0 = month 0, M1 = month 1, M2 = month 2, M3 = month 3, M4 = month 4)

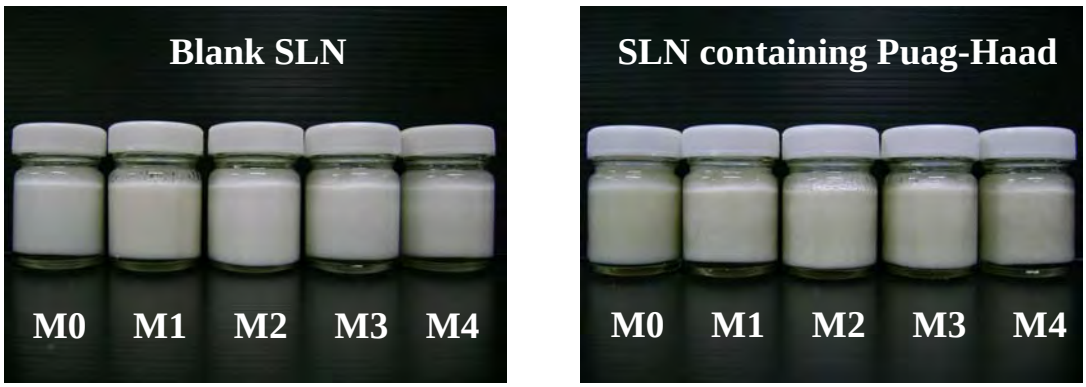


Figure 85 Physical stability of SLN formulations by w/o/w double emulsion method using citrate buffer pH 5.5 as vehicle at 30±2°C (MGB) (Left : Blank SLN, Right : SLN containing Puag-Haad) (M0 = month 0, M1 = month 1, M2 = month 2, M3 = month 3, M4 = month 4)

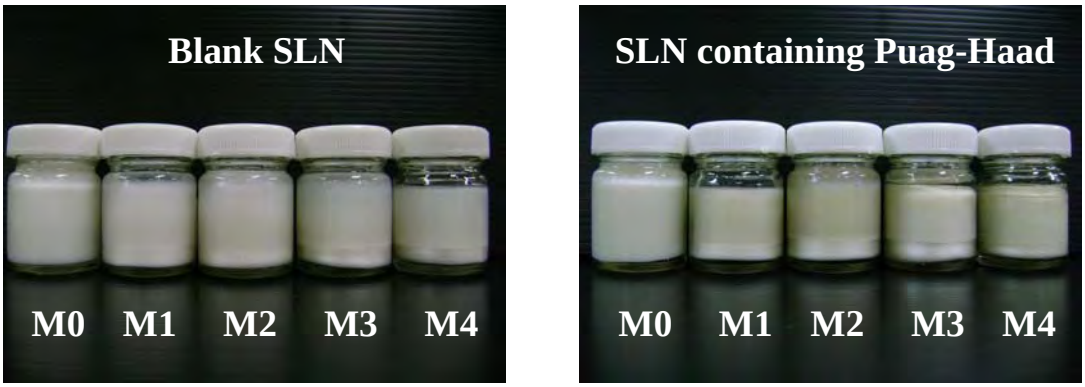


Figure 86 Physical stability of SLN formulations by cold homogenization method using water as vehicle at 30±2°C (CGW) (Left : Blank SLN, Right : SLN containing Puag-Haad) (M0 = month 0, M1 = month 1, M2 = month 2, M3 = month 3, M4 = month 4)

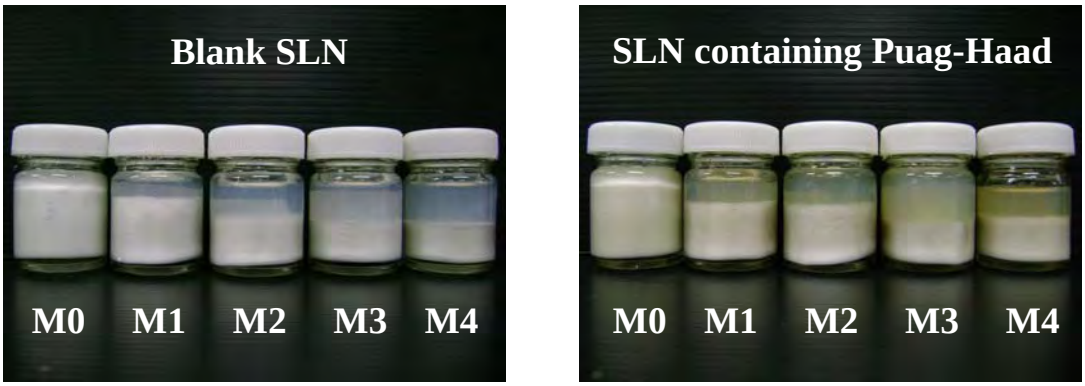


Figure 87 Physical stability of SLN formulations by cold homogenization method using citrate buffer pH 5.5 as vehicle at 30±2°C (CGB) (Left : Blank SLN, Right : SLN containing Puag-Haad) (M0 = month 0, M1 = month 1, M2 = month 2, M3 = month 3, M4 = month 4)

For MGW containing Puag-Haad, it was noticed that the change to more yellow color slightly increased with time especially at month 4. In contrast, for MGB containing Puag-Haad, the change in yellow color did not occur. The gel formation was formed at some parts of all formulations (blank SLN and SLN containing Puag-Haad) resulting in increased viscosity. This result was observed in both formulations

(MGW and MGB) that were prepared by w/o/w double emulsion method as shown in Table 55.

For CGW, this formulation (both blank SLN and SLN containing Puag-Haad) was stable during the study period (4 month). No discoloration, phase separation, gelation or caking occurred. However, it was observed that the pearl-like appearance was formed at the bottom of the bottle. It was like loose coalescent of the particles, so it could be easily dispersed. In contrast, the phase separation occurred for CGB even in the first month for both blank and SLN containing Puag-Haad. It could be easily dispersed but no gelation or caking was observed. The physical stability of CGW and CGB is shown in Table 55.

Moreover, the physical instability of SLN formulations did not seem to associate with the incorporation of Puag-Haad. These were more related to their SLN base formulations because the instability also occurred with the blank SLN.

Table 55 The physical stability of SLN formulations at 30±2°C for 4 months

Month	MGW		MGB		CGW		CGB	
	B	P	B	P	B	P	B	P
0	S	S	S	S	S	S	S	S
1	S	S	S	S	S	S	PS (0.83)	PS (0.62)
2	S	S	S	S	S	S	PS (0.64)	PS (0.60)
3	GF	GF	GF	GF	S	S	PS (0.58)	PS (0.55)
4	GF	GF	GF	GF	S	S	PS (0.46)	PS (0.45)

B = Blank SLN, P = SLN containing Puag-Haad, S = Stable, GF = Gelation but flowable, PS = Phase separation

It was noticed that the gelation occurred in SLN by w/o/w double emulsion method. Gelation was the transformation in viscosity of SLN formulations to a viscous gel. This process appear to occur very rapidly, unpredictably and irreversibly which involved the loss of the colloidal size. It was suggested that gel formation is connected with crystallization processes (Mehnert and Mäder, 2001). For w/o/w double emulsion method, the samples were subjected to heat all over the production process. High temperature and energy input could lead to changes in the crystalline structure of lipids resulting in the subsequent and gradual gelation (Freitas and Muller, 1998). This observation indicated the need for alternative methods to store the nanoparticles. Therefore, transformation of SLN dispersions into a dry powder (such as lyophilized powder) may be able to prevent aggregation and gelation of nanoparticles and improve their physical stability (Mehnert and Mäder, 2001). Moreover, the dilution by incorporation of SLN into the cream base might reduce the probability of SLN particles collision and increase further physical stability (Dingler et al., 1999).

1.2 pH

The pH of all SLN formulations were relatively constant with time regardless of the method and vehicle type as shown in Table 56.

Table 56 The pH measurements of SLN containing Puag-Haad during storage at 30±2°C (month 0, 1, 2, 3 and 4)

No	Code	pH measurement (Mean±SD)				
		Month 0	Month 1	Month 2	Month 3	Month 4
1	MGW	6.06±0.02	6.06±0.01	6.00±0.08	5.93±0.23	5.91±0.12
2	MGB	5.51±0.01	5.50±0.00	5.48±0.01	5.50±0.02	5.49±0.01
3	CGW	6.07±0.02	6.04±0.02	6.04±0.01	6.04±0.02	6.02±0.06
4	CGB	5.50±0.00	5.49±0.01	5.48±0.00	5.48±0.01	5.48±0.01

1.3 Size and size distribution

The particle size and size distribution of SLN containing Puag-Haad at each time are shown in Table 57.

From Table 57, the particle size of all formulations increased with time. Especially, the particle size of SLN by w/o/w double emulsion method (MGW and MGB) increased rapidly because of the presence of gelation. CGW showed the best physical stability because its particle size remained in the nano-size range during the study period (4 months).

Table 57 The particle size and size distribution measurement of SLN containing Puag-Haad during storage at 30±2°C (month 0, 1, 2, 3 and 4)

Time (months)	MGW		MGB		CGW		CGB	
	Size (nm)	PI	Size (nm)	PI	Size (nm)	PI	Size (nm)	PI
0 (SD)	155.9 (3.07)	0.212 (0.01)	186.9 (5.74)	0.259 (0.02)	253.3 (18.52)	0.174 (0.07)	312.2 (10.63)	0.156 (0.02)
1 (SD)	855.7 (43.48)	0.264 (0.01)	1188.2 (85.63)	0.237 (0.02)	359.6 (22.75)	0.193 (0.05)	683.5 (9.07)	0.150 (0.12)
2 (SD)	1163.0 (51.34)	0.155 (0.13)	1368.5 (66.81)	0.005 (0.00)	564.3 (28.03)	0.149 (0.04)	803.5 (76.49)	0.005 (0.00)
3 (SD)	1522.3 (72.56)	0.103 (0.09)	1699.3 (55.58)	0.005 (0.00)	658.8 (17.58)	0.078 (0.02)	987.8 (31.37)	0.005 (0.00)
4 (SD)	1825.3 (56.22)	0.005 (0.00)	2120.7 (63.31)	0.005 (0.00)	724.3 (16.71)	0.093 (0.02)	1178.8 (60.27)	0.005 (0.00)

1.4 Drug remaining

The total amount of oxyresveratrol remaining in the formulations was analyzed by HPLC method. Table 58 shows the percentage of oxyresveratrol remaining in the formulations at 30±2°C for 4 months.

The percentage of oxyresveratrol remaining in all formulations decreased with time. Especially since month 3, there was on obvious decrease in the oxyresveratrol content remaining in the formulations. The SLN by cold homogenization method showed better chemical stability than SLN by w/o/w double emulsion method especially when using water as vehicle. Furthermore, using citrate buffer pH 5.5 as vehicle could further improve chemical stability of both methods. This was in agreement with previous results by Manatchaya Wanawatanakun (2006) who reported that citrate buffer pH 5.5 was able to stabilize the content of oxyresveratrol in aqueous solutions.

Table 58 Normalized percentage of total oxyresveratrol remaining in SLN containing Puag-Haad formulations at 30±2°C for 4 months

Time (months)	% Oxyresveratrol remaining			
	MGW	MGB	CGW	CGB
0	100.00	100.00	100.00	100.00
(SD)	(0.00)	(0.00)	(0.00)	(0.00)
1	97.67	97.57	97.59	98.32
(SD)	(1.35)	(1.40)	(1.27)	(1.63)
2	96.00	96.31	96.69	96.76
(SD)	(1.40)	(1.02)	(2.81)	(2.32)
3	88.50	92.70	90.02	93.34
(SD)	(2.23)	(2.07)	(3.14)	(1.82)
4	79.41	88.07	85.08	89.39
(SD)	(2.93)	(1.46)	(2.59)	(2.76)
Decrease in % Oxyresveratrol after 4 months	20.59	11.93	14.92	10.61

1.5 The percentage of entrapment efficiency

The SLN containing Puag-Haad formulations were purified by ultrafiltration method. The amount of entrapped and unentrapped oxyresveratrol

was analyzed by HPLC method. The percentages of entrapment efficiency, unentrapped oxyresveratrol and recovery are shown in Tables 59 – 61 and Figures 88 – 93.

Table 59 The percentage of entrapment efficiency of SLN containing Puag-Haad formulations at 30±2°C for 4 months

Time (months)	% Entrapment efficiency			
	MGW	MGB	CGW	CGB
0	85.48	85.63	68.36	71.82
(SD)	(1.15)	(1.19)	(0.97)	(0.87)
1	84.54	83.10	62.98	67.91
(SD)	(0.60)	(0.84)	(1.01)	(1.01)
2	77.89	78.46	60.50	62.53
(SD)	(2.05)	(2.33)	(3.27)	(1.01)
3	69.50	73.64	49.59	59.57
(SD)	(2.61)	(0.72)	(1.08)	(1.30)
4	65.78	70.95	46.02	58.00
(SD)	(2.23)	(1.27)	(0.26)	(1.09)
Decrease in % Entrapment efficiency after 4 months	19.70	14.68	22.34	13.82

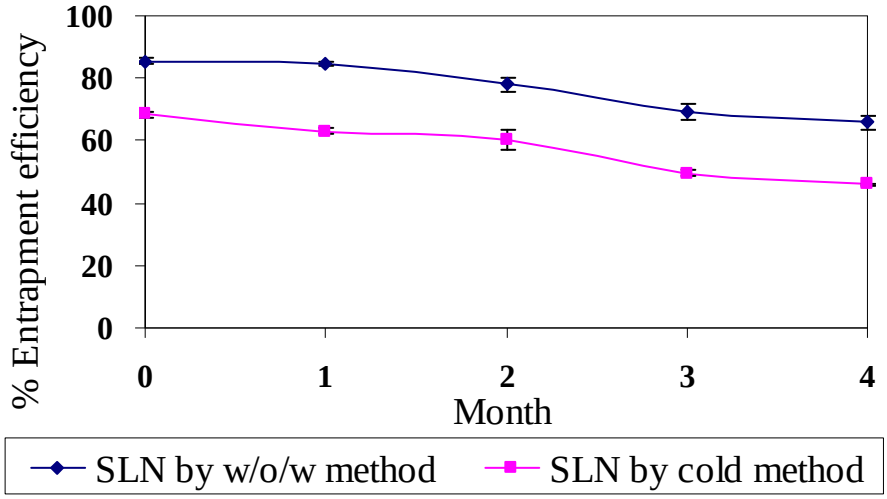


Figure 88 The percentage of entrapment efficiency of SLN containing Puag-Haad formulations using water as vehicle at 30±2°C for 4 months

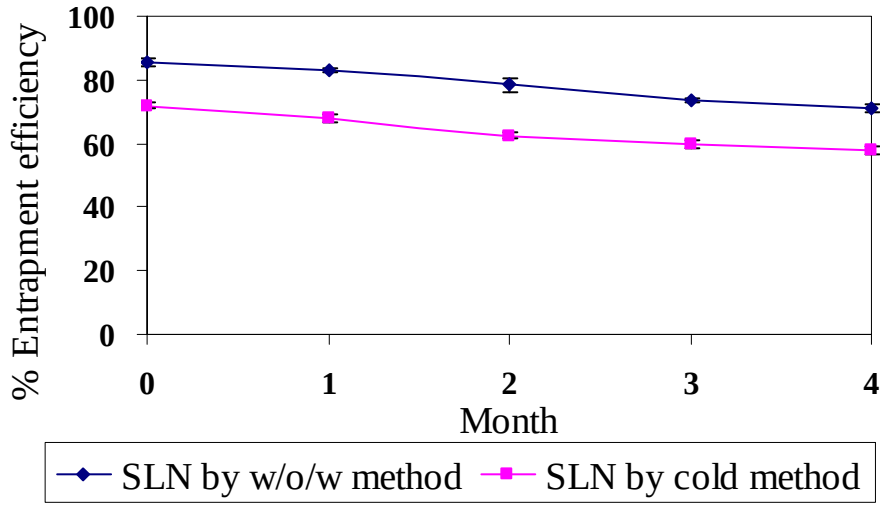


Figure 89 The percentage of entrapment efficiency of SLN containing Puag-Haad formulations using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

Table 60 The percentage of unentrapped oxyresveratrol of SLN containing Puag-Haad formulations at 30±2°C for 4 months

Time (months)	% Unentrapped oxyresveratrol			
	MGW	MGB	CGW	CGB
0	14.58	13.20	18.64	20.55
(SD)	(0.61)	(0.50)	(0.57)	(0.29)
1	8.50	8.38	19.76	17.21
(SD)	(0.54)	(0.60)	(3.17)	(0.36)
2	9.89	6.77	19.65	17.96
(SD)	(2.91)	(0.17)	(3.61)	(0.42)
3	16.53	7.33	27.55	16.30
(SD)	(4.25)	(0.15)	(0.74)	(0.22)
4	13.50	6.69	27.39	16.64
(SD)	(4.14)	(0.48)	(3.31)	(0.28)
Changes in % Unentrapped oxyresveratrol after 4 months	Decrease 1.08	Decrease 6.51	Increase 8.75	Decrease 3.91

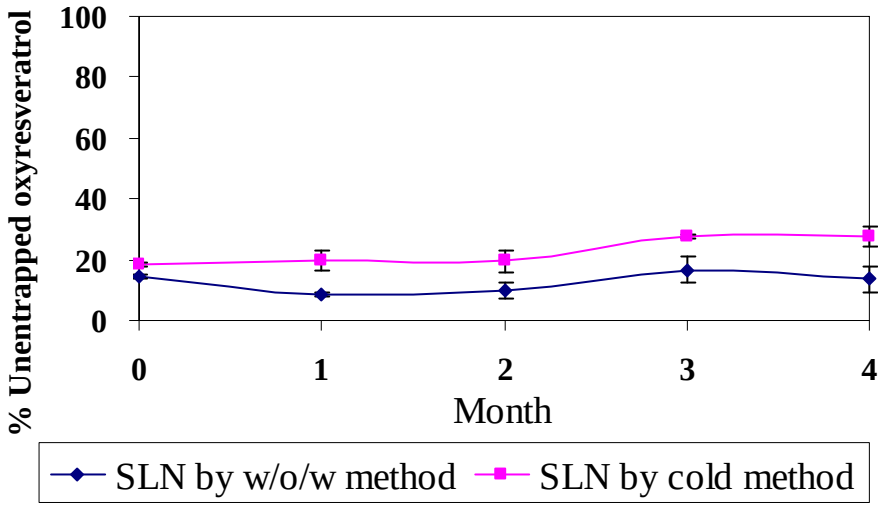


Figure 90 The percentage of unentrapped oxyresveratrol of SLN containing Puag-Haad formulations using water as vehicle at 30±2°C for 4 months

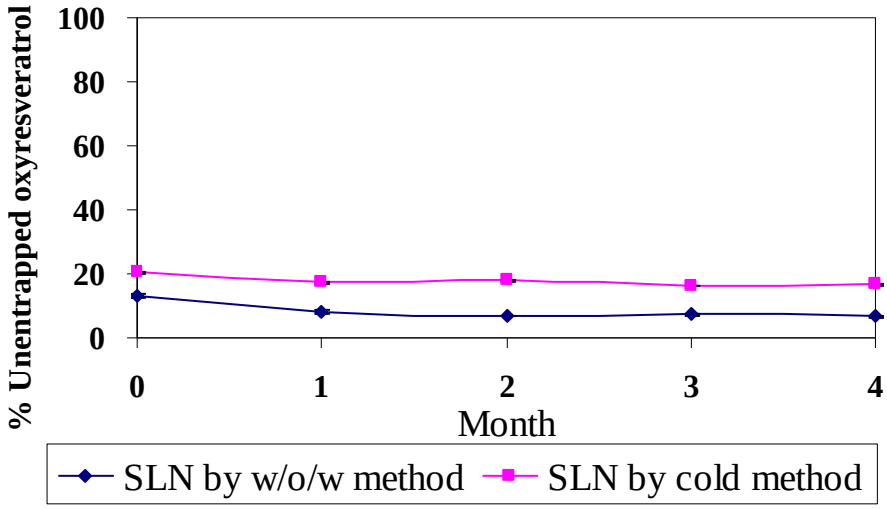


Figure 91 The percentage of unentrapped oxyresveratrol of SLN containing Puag-Haad formulations using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

Table 61 The percentage of recovery of SLN containing Puag-Haad formulations at 30±2°C for 4 months

Time (months)	% Recovery			
	MGW	MGB	CGW	CGB
0 (SD)	100.06 (1.57)	98.82 (0.77)	87.00 (1.13)	92.37 (1.17)
1 (SD)	93.04 (1.12)	91.47 (1.09)	82.42 (1.15)	85.12 (1.22)
2 (SD)	87.89 (1.43)	85.23 (2.25)	80.38 (1.23)	80.49 (1.30)
3 (SD)	86.02 (3.21)	80.97 (0.82)	77.14 (0.81)	75.87 (1.09)
4 (SD)	79.28 (3.81)	77.64 (1.69)	73.41 (3.47)	74.64 (1.00)
Decrease in % Recovery after 4 months	20.78	21.18	13.59	17.73

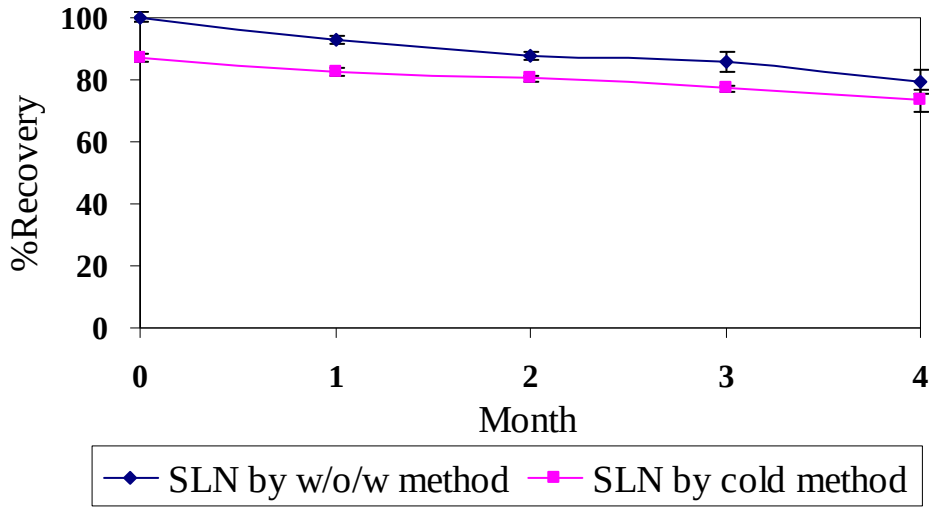


Figure 92 The percentage of recovery of SLN containing Puag-Haad formulations using water as vehicle at 30±2°C for 4 months

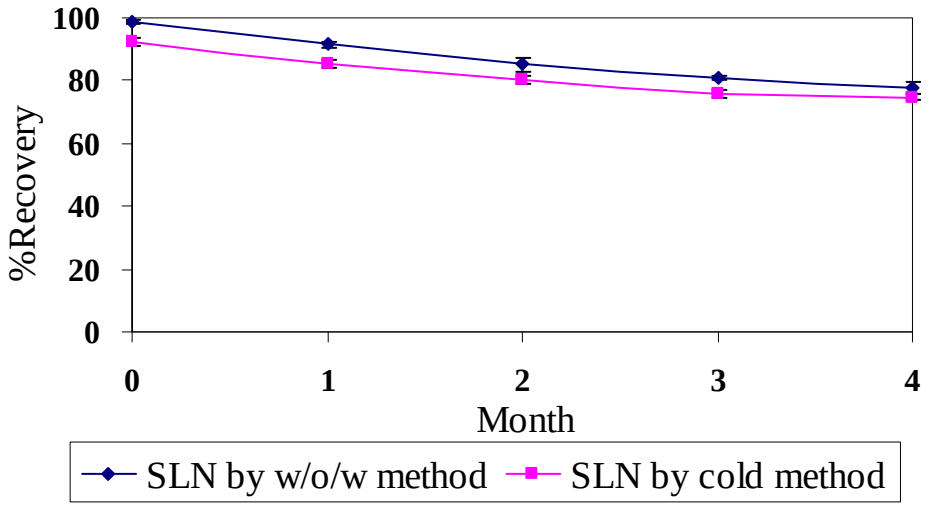


Figure 93 The percentage of recovery of SLN containing Puag-Haad formulations using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

From Tables 59 and Figures 88 – 89, it was found that oxyresveratrol was expelled from lipid matrix into the external aqueous phase resulting in the marked reduction of the entrapment efficiency. Because GMS was a monostearin, which

formed highly crystalline particles with a perfect lattice, this led to drug expulsion. The amount of the unentrapped or free oxyresveratrol in the external aqueous phase of all formulations (Table 60, Figures 90 – 91) depended on the rate of drug expulsion from the lipid matrix as well as the rate of drug degradation in the external aqueous system. The percentage of recovery, the sum of entrapped and free oxyresveratrol, of all formulations (Table 61, Figures 92 – 93) decreased with time which corresponded with the total oxyresveratrol remaining. It was possible that the degradation of oxyresveratrol might occur with the unentrapped oxyresveratrol in the external aqueous phase rather than in the lipid matrix.

Drug expulsion from the lipid matrix was common problem during storage of SLN dispersions as well as the aggregation, phase separation and gelation.

The SLN dispersions increased in viscosity during storage, finally leading to a viscous gel after a few weeks especially with the w/o/w double emulsion method. The gel formation limited the amount of drug to be incorporated in the lipid matrix of the SLN formulations stabilized with only one surfactant. In the present study, only S-40 was used as the external surfactant to prepare SLN by w/o/w double emulsion method for the stability evaluation. However, the gelation could be avoided by using binary (or ternary) surfactant mixtures (Schwarz and Mehnert, 1999).

To further study the effect of storage temperature on the physical and chemical stability of SLN containing Puag-Haad, all formulations were divided into two groups. The first group was stored at 30±2°C and the second was kept at 4 – 8°C. Both groups were evaluated for the physical and chemical stability when stored for 9 months to compare with each other. The parameters that were analyzed included the particle size and size distribution as well as the zeta potential which are shown in Table 62. Furthermore, the percentage of total oxyresveratrol remaining, the percentage of entrapment efficiency, the percentage of unentrapped oxyresveratrol and the percentage of recovery are shown in Table 63.

From Table 62, the particle size and size distribution of all SLN formulations after storage at 30±2°C for 9 months could not be measured because their size were over limit of detection of PCS (>3 μm) and gelation occurred. PCS was limited to detection of particles undergoing Brownian motion and particles larger than about 3 μm were not detectable (Souto and Müller, 2005). The particle size and size

distribution of all SLN formulations after storage at 4 – 8°C for 9 months also increased considerably upon storage but not so much as at 30±2°C. No phase separation, caking or gelation occurred at this temperature.

At higher temperature, the growth of particles occurred at a faster rate than at lower temperature. The temperature at 4°C was generally the most favorable storage temperature (Freitas and Muller, 1998). At lower temperature, a high film rigidity of the emulsifier (microviscosity) avoided fusion of the film layers after particle contact. Microviscosity was a temperature dependent factor. Temperature increase caused a microviscosity decrease leading to destabilization of the SLN dispersion.

The measurement of the zeta potential allowed the predictions about the storage stability of SLN dispersions. In general, particle aggregation is less likely to occur for charged particles (high zeta potential) due to electric repulsion (Müller, Mäder, and Gohla, 2000). From Table 62, it was found that all SLN formulations (month 0) possessed a potential as low as ±30 mV. This was at the lower edge of the zeta potential range required for adequate stabilization (Müller and Heinemann, 1994). The decrease in zeta potential (near zero value) corresponded with the increase in the particle size of the SLN formulations due to the lack of electrostatic repulsion. This was observed in all SLN formulations regardless of the type of vehicle and their initial charge (negative or positive). However, for CGW when stored at different temperatures, it was found that the zeta potential of CGW at 4 – 8°C was still markedly negative (-20.84±1.44 mV). In contrast, at 30±2°C, the zeta potential of all SLN was near zero (almost no charge). Thus, it was apparent that the storage temperature influenced the zeta potential of the SLN. The higher temperature may increase the kinetic energy of a system. This energy input could lead to changes in the crystalline structure of the lipid. Crystalline re-orientation could result in changes of the charges on the particle surface (Nernst potential) and subsequently the measured zeta potential (Siekmann and Westesen, 1992). For buffer system, the zeta potential of all SLN formulations (MGB and CGB) at both storage temperatures was near zero. The explanation that could describe this result was still not available at present. But it should be noted that citrate species may have had significant influence on the initial particle size as well as the initial positive values of zeta potential. The presence of the

citrate buffer tended to increase the initial particle size as previously observed in both methods.

From Table 63, it was found that the percentage of total oxyresveratrol remaining and the entrapment efficiency decreased upon storage. At lower temperature (4 – 8°C) the results showed better chemical stability than at higher temperature (30±2°C). Like the particle size and zeta potential, the entrapment efficiency of all SLN formulations depended on the storage temperature. The high storage temperature (high energy) induced the more rapid change in the crystalline structure of lipid to more stable form resulting in drug expulsion and subsequent decrease in % entrapment, leading to more drug in the external phase which then underwent further degradation.

Table 62 The physical stability (particle size, size distribution and zeta potential) of all SLN containing Puag-Haad formulations after storage at 4 – 8°C and 30±2°C for 9 months

Sample	Particle size and size distribution (nm, PI)			Zeta potential (mV)		
	Month 0	Month 9 (4 – 8°C)	Month 9 (30±2°C)	Month 0	Month 9 (4 – 8°C)	Month 9 (30±2°C)
MGW (SD)	155.9, 0.212 (3.07, 0.01)	1295.56, 0.291 (163.03, 0.037)	> 3 μm	-20.32 (0.89)	-9.02 (1.68)	0.25 (3.20)
MGB (SD)	186.9, 0.259 (5.74, 0.02)	2047.33, 0.288 (340.46, 0.044)	> 3 μm	28.57 (3.61)	0.86 (0.68)	0.18 (4.11)
CGW (SD)	253.3, 0.174 (18.52, 0.07)	397.70, 0.305 (15.98, 0.032)	> 3 μm	-25.31 (3.30)	-20.84 (1.44)	1.14 (2.35)
CGB (SD)	312.2, 0.156 (10.63, 0.02)	491.46, 0.235 (74.06, 0.024)	> 3 μm	19.20 (2.25)	1.37 (0.49)	0.32 (2.88)

Table 63 The chemical stability (% Total oxyresveratrol remaining, % Entrapment efficiency, % Unentrapped oxyresveratrol and % Recovery) of all SLN containing Puag-Haad formulations after storage at 4 – 8°C and 30±2°C for 9 months

Sample	% Oxyresveratrol remaining			% Entrapment efficiency			% Unentrapped oxyresveratrol			% Recovery		
	Month 0	Month 9 (4– 8°C)	Month 9 (30±2°C)	Month 0	Month 9 (4 – 8°C)	Month 9 (30±2°C)	Month 0	Month 9 (4 – 8°C)	Month 9 (30±2°C)	Month 0	Month 9 (4 – 8°C)	Month 9 (30±2°C)
MGW (SD)	100.00 (0.00)	79.81 (1.68)	40.53 (2.23)	85.48 (1.15)	63.18 (1.22)	30.77 (1.29)	14.58 (0.61)	14.12 (1.82)	4.44 (0.93)	100.06 (1.57)	77.30 (1.50)	35.21 (1.66)
MGB (SD)	100.00 (0.00)	84.25 (1.77)	45.56 (2.41)	85.63 (1.19)	67.67 (1.67)	33.19 (0.64)	13.20 (0.50)	12.44 (3.33)	5.03 (1.29)	98.82 (0.77)	80.12 (1.68)	38.21 (1.25)
CGW (SD)	100.00 (0.00)	83.96 (1.46)	43.81 (2.15)	68.36 (0.97)	45.60 (1.17)	25.02 (1.48)	18.64 (0.57)	25.33 (0.55)	10.16 (0.17)	87.00 (1.13)	70.93 (1.31)	35.18 (1.32)
CGB (SD)	100.00 (0.00)	87.34 (1.55)	47.72 (2.27)	71.82 (0.87)	55.17 (1.55)	28.56 (1.30)	20.55 (0.29)	23.35 (0.15)	8.25 (1.01)	92.37 (1.17)	78.52 (1.42)	36.81 (1.46)

1.6 Thermal analysis by DSC

The DSC thermograms of SLN containing Puag-Haad were determined at months 0, 2 and 4 as shown in Figures 94 – 97. The recorded DSC parameters are present in Table 64.

Table 64 DSC results of SLN containing Puag-Haad during storage at 30±2°C (month 0, 2 and 4)

No	Code	Month	Endotherm				RI (%)
			Enthalpy (J/g)	Peak (°C)	Onset (°C)	End set (°C)	
1	Bulk lipid (GMS)	0	194.27	73.29	69.67	75.68	-
2	MGW	0	45.48	64.26	56.61	67.44	38.63
		2	52.16	67.40	61.78	70.04	44.30
		4	55.44	68.18	63.28	70.58	47.09
3	MGB	0	46.97	65.68	60.58	67.69	39.89
		2	49.51	67.55	61.80	69.59	42.05
		4	51.59	68.00	61.88	70.15	43.82
4	CGW	0	93.74	69.11	62.90	70.58	79.62
		2	98.84	70.98	65.46	72.66	83.95
		4	99.59	72.30	66.71	74.55	84.59
5	CGB	0	87.02	68.20	64.26	70.98	73.91
		2	93.02	70.85	62.48	72.99	79.00
		4	96.07	71.67	62.70	75.00	81.60

From Table 64, all formulations showed the increase in melting point and enthalpy endotherm. This result could be correlated with the more ordered crystal lattice, in which the substance required more energy than the less ordered crystal lattice to melt. Crystalline structure that was related to the chemical nature of lipid was a key factor in determining whether a drug will be expelled or firmly incorporated in the long term. During the storage condition, the formation of the more

stable modifications increased. The lattice was getting more perfect and the number of imperfection decreased resulting in the drug expulsion. These DSC results were in agreement with the results of the entrapment efficiency that showed the decrease in drug amount in the lipid phase due to drug expulsion.

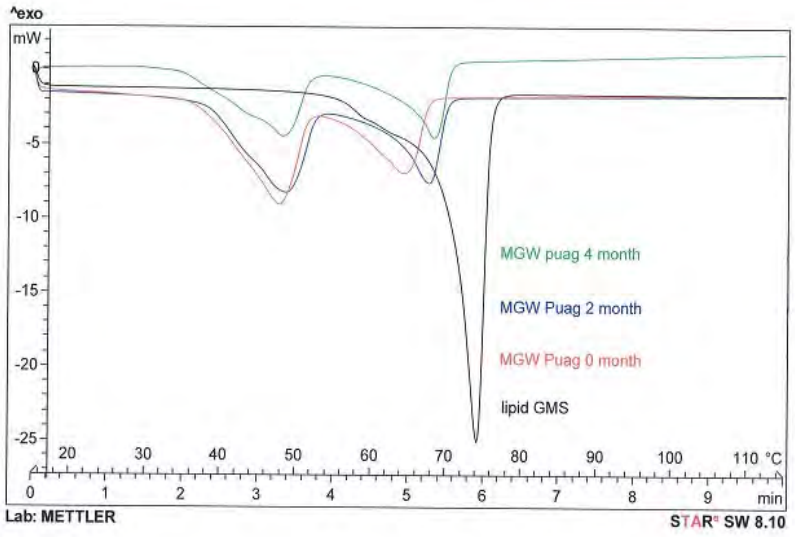


Figure 94 The DSC thermograms of bulk lipid (GMS) and SLN containing Puag-Haad by w/o/w double emulsion method using water as vehicle at 30±2°C for 4 months

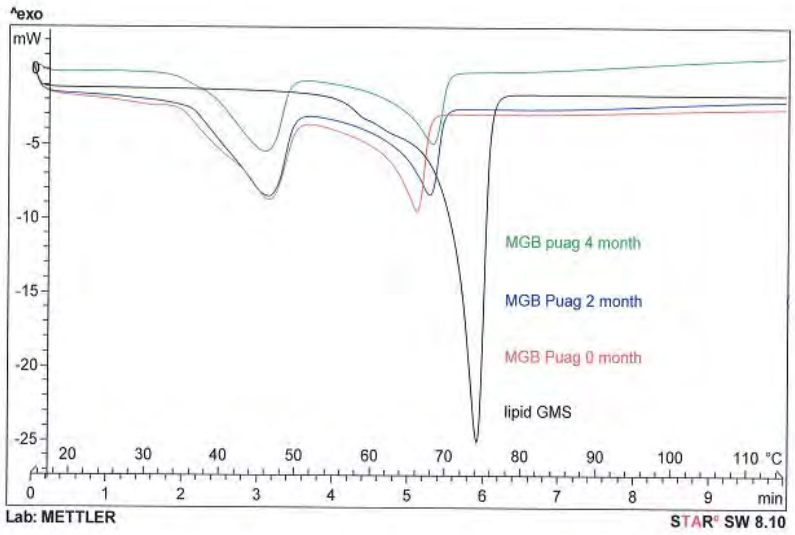


Figure 95 The DSC thermograms of bulk lipid (GMS) and SLN containing Puag-Haad by w/o/w double emulsion method using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

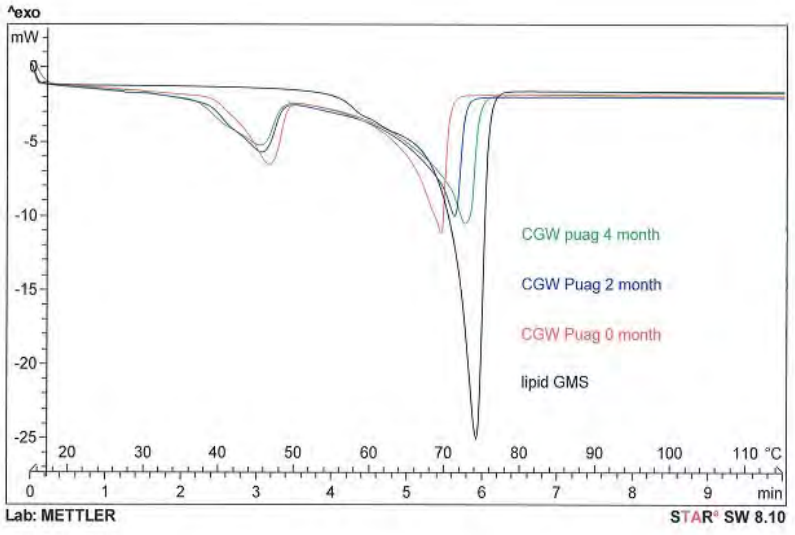


Figure 96 The DSC thermograms of bulk lipid (GMS) and SLN containing Puag-Haad by cold homogenization method using water as vehicle at 30±2°C for 4 months

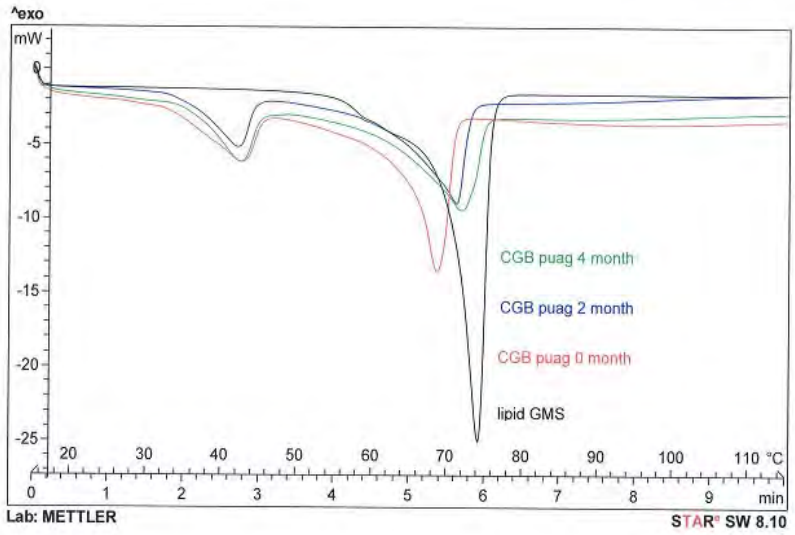


Figure 97 The DSC thermograms of bulk lipid (GMS) and SLN containing Puag-Haad by cold homogenization method using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

2. Stability study of aqueous solutions of Puag-Haad

The aqueous solutions of Puag-Haad were 0.25% Puag-Haad in 3% w/v polyoxyethylene 40 stearate (S-40) micellar solution, with and without citrate buffer; and 0.25% Puag-Haad in 20% v/v aqueous propylene glycol solution, with and without citrate buffer.

For convenience, the code was designated as follows:

"1 2 3" The first letter was "S". It represented that this formulation was solution.

The second letter was "S" or "P".

"S" represented that S-40 was used as surfactant in this formulation.

"P" represented that propylene glycol was used as co-solvent in this formulation.

The third letter represented type of vehicle (water or buffer).

The formulations are shown in Table 65.

Table 65 The formulations of Puag-Haad solutions used in the physical and chemical stability study

No.	Code	%Loading	Type of surfactant or cosolvent	Vehicle
1	SSW	0.25% w/v	3% w/v S-40	Water
2	SSB	0.25% w/v	3% w/v S-40	Citrate buffer pH 5.5
3	SPW	0.25% w/v	20% v/v propylene glycol	Water
4	SPB	0.25% w/v	20% v/v propylene glycol	Citrate buffer pH 5.5

All formulations in Table 65 were evaluated according to the topics below.

2.1 Physical appearance

The physical appearances such as color and color changes were visually observed. All formulations were freshly prepared and compared with their own samples every month. The photographs of physical stability study are shown in Figures 98 – 99.

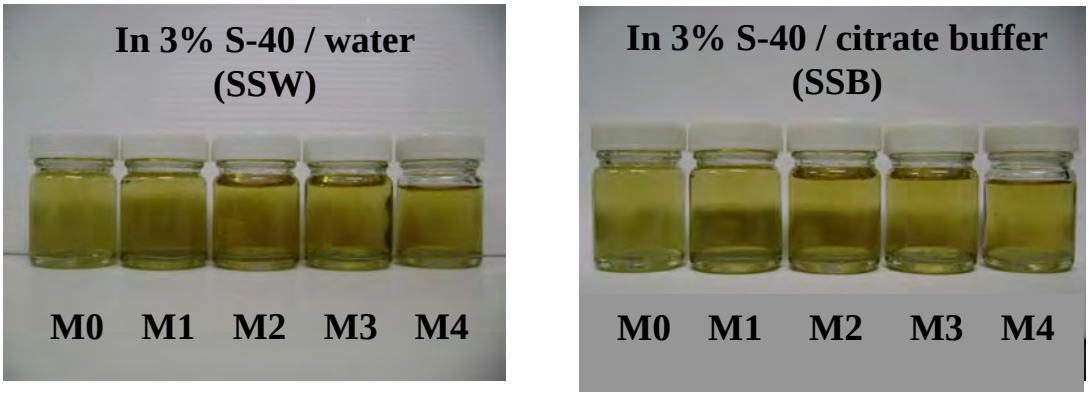


Figure 98 Physical stability of aqueous solutions of Puag-Haad using 3% w/v S-40 as surfactant at 30±2°C. Left : using water as vehicle (SSW). Right : using citrate buffer pH 5.5 as vehicle (SSB). (M0 = month 0, M1 = month 1, M2 = month 2, M3 = month 3, M4 = month 4)

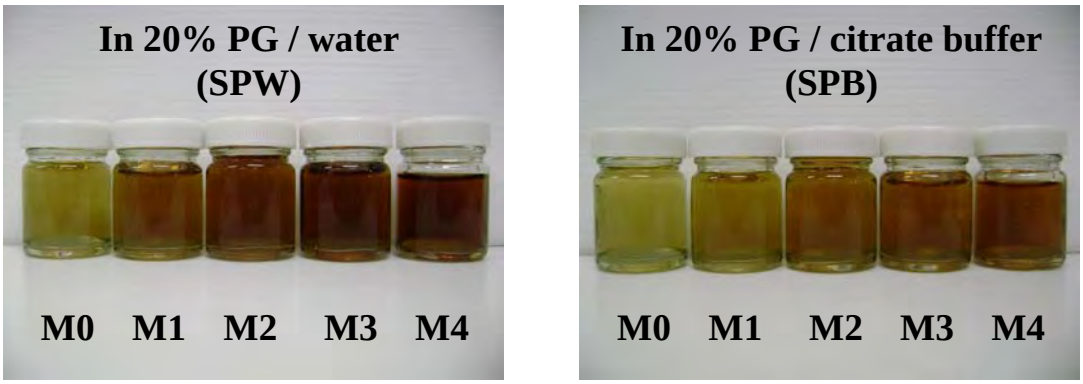


Figure 99 Physical stability of aqueous solutions of Puag-Haad using 20% v/v propylene glycol as co-solvent at 30±2°C. Left : using water as vehicle (SPW). Right : using citrate buffer pH 5.5 as vehicle (SPB). (M0 = month 0, M1 = month 1, M2 = month 2, M3 = month 3, M4 = month 4)

From Figure 98, there was a slight increase in yellow color with time for both solutions in 3% S-40 when using water and citrate buffer pH 5.5 as vehicle. In contrast, from Figure 99, there was a marked discoloration from yellow to brown color with time for both solutions in 20% propylene glycol when using water and citrate buffer pH 5.5 as vehicle. The result seemed to suggest that the presence of S-40 surfactant was able to stabilize the color of Puag-Haad better than the propylene glycol system. Considering the effect of citrate buffer pH 5.5, it was also able to improve physical appearance of Puag-Haad by delaying the change from yellow to brown color especially in the case of 20% propylene glycol. This result agreed with Manatchaya Wanawatanakun (2006) who studied the physical and chemical stability of Puag-Haad in various buffer systems. She found that the addition of buffer, particularly citrate buffer pH 5.5 was able to protect 0.1% Puag-Haad solution from increased coloration after prolonged storage. However, the addition of citrate buffer pH 5.5 to 3% S-40 did not show a marked improvement in color stability from water system. S-40 might be able to adsorb and already stabilize Puag-Haad molecules within its micelles resulting in protection of Puag-Haad from physical and chemical degradation such that the stabilizing effect by the buffer was less dominant.

2.2 pH

The pH values of the solutions of Puag-Haad at each time are shown in Table 66.

Table 66 The pH measurements of Puag-Haad solutions during storage at 30±2°C (month 0, 1, 2, 3 and 4)

No	Code	pH measurement (Mean±SD)				
		Month 0	Month 1	Month 2	Month 3	Month 4
1	SSW	6.08±0.02	6.03±0.02	6.00±0.01	5.93±0.02	5.88±0.03
2	SSB	5.49±0.00	5.49±0.00	5.49±0.00	5.48±0.01	5.49±0.01
3	SPW	6.54±0.02	6.07±0.01	5.98±0.01	5.88±0.01	5.82±0.01
4	SPB	5.74±0.01	5.72±0.00	5.74±0.00	5.72±0.00	5.72±0.01

The pH of formulation in 3% S-40 in water (SSW) slightly decreased (about 0.20 drop after 4 months) whereas the formulation in 20% propylene glycol in water without surfactant (SPW) showed a larger drop in pH (about 0.72 drop after 4 months). When the citrate buffer was added, the pH of the formulations (both SSB and SPB) hardly changed with time. This result was also in agreement with Manatchaya Wanawatanakun (2006) who reported that the citrate buffer pH 5.5 possessed a good buffering capacity and was able to stabilize the pH of Puag-Haad aqueous solutions in propylene glycol. Results from this study further suggested that the micellar solution of S-40 was also able to stabilize the pH of Puag-Haad to some extent since SPW gave greater drop in pH than SSW.

To compare the change in pH values between SLN and solutions (in 20% PG and in 3% S-40), Figures 100 and 101 show the change in pH values of all formulations at 30±2°C for 4 months.

From Figures 100 and 101, it indicated that the addition of either S-40 or citrate buffer pH 5.5 could stabilize the pH of Puag-Haad in the solution as well as the preparation of SLN. However, citrate buffer obviously had better buffering capacity than S-40 probably due to its direct interaction with the hydronium ions in the

environment. Stabilization of pH change by S-40 was probably due to its indirect effect which delayed degradation of oxyresveratrol.

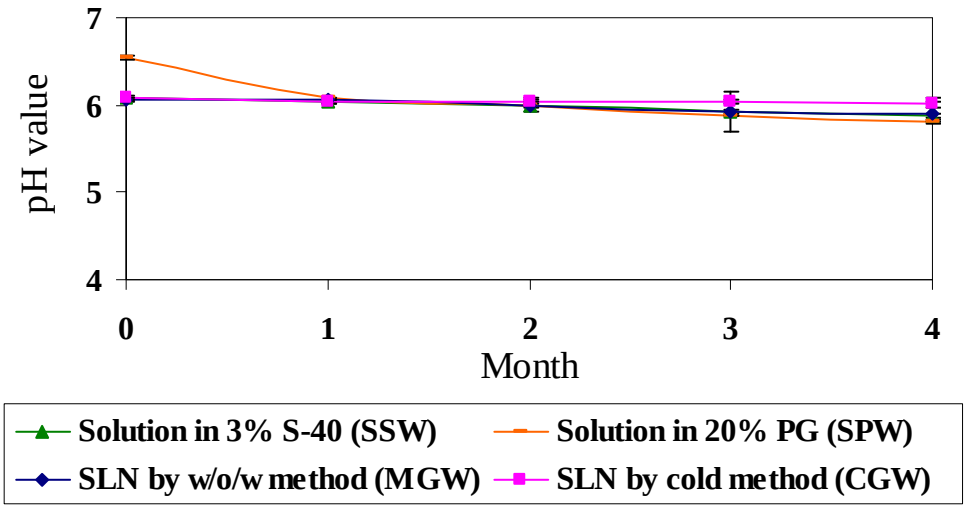


Figure 100 Changes in pH values of SLN containing Puag-Haad in comparison with aqueous solutions of Puag-Haad when using water as vehicle at 30±2°C for 4 months

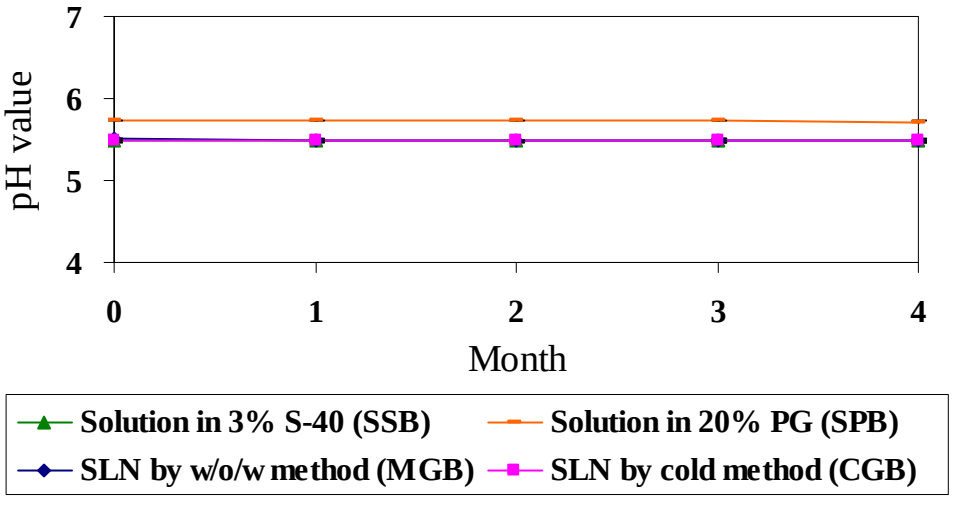


Figure 101 Changes in pH values of SLN containing Puag-Haad in comparison with aqueous solutions of Puag-Haad when using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

2.3 Drug remaining

The total amount of oxyresveratrol remaining in the formulations was analyzed by HPLC method. Table 67 shows the percentage of oxyresveratrol remaining in solutions at 30±2°C for 4 months.

From Table 67, the percentage of oxyresveratrol remaining in all formulations decreased with time. The solution in 3% S-40 in citrate buffer pH 5.5 (SSB) showed the best chemical stability of oxyresveratrol in the solution. On the other hand, the solution in 20% PG in water (SPW) showed the lowest amount of oxyresveratrol remaining. Considering the physical and chemical stability of all solutions in Figures 98 – 99 and Table 67, the increase in yellow color was associated with the reduction in the percentage of total oxyresveratrol especially observed in the simple solution with cosolvent, 20% PG (SPW and SPB). These results agreed with Manatchaya Wanawatanakun (2006) who studied the physical and chemical stability of Puag-Haad solutions.

To compare the chemical stability between SLN and solutions (in 20% PG and in 3% S-40), Table 58 and 67 were combined to Table 68. Table 68 shows the percentage of oxyresveratrol remaining in all formulations after storage at 30±2°C for 4 months.

Table 67 Normalized percentage of total oxyresveratrol remaining in solutions of Puag-Haad formulations at 30±2°C for 4 months

Time (months)	% Oxyresveratrol remaining			
	SSW	SSB	SPW	SPB
0	100.00	100.00	100.00	100.00
(SD)	(0.00)	(0.00)	(0.00)	(0.00)
1	94.72	96.65	82.78	89.10
(SD)	(0.96)	(0.98)	(1.31)	(0.78)
2	91.54	93.07	72.44	77.88
(SD)	(0.98)	(1.13)	(1.11)	(0.63)
3	87.16	90.36	62.57	73.13
(SD)	(3.07)	(1.08)	(0.96)	(1.14)
4	70.98	86.92	54.69	65.44
(SD)	(3.13)	(1.25)	(0.45)	(0.90)
Decrease in % Oxyresveratrol after 4 months	29.02	13.08	45.31	34.56

Table 68 Normalized percentage of total oxyresveratrol remaining in formulations at 30±2°C at 4 months

Formulation	% Oxyresveratrol remaining			
Solution	SPW	SPB	SSW	SSB
%	54.69	65.44	70.98	86.92
(SD)	(0.45)	(0.90)	(3.13)	(1.25)
SLN	MGW	MGB	CGW	CGB
%	79.41	88.07	85.08	89.39
(SD)	(2.93)	(1.46)	(2.59)	(2.76)

From Table 68, the percentage of oxyresveratrol remaining in the simple solution with cosolvent, 20% PG in water system (SPW) was 54.69% in comparison with buffer system (SPB) that was 65.44%. SPW showed the most rapid reduction in total oxyresveratrol remaining. When the buffer was added (SPB), chemical stability was further improved. Furthermore, the effect of surfactant on the chemical stability of the formulations was evaluated. The percentage of oxyresveratrol remaining in the micellar solution with 3% S-40 in water (SSW) was 70.98% in comparison with SPW. 3% S-40 in the system could stabilize Puag-Haad probably by protecting Puag-Haad components within its micelles. In addition, the percentage of oxyresveratrol remaining in the micellar solution with 3% S-40 in citrate buffer pH 5.5 (SSB) was 86.92% that was higher than SSW due to stabilizing by citrate buffer. The efficiency to stabilize Puag-Haad was increased when using the surfactant with buffer system. It was suggested that the surfactant used in this study (S-40) may have synergistic effect with the citrate buffer in stabilizing Puag-Haad components. To understand easily, the diagram is shown in Figure 102.

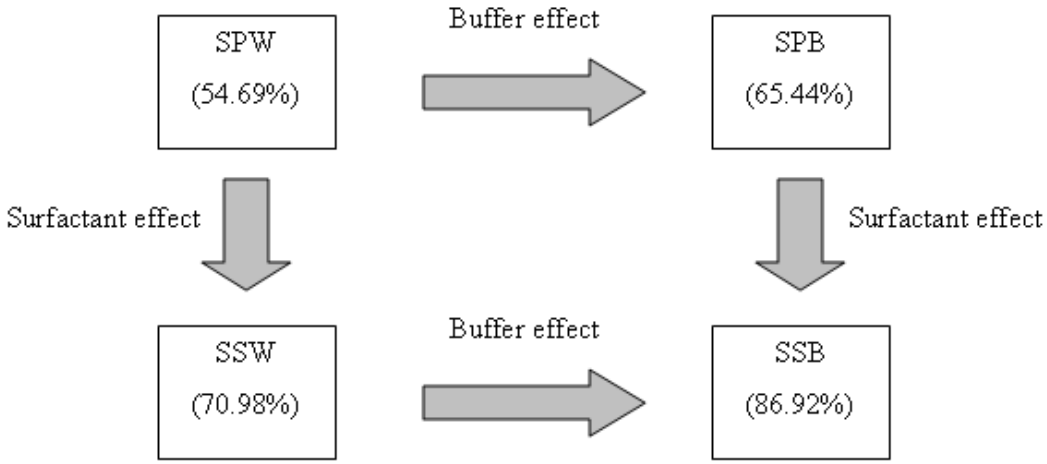


Figure 102 Diagram comparing the chemical stability of the solution formulations

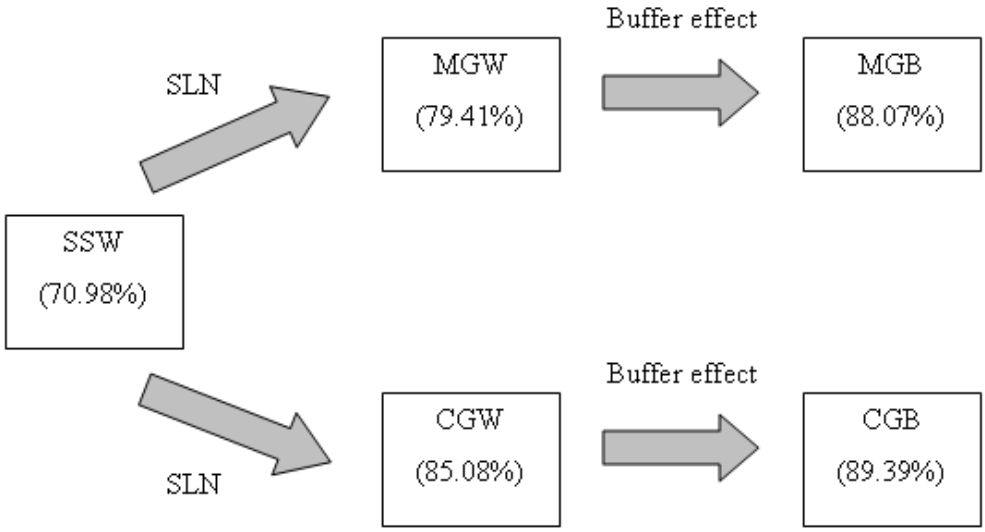


Figure 103 Diagram comparing the chemical stability of the SLN formulations

When SSW was formulated into SLN by the two methods (MGW and CGW), the percentage of oxyresveratrol remaining in the formulations were 79.41% and 85.08%, respectively. Considering SSW in comparison with MGW and CGW, the incorporation of Puag-Haad in the lipid component of the SLN formulations could improve the chemical stability over the micellar solution alone. The same trend of buffer effect on the stability of Puag-Haad in the SLN formulation was also observed when using citrate buffer pH 5.5 as vehicle (the percentage of oxyresveratrol remaining in MGB and CGB were 88.07% and 89.39%, respectively). To understand easily, the diagram is shown in Figure 103.

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Figures 104 – 105 show the percentage of oxyresveratrol remaining in SLN containing Puag-Haad formulations in comparison with aqueous solutions of Puag-Haad at 30±2°C for 4 months.

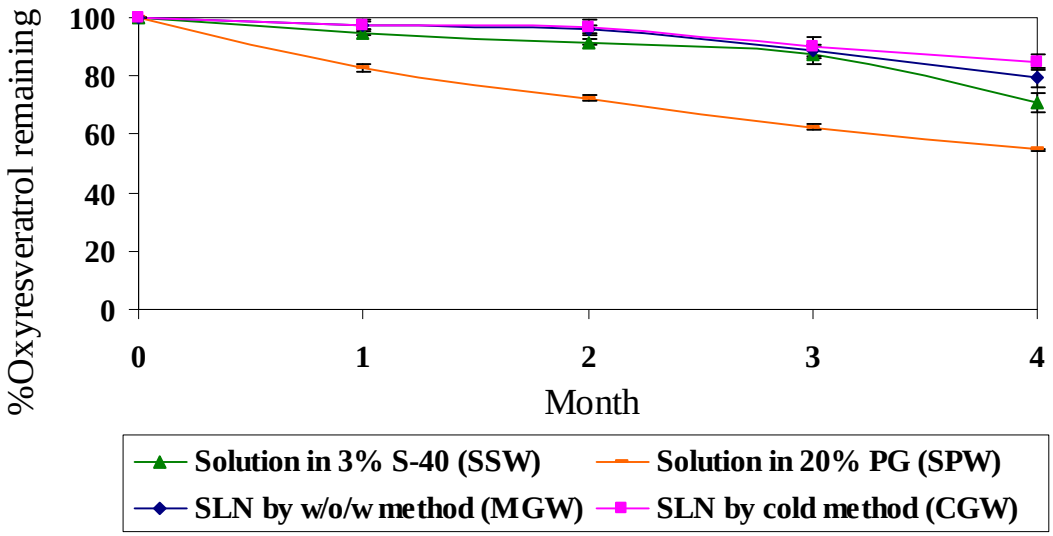


Figure 104 The percentage of oxyresveratrol remaining (relative to initial value) in SLN containing Puag-Haad formulations in comparison with aqueous solutions of Puag-Haad when using water as vehicle at 30±2°C for 4 months

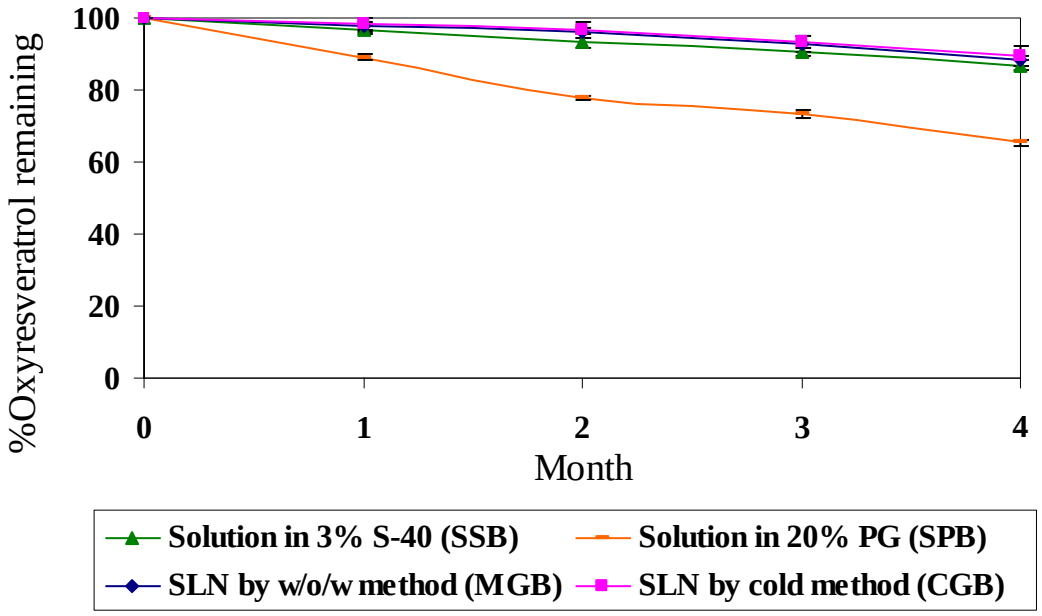
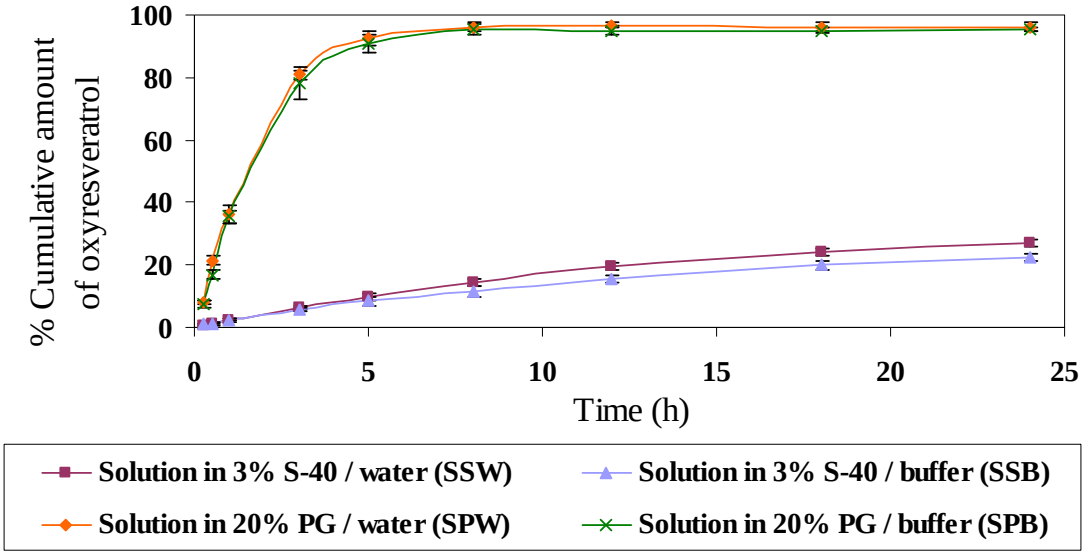


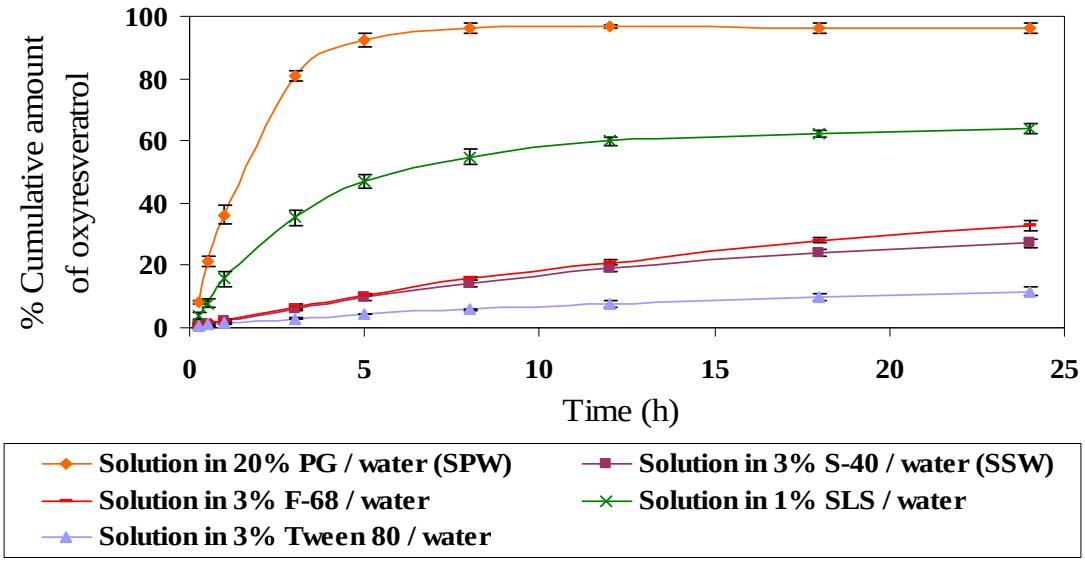
Figure 105 The percentage of oxyresveratrol remaining (relative to initial value) in SLN containing Puag-Haad in comparison with aqueous solutions of Puag-Haad when using citrate buffer pH 5.5 as vehicle at 30±2°C for 4 months

From the stability of the micellar solution with 3% S-40, it was found that S-40 could stabilize Puag-Haad components both physically and chemically. It is possible that Puag-Haad molecules were entrapped within the micelles of S-40 resulting in the improvement of both physical and chemical stability of Puag-Haad. Entrapment of Puag-Haad component, especially oxyresveratrol, within the micelles may also have an effect on the release characteristics of oxyresveratrol. Therefore, the release of the micellar solution of Puag-Haad was also evaluated as shown in Figure 106.

From Figure 106, it was found that the micellar solutions of Puag-Haad with 3% S-40 showed sustained release profiles of oxyresveratrol. The result of this study thus corresponded with the above assumption that the incorporation of Puag-Haad (oxyresveratrol) within the micelles of S-40 could stabilize and protect Puag-Haad from physical and chemical degradation and result in the sustained release of active component.



(a)



(b)

Figure 106 The release profiles of the control solutions (in 20% PG) of Puag-Haad and the micellar solutions of Puag-Haad, (a) the micellar solutions (in 3% S-40) using water and citrate buffer pH 5.5 as vehicle (medium is also used the same as vehicle of the formulation), (b) the micellar solutions (in 3% S-40, 3% F-68, 1% SLS and 3% Tween 80) using water as vehicle (medium also = water) (F-68 = poloxamer 188, SLS = sodium lauryl sulfate)

When formulated into SLN, Puag-Haad molecules were entrapped both in the SLN and inside the micelles of S-40. Considering the entrapment efficiency of all SLN formulations after storage at 30±2°C for 4 months as discussed above, the entrapment efficiency of all SLN formulations was rapidly decreased with time due to the drug expulsion from the lipid matrix. Although Puag-Haad molecules could not be protected by the lipid matrix of SLN, the Puag-Haad molecules were still protected by the surrounding micelles of S-40 resulting in the high percentage of total oxyresveratrol remaining in all formulations (more than 79% when stored at 30±2°C for 4 months).

In this present work, it could not define the percentage of entrapment efficiency in each part of formulation (either SLN or micelles). In this study, Centriprep® with membrane MWCO = 50 K could separate SLN (lipid pellets) from filtrate (both micelles and free drug). Both micelles and free drug could pass this membrane. So, the calculated entrapment efficiency was the entrapped drug in SLN only. To find the entrapped drug in micelles only, it was necessary to select the Centriprep® with smaller MWCO membrane to separate only free drug from SLN and micelles. Then, the entrapment of drug in micelles only was calculated from the equation below:

% Entrapment in micelles = % Entrapment in both SLN and micelles (derived from Centriprep® with smaller MWCO) – % Entrapment in SLN only (derived from Centriprep® with MWCO = 50K)

In addition, the micellar solution of Puag-Haad was prepared to find and confirm its entrapment efficiency.

Although the formulations of SLN developed in this study showed rapid increase in particle size and decrease in percentage drug entrapment due to insufficient stability of the lipid phase which resulted in particle fusion and drug expulsion, SLN formulations were still able to protect Puag-Haad and oxyresveratrol from physical and chemical degradation better than the solution systems, especially when water was used as vehicle. Both the citrate buffer pH 5.5 and surfactant (S-40) also played a significant role in improving the physical and chemical stability of Puag-Haad and their protective effects appeared to be additive.

Part E. Evaluation of whitening and anti-wrinkle efficacy of SLN containing Puag-Haad in human volunteers

The purpose of this part was to demonstrate the in vivo whitening and anti-wrinkle efficacy of SLN containing Puag-Haad in comparison with the same concentration of Puag-Haad in micellar solution. The micellar solution was composed of 3% w/v S-40 to dissolve Puag-Haad (0.25%). The type and amount of surfactant, 3% S-40, was the same surfactant used in the SLN preparation. Since the surfactant in the formulations may be able to interact with the skin and enhance drug penetration, the Puag-Haad micellar solution was thus used as a control sample for this study to eliminate the confounding effect from the surfactant. The compositions of the reference and test formulations are as shown in Table 69.

Table 69 The composition of formulations to evaluate whitening and anti-wrinkle efficacy in woman volunteers

Sample	Formulation	Type and % of lipid	Type and % of surfactant	% Puag-Haad loading	Vehicle
Reference (SSW)	Solution	-	3% w/v S-40	0.25% w/v	Water
Test (CGW)	SLN	5% w/v GMS	3% w/v S-40	0.25% w/v	Water

The reason for selecting the concentration of Puag-Haad at 0.25% was based on the study of Parkpoom Tengamnuay et al. (2006) and Manatchaya Wanawatanakun (2006). This concentration showed the efficacy in woman volunteers of both skin whitening and anti wrinkle when prepared as solution (in 20% propylene glycol).

Since CGW has demonstrated a good appearance as well as acceptable physical and chemical stability (from part D), it was chosen as a representative SLN formulation in this part.

Twenty six woman volunteers participated in this study. One forearm was micellar solution and the other forearm was SLN. Before starting the experiment,

patch tests were performed in all subjects. Each subject was given both solution and SLN to the inner side of their upper forearms. The right side was SLN and the left side was solution. After 24 hours and 3 days, no subjects had adverse reactions for both cycles (acute and delayed hypersensitivity test). Therefore, all subjects (26 persons) were included in this study. Moreover, no skin irritation or hypersensitivity reactions were observed in all cases and none of the subjects withdrew from the study.

Before application of the samples, all subjects stopped using any cosmetics on the application areas for at least two weeks and then each of them was measured for their baseline parameters at week 0. All parameters that were evaluated in this study were as follows:

1. Skin whitening as melanin index values
2. Skin roughness as mean roughness (Horizontal and vertical lines) and circular roughness values
3. Skin elasticity as Young's modulus values
4. Skin hydration as skin hydration values

For Sebum, no values could be evaluated. All values showed 0 for all subjects during the experiment. Because the skin of the forearms was relatively dry, the measurement of sebum at this area may not be sufficiently sensitive by the current instrument.

The initial baseline values for both solution and SLN sides were tested by Paired t-test to make sure that the starting values were the same before product application ($P > 0.05$). The baseline characteristics of the subjects are shown in Table 70.

It was found that no significant difference was found between the two sides indicating that the baseline integrity with respect to the application side was observed for all parameters.

Table 70 Baseline characteristics of the subjects (at week 0)

Characteristic	Solution side (mean±SD)	SLN side (mean±SD)	P-value
1 Skin whitening (Melanin index)	36.80±2.84	36.66±2.83	0.664 (NS)
2 Skin roughness			
Mean roughness (H + V)	39.35±7.52	39.82±7.88	0.658 (NS)
2.2 Circular roughness	40.36±7.90	40.75±8.20	0.705 (NS)
3 Skin elasticity (Young's modulus)	9.48±2.46	10.01±2.47	0.379 (NS)
4 Skin hydration	23.34±12.45	23.38±12.63	0.984 (NS)

NS = not significantly different ($P > 0.05$)

The efficacy of formulations was evaluated using Paired t-test. It compared the difference between the solution and the SLN sides at each follow-up week, and between the baseline and the value at each follow up week of the same side. All the data are shown in Table 71.

Furthermore, since many studies took the form of baseline measurement followed by observations at more than one point in time, which is commonly known as "Repeated measures or Split-plot design", thus, the Repeated measures ANOVA was also chosen to evaluate the difference in overall efficacy between the solution and the SLN formulations over time at the same significance level ($\alpha = 5\%$).

Table 71 Efficacy parameters of SLN containing Puag-Haad in comparison with micellar solution of Puag-Haad

Week	Mean±SD									
	Skin whitening		Mean roughness		Circular roughness		Skin elasticity		Skin hydration	
	Solution	SLN	Solution	SLN	Solution	SLN	Solution	SLN	Solution	SLN
0	36.80±2.84	36.66±2.83	39.35±7.52	39.82±7.88	40.36±7.90	40.75±8.20	9.48±2.46	10.01±2.47	23.34±12.45	23.38±12.63
2	36.22±3.06 #(P=0.008)	36.12±3.19 #(P=0.013)	37.80±6.76	35.41±4.38 *(P=0.027) #(P=0.004)	38.99±7.31	36.38±4.51 *(P=0.029) #(P=0.005)	9.16±2.07	9.68±2.42	22.41±12.68	28.72±8.34 *(P=0.020) #(P=0.039)
4	35.57±2.72 #(P=0.000)	35.68±2.70 #(P=0.000)	33.46±5.93 #(P=0.000)	31.80±4.02 *(P=0.015) #(P=0.000)	34.37±5.93 #(P=0.000)	32.71±4.37 *(P=0.009) #(P=0.000)	8.86±1.85	8.92±2.13 #(P=0.029)	26.88±6.95	40.14±12.44 *(P=0.000) #(P=0.000)
6	35.88±2.75 #(P=0.001)	35.59±2.54 #(P=0.000)	35.31±4.88 #(P=0.005)	33.32±4.64 *(P=0.001) #(P=0.000)	35.88±4.75 #(P=0.004)	34.03±4.36 *(P=0.003) #(P=0.000)	9.04±2.02	8.14±2.29 *(P=0.030) #(P=0.001)	25.47±7.94	34.05±8.94 *(P=0.000) #(P=0.000)
8	32.60±3.18 #(P=0.000)	32.54±3.21 #(P=0.000)	33.13±4.37 #(P=0.005)	30.87±3.67 *(P=0.000) #(P=0.000)	34.04±4.54 #(P=0.000)	31.59±3.99 *(P=0.000) #(P=0.000)	8.93±2.17	8.35±2.79 #(P=0.000)	23.76±6.95	32.08±7.21 *(P=0.000) #(P=0.000)

* $P < 0.05$ by paired t-test, compared with solution side at the same week

\# $P < 0.05$ by paired t-test, compared with baseline (week 0) of the same side

1. Skin whitening

Table 72 shows the mean melanin index values in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks.

Table 72 The mean melanin index values in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks (n = 26 subjects)

Formulation	Melanin index values				
	Week 0	Week 2	Week 4	Week 6	Week 8
Solution (SD)	36.80 (2.84)	36.22# (3.06)	35.57# (2.72)	35.88# (2.75)	32.60# (3.18)
SLN (SD)	36.66 (2.83)	36.12# (3.19)	35.68# (2.70)	35.59# (2.54)	32.54# (3.21)
P-value At the same week	0.664	0.765	0.633	0.191	0.804

* $P < 0.05$ by paired t-test, compared with solution side at the same week

\# $P < 0.05$ by paired t-test, compared with baseline (week 0) of the same side

The melanin index values are graphically represented for each formulation in Figure 107.

From Table 72 and Figure 107, it can be seen that the melanin index values of both formulations changed from the initial values in both sides. They gradually decreased with time and showed significant difference from their corresponding baselines at week 2 and until the end of the study (week 8). However, no significant difference in the melanin index values between the solution and the SLN sides at the same week, indicating that both formulations were effective in reducing melanin content with time.

The whitening efficacy of Puag-Haad could be related to oxyresveratrol, the main constituent that showed the potent anti-tyrosinase activity with IC_{50} of 0.83

μg/ml. Parkpoom Tengamnuay et al. (2006) studied the whitening efficacy of 0.25% Puag-Haad solution (in 20% w/v propylene glycol) in woman volunteers. They found that this solution was significantly different in the mean % whitening values when compared to its self control arms (only propylene glycol without Puag-Haad) after 4 weeks of application and until the end of the 12-week study. It was noticed that at the same concentration of 0.25% Puag-Haad solution, the solution with surfactant evaluated in this study appeared to be more effective than the simple solution with propylene glycol with respect to the rate of whitening. It was probably due to the effect of surfactant that acted as a skin penetration enhancer. However, the optimum concentration of surfactant must be carefully considered so that it would not cause skin reaction or hypersensitivity.

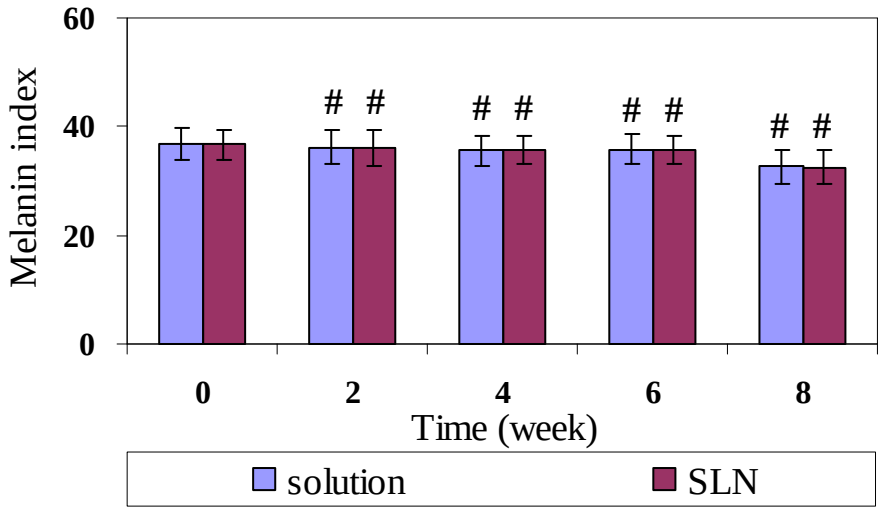


Figure 107 Skin whitening values (Melanin index) after applying 0.25% Puag-Haad formulations for different times. Data = mean ± SD (n = 26)

* P < 0.05 by paired t-test, compared with solution side at the same week

\# P < 0.05 by paired t-test, compared with baseline (week 0) of the same side

The data were also further analyzed by Repeated measures ANOVA after baseline correction. By subtracting the baseline measurement from each observation, the measurements would represent changes from baseline. Since the melanin index

values generally decreased with application times, the changes from baseline in this case were calculated from the formula: Melanin index (week 0) – Melanin index (week 2, 4, 6 or 8) to obtain positive values. Table 73 shows the changes from baseline of melanin index values after applying Puag-Haad formulations for 8 weeks.

Table 73 The changes from baseline (week 0) of melanin index values over 8 weeks

Formulation	Changes from baseline of melanin index values				
	Week 2	Week 4	Week 6	Week 8	Overall
Solution					
Sum	14.94	31.82	23.94	109.18	179.88
Mean	0.57	1.22	0.92	4.20	1.73
(SD)	(1.02)	(1.09)	(1.22)	(1.69)	
SLN					
Sum	14.03	25.32	27.74	107.12	174.21
Mean	0.54	0.97	1.07	4.12	1.68
(SD)	(1.03)	(0.99)	(1.04)	(1.46)	

The final analysis combined the separate two-way ANOVAs and had two new terms, “weeks × drugs” interaction and “drugs. The final ANOVA Table is shown in Table 74.

Table 74 Repeated measures ANOVA on the melanin index values

Source of variance	df	SS	MS	F	P-value
Subjects	50	204.36	4.09		
Weeks	3	427.23	142.41		
Drugs	1	602.94	602.94	147.52	0.0000
Weeks × Drugs	3	1.03	0.34	0.57	0.6358
Error	150	90.68	0.60		
Total	207	1326.24			

The terms of most interest were the “drugs” and “weeks × drugs” components of the ANOVA. “Drugs” measured the difference between the overall averages of the two treatment groups. The overall average change from baseline for SLN was 1.68 whereas it was 1.73 for solution. The F test for “drugs” difference was 147.52 which was greater than critical $F_{1, 50}$ of 4.03. This difference was significant ($P < 0.05$). The significant result indicated that on the average, the solution was superior to the SLN with regard to lowering the melanin index values.

“Weeks × Drugs” was a measure of interaction. This test compared the parallelism of the two “change from baseline” curves as shown in Figure 108. The F test for “weeks × drugs” differences was 0.57 which was less than critical $F_{3, 150}$ of 2.66. This non significant result ($P > 0.05$) suggested that the pattern of response was constant for solution and SLN for the entire period of study.

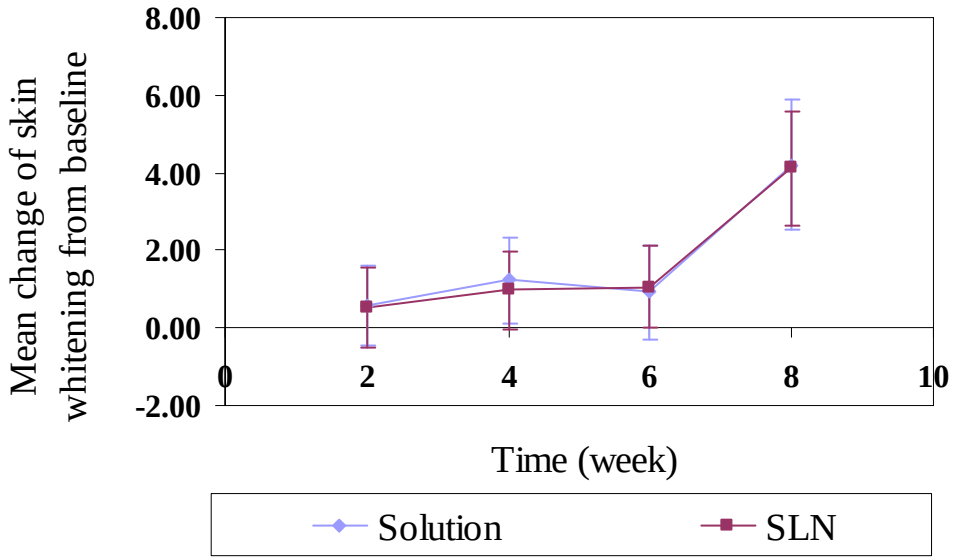


Figure 108 The changes from baseline of melanin index values over 8 weeks

Although results from Repeated measures ANOVA indicated that surfactant solution of Puag-Haad was more effective than SLN with respect to the skin whitening effect. However, the difference between the two formulations was very small as seen from Figure 108. In addition, paired t-test had previously shown that the two formulas were similarly effective.

2. Skin roughness

2.1 Mean roughness (horizontal and vertical lines, H + V)

Table 75 shows the mean roughness values (H + V) in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks.

Table 75 The mean roughness values (H + V) in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks (n = 26 subjects)

Formulation	Mean roughness values				
	Week 0	Week 2	Week 4	Week 6	Week 8
Solution (SD)	39.35 (7.52)	37.80 (6.76)	33.46# (5.93)	35.31# (4.88)	33.13# (4.37)
SLN (SD)	39.82 (7.88)	35.41# (4.38)	31.80# (4.02)	33.32# (4.64)	30.87# (3.67)
P-value At the same week	0.658	0.027*	0.015*	0.001*	0.000*

* $P < 0.05$ by paired t-test, compared with solution side at the same week

\# $P < 0.05$ by paired t-test, compared with baseline (week 0) of the same side

The mean roughness values (H + V) are graphically represented for each formulation in Figure 109.

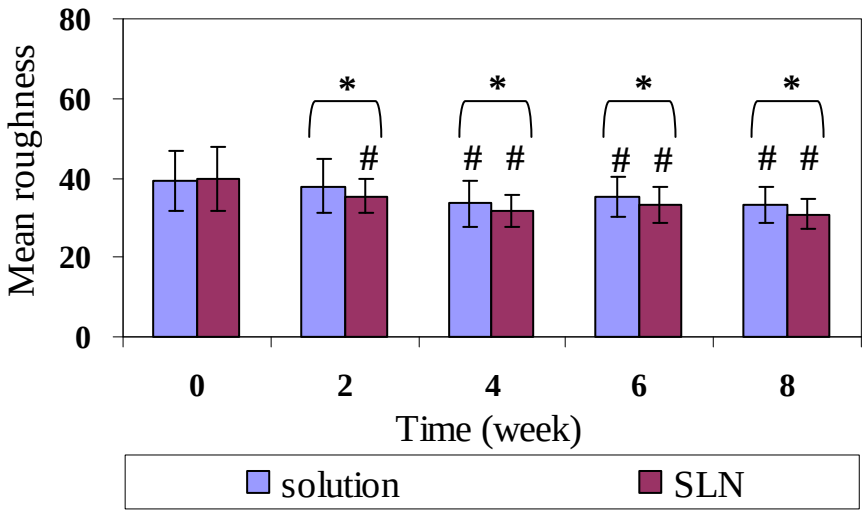


Figure 109 Mean roughness values after applying 0.25% Puag-Haad formulations for different times. Data = mean ± SD (n = 26)

* P < 0.05 by paired t-test, compared with solution side at the same week

\# P < 0.05 by paired t-test, compared with baseline (week 0) of the same side

From Table 75 and Figure 109, it can be seen that the mean roughness values of both formulations changed from the initial values in both sides. For the solution side, the mean roughness values significantly decreased from baseline after 4 weeks of application. In contrast, the mean roughness values of SLN side significantly decreased from baseline after only 2 weeks and were also significantly different from solution side at week 2 and onwards.

Manatchaya Wanawatanakun (2006) studied the anti-wrinkle efficacy of 0.25% Puag-Haad solution (in 20% propylene glycol) in woman volunteers. She found that this solution gave significant decrease in mean roughness than the control side of propylene glycol after 8 weeks. So, the type of formulations was important factors that may influence both the extent and onset of the desired effects of the active ingredients.

The data were also further analyzed by Repeated measures ANOVA after baseline correction. By subtracting the baseline measurement from each observation, the measurements would represent changes from baseline. Since the mean roughness

values generally decreased with application times, the changes from baseline in this case were calculated from the formula: Mean roughness (week 0) – Mean roughness (week 2, 4, 6 or 8) to obtain positive values. Table 76 shows the changes from baseline of mean roughness values after applying Puag-Haad formulations for 8 weeks.

Table 76 The changes from baseline (week 0) of mean roughness values over 8 weeks

Formulation	Changes from baseline of mean roughness values				
	Week 2	Week 4	Week 6	Week 8	Overall
Solution					
Sum	40.25	153.00	105.00	161.75	460.00
Mean	1.55	5.88	4.04	6.22	4.42
(SD)	(6.12)	(5.05)	(6.74)	(6.18)	
SLN					
Sum	114.50	208.50	169.00	232.75	724.75
Mean	4.40	8.02	6.50	8.95	6.97
(SD)	(7.04)	(7.32)	(7.08)	(6.50)	

The final ANOVA Table is shown in Table 77.

Table 77 Repeated measures ANOVA on the mean roughness values

Source of variance	df	SS	MS	F	P-value
Subjects	50	6602.20	132.04		
Weeks	3	662.08	220.69		
Drugs	1	7085.22	7085.22	53.66	0.0000
Weeks × Drugs	3	3.98	1.33	0.10	0.9586
Error	150	1949.08	12.99		
Total	207	16302.56			

The overall average change from baseline for SLN was 6.97 whereas it was 4.42 for solution. The F test for “drugs” difference was 53.66 which was greater than critical $F_{1, 50}$ of 4.03. This difference was significant ($P < 0.05$). The significant result indicated that on the average, the SLN was superior to the solution with regard to lowering the mean roughness values.

The F test for “weeks × drugs” differences was 0.10 which was less than critical $F_{3, 150}$ of 2.66. This non significant result ($P > 0.05$) suggested that the pattern of response was constant for solution and SLN for the entire period of study. The curves of weeks × drugs interaction are shown as in Figure 110.

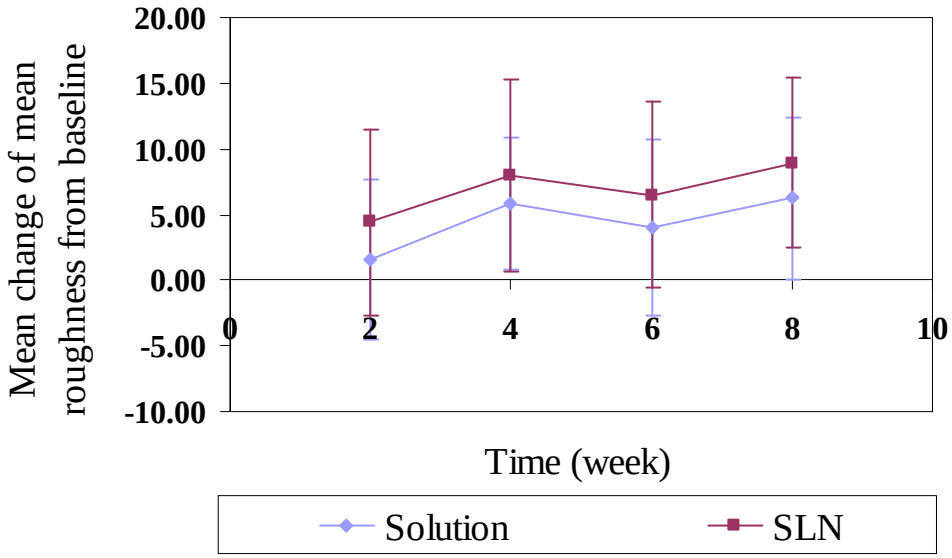


Figure 110 The changes from baseline of mean roughness values over 8 weeks

2.2 Circular roughness

Table 78 shows the circular roughness values in woman volunteers after applying Puag-Haad formulations for 8 weeks.

Table 78 The circular roughness values in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks (n = 26 subjects)

Formulation	Circular roughness values				
	Week 0	Week 2	Week 4	Week 6	Week 8
Solution (SD)	40.36 (7.90)	38.99 (7.31)	34.37# (5.93)	35.88# (4.75)	34.04# (4.54)
SLN (SD)	40.75 (8.20)	36.38# (4.51)	32.71# (4.37)	34.03# (4.36)	31.59# (3.99)
P-value At the same week	0.705	0.029*	0.009*	0.003*	0.000*

* P < 0.05 by paired t-test, compared with solution side at the same week

\# P < 0.05 by paired t-test, compared with baseline (week 0) of the same side

The circular roughness values are graphically represented for each formulation in Figure 111.

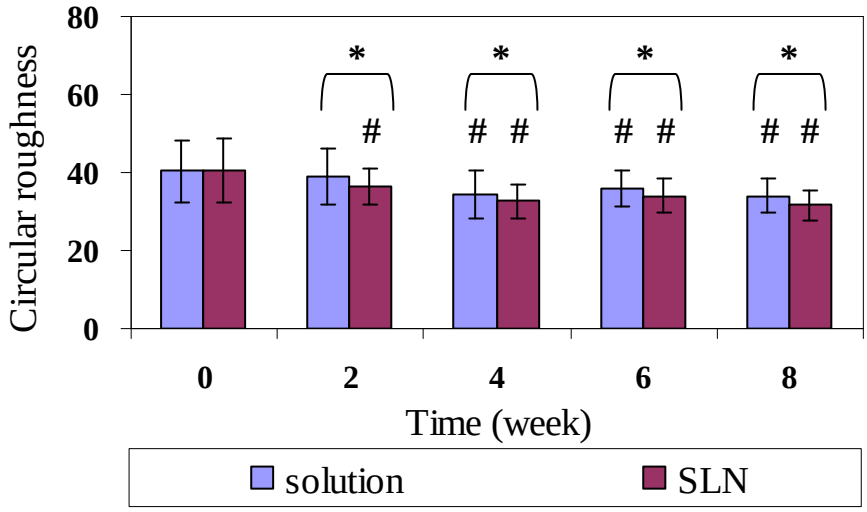


Figure 111 Circular roughness values after applying 0.25% Puag-Haad formulations for different times. Data = mean ± SD (n = 26)

* P < 0.05 by paired t-test, compared with solution side at the same week

\# P < 0.05 by paired t-test, compared with baseline (week 0) of the same side

From Table 78 and Figure 111, it can be seen that the circular roughness values of both formulations changed from the initial values in both sides. The circular roughness showed the same trend as the mean roughness. For the solution side, the circular roughness values significantly decreased from baseline after 4 weeks of application. In contrast, the circular roughness values of SLN side significantly decreased from baseline after only 2 weeks and were also significantly different from solution side at week 2 and onwards.

The data were also further analyzed by Repeated measures ANOVA after baseline correction. By subtracting the baseline measurement from each observation, the measurements would represent changes from baseline. Since the circular roughness values generally decreased with application times, the changes from baseline in this case were calculated from the formula: Circular roughness (week 0) – Circular roughness (week 2, 4, 6 or 8) to obtain positive values. Table 79 shows the changes from baseline of circular roughness values after applying Puag-Haad formulations for 8 weeks.

Table 79 The changes from baseline (week 0) of circular roughness values over 8 weeks

Formulation	Changes from baseline of circular roughness values				
	Week 2	Week 4	Week 6	Week 8	Overall
Solution					
Sum	35.50	155.75	116.25	164.25	471.75
Mean	1.37	5.99	4.47	6.32	4.54
(SD)	(6.41)	(5.61)	(7.07)	(6.68)	
SLN					
Sum	113.50	209.00	174.75	238.25	735.5
Mean	4.37	8.04	6.72	9.16	7.07
(SD)	(7.13)	(7.60)	(7.00)	(6.85)	

The final ANOVA Table is shown in Table 80.

Table 80 Repeated measures ANOVA on the circular roughness values

Source of variance	df	SS	MS	F	P-value
Subjects	50	7320.03	146.40		
Weeks	3	722.46	240.82		
Drugs	1	7341.43	7341.43	50.15	0.0000
Weeks × Drugs	3	8.21	2.74	0.21	0.8908
Error	150	1973.70	13.16		
Total	207	17365.81			

The overall average change from baseline for SLN was 7.07 whereas it was 4.54 for solution. The F test for "drugs" difference was 50.15 which was greater than critical $F_{1, 50}$ of 4.03. This difference was significant ($P < 0.05$). The significant result indicated that on the average, the SLN was superior to the solution with regard to lowering the circular roughness values.

The F test for "weeks × drugs" differences was 0.21 which was less than critical $F_{3, 150}$ of 2.66. This non significant result ($P > 0.05$) suggested that the pattern of response was constant for solution and SLN for the entire period of study. The curves of weeks × drugs interaction are shown as in Figure 112.

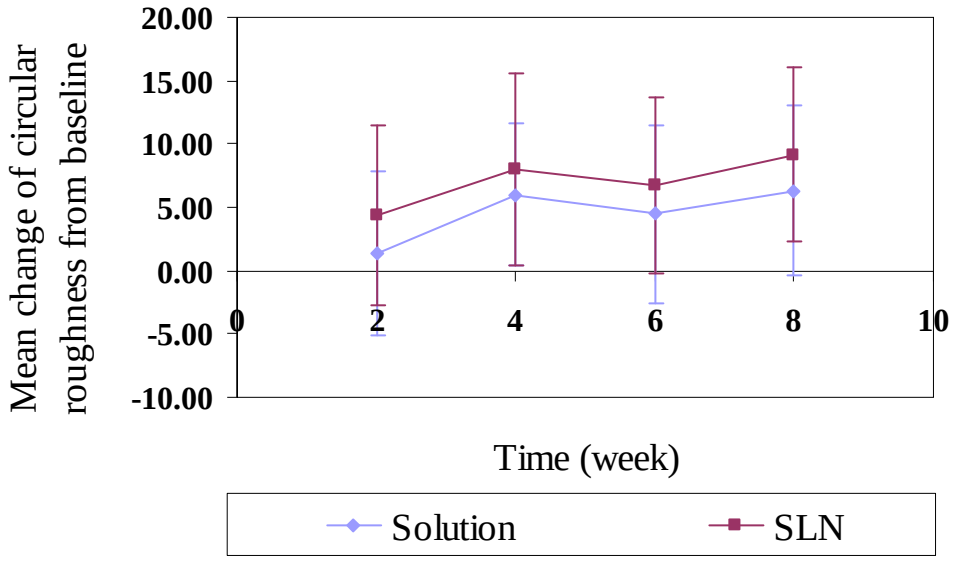


Figure 112 The changes from baseline of circular roughness values over 8 weeks

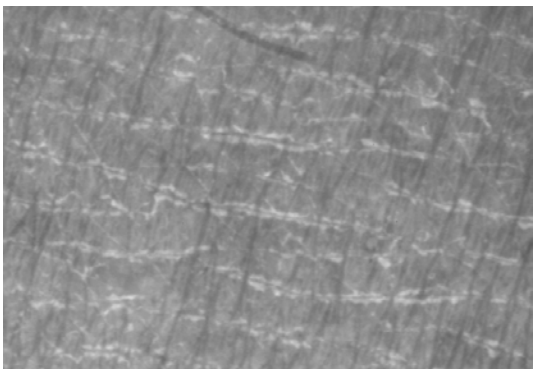
The actual skin surface of each subject was also photographed to determine the correlation between the skin surface picture and the calculated roughness values**.** Figures 113 – 118 show the representative skin surface of 3 subjects after applying each formulation for different times in comparison with the initial measurement.

The skin surface pictures at week 2 were selected to present because there was a marked change in the mean roughness and circular roughness values from initial measurement (week 0) for both formulations. However, the SLN formulation showed greater change in roughness values than the micellar solution.

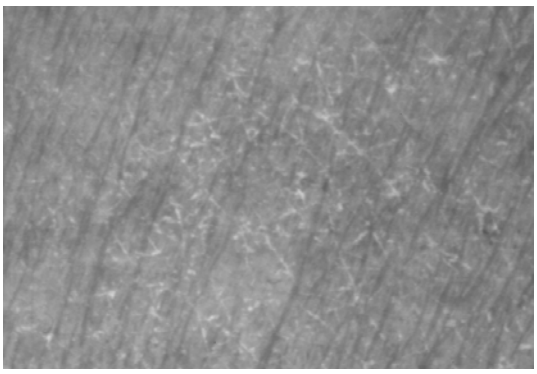
For example (Figure 113), after applying the micellar solution for 2 weeks, the mean roughness and circular roughness of subject number 13 decreased from 47.75 to 41.75 and 51.00 to 44.25, respectively, with the differences of 6 and 6.75. These showed that both parameters only slightly decreased with time and these numerical changes also were in agreement with the skin topography, in which the skin roughness and dryness still remained clearly visible from the photographs after 2 weeks.

On the other hand, data from the same subject receiving SLN revealed that after applying the SLN for 2 weeks (Figure 114), the mean roughness and circular roughness decreased from 61.50 to 33.50 and 62.75 to 33.75, corresponding to the differences of 28 and 29, respectively. These numerical changes also agreed with the visual observation in that the skin surface became smoother without apparent dryness (usually present as white streaks) after 2 weeks, indicating that the skin wrinkles were more shallow and hydrated. However, the improvement of skin dryness would be further evaluated to confirm the effect on skin hydration.

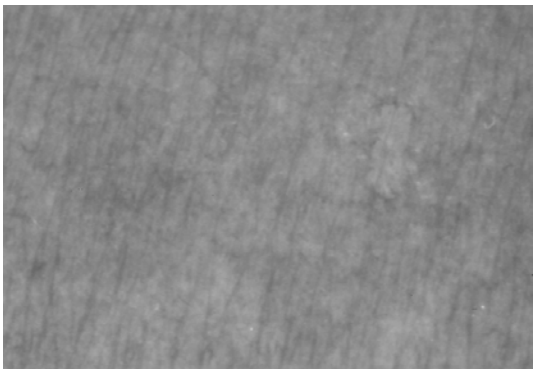
Similar changes in roughness values and skin topography were also observed in other subjects as shown in Figure 115 – 118.



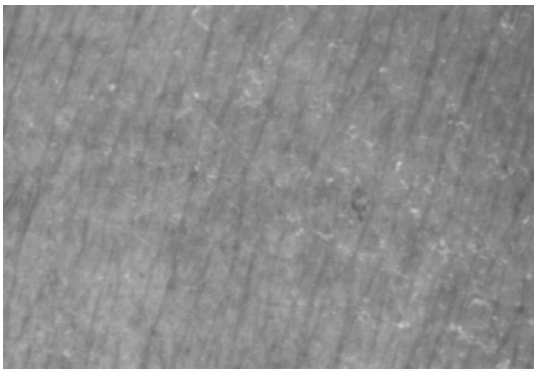
A Mean roughness = 47.75
Circular roughness = 51.00



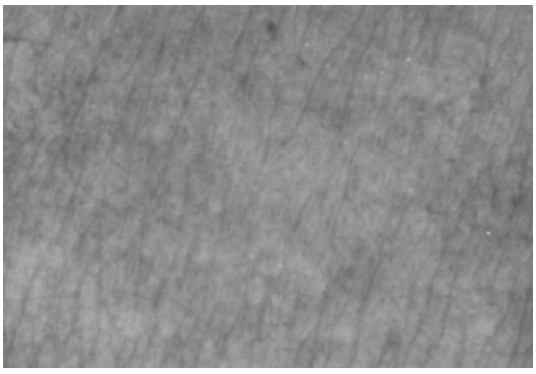
B Mean roughness = 41.75
Circular roughness = 44.25



C Mean roughness = 25.75
Circular roughness = 26.00

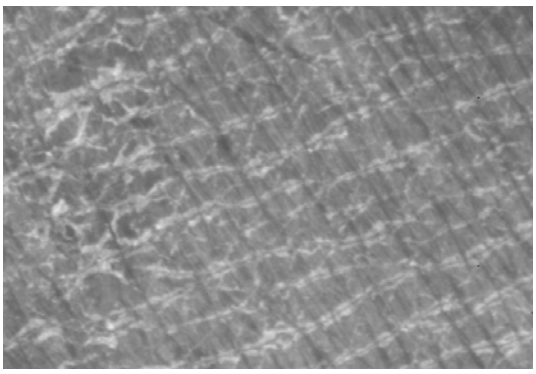


D Mean roughness = 36.25
Circular roughness = 37.25

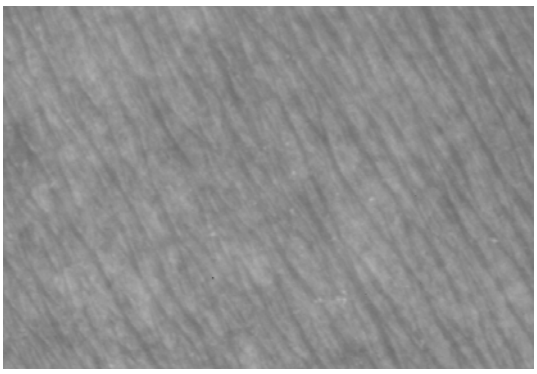


E Mean roughness = 32.75
Circular roughness = 33.75

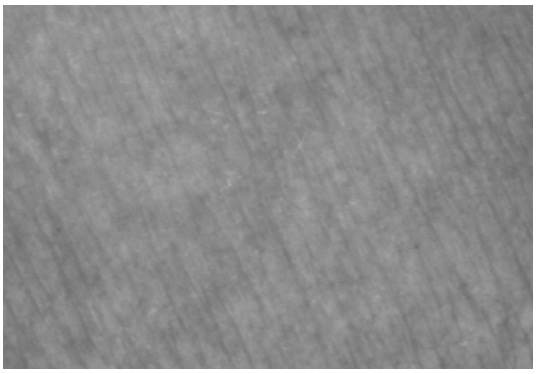
Figure 113 Representative skin surface of subject number 13 after applying the micellar solution of Puag-Haad for different times in comparison with the initial or baseline measurement (A = week 0, B = week 2, C = week 4, D = week 6 and E = week 8)



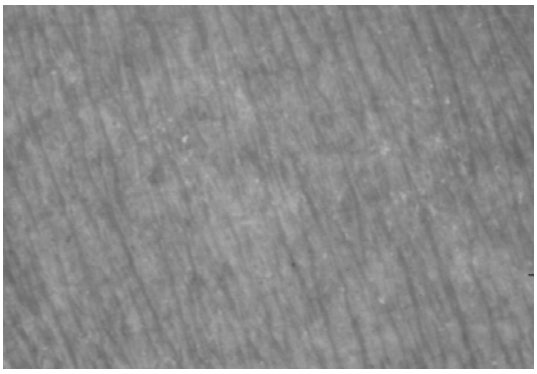
A Mean roughness = 61.50
Circular roughness = 62.75



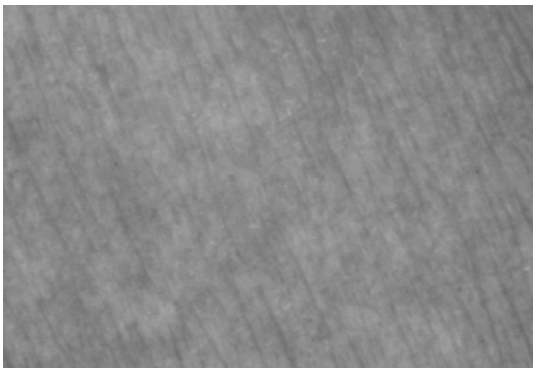
B Mean roughness = 33.50
Circular roughness = 33.75



C Mean roughness = 24.50
Circular roughness = 25.00

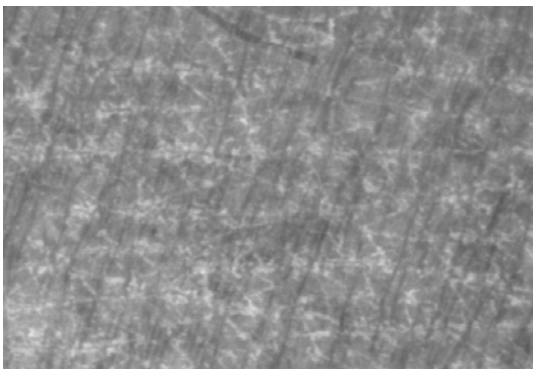


D Mean roughness = 33.25
Circular roughness = 33.75

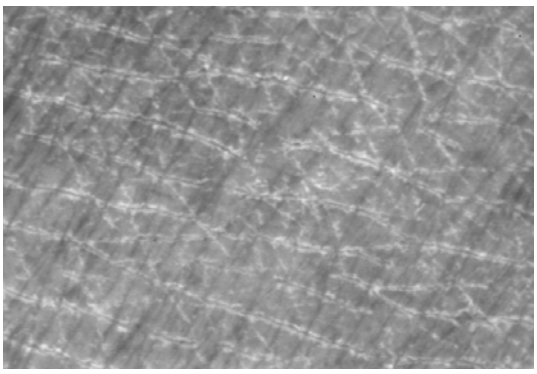


E Mean roughness = 31.00
Circular roughness = 30.75

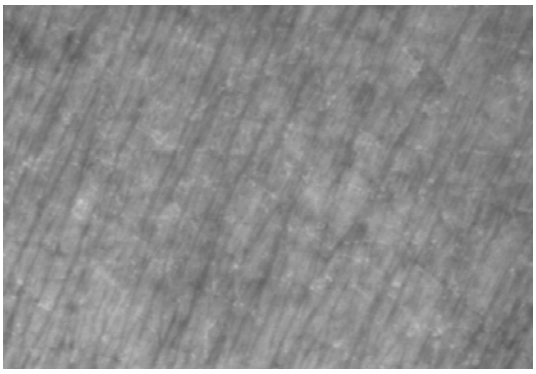
Figure 114 Representative skin surface of subject number 13 after applying the SLN containing Puag-Haad for different times in comparison with the initial or baseline measurement (A = week 0, B = week 2, C = week 4, D = week 6 and E = week 8)



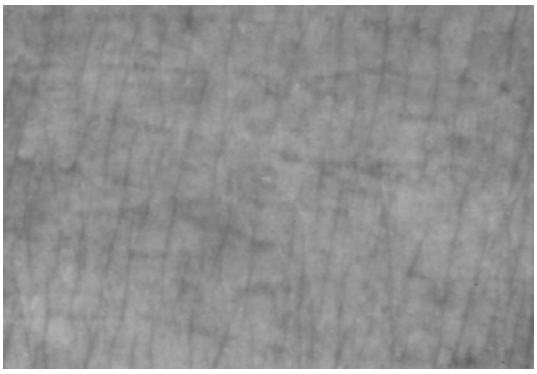
A Mean roughness = 55.25
Circular roughness = 57.25



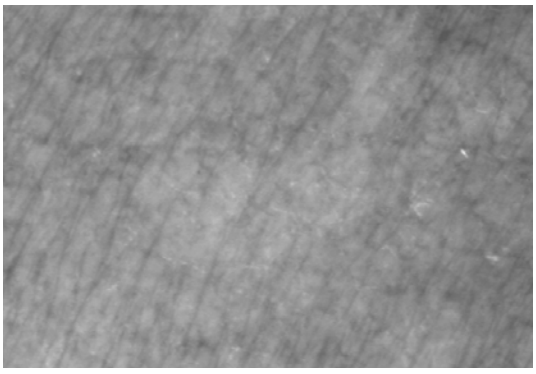
B Mean roughness = 61.50
Circular roughness = 63.25



C Mean roughness = 47.75
Circular roughness = 48.75

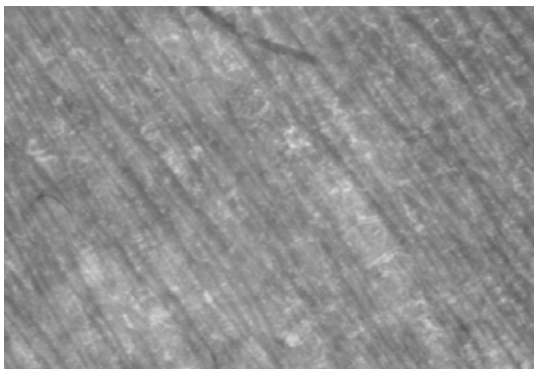


D Mean roughness = 31.75
Circular roughness = 32.50

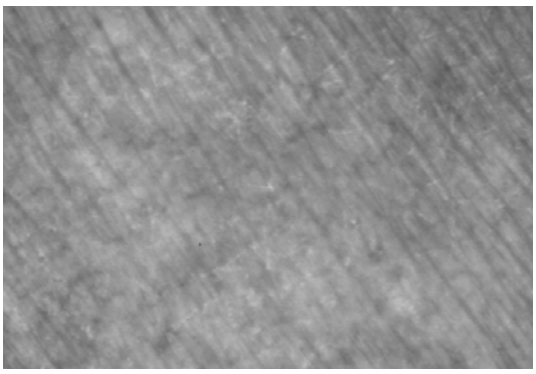


E Mean roughness = 36.75
Circular roughness = 38.00

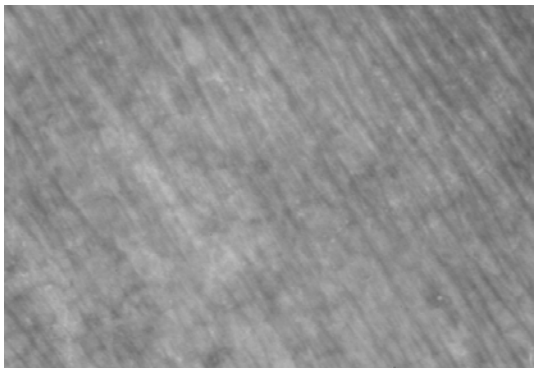
Figure 115 Representative skin surface of subject number 5 after applying the micellar solution of Puag-Haad for different times in comparison with the initial or baseline measurement (A = week 0, B = week 2, C = week 4, D = week 6 and E = week 8)



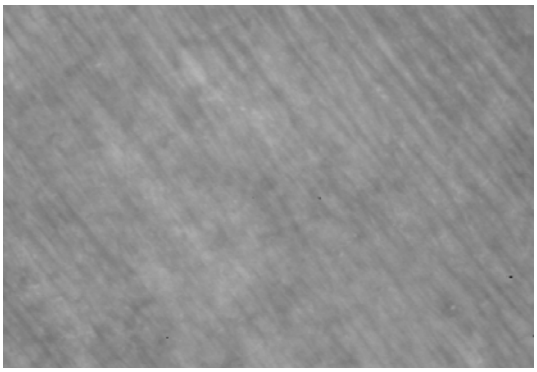
A Mean roughness = 51.00
Circular roughness = 50.75



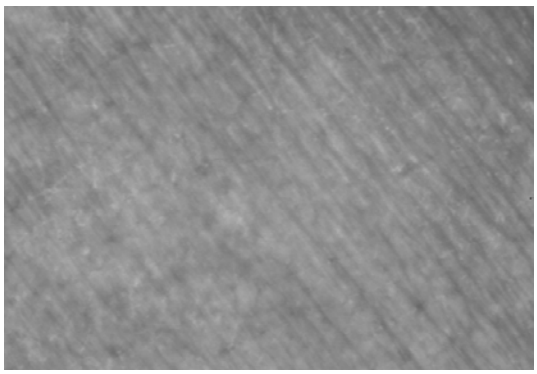
B Mean roughness = 44.50
Circular roughness = 46.00



C Mean roughness = 39.00
Circular roughness = 40.75

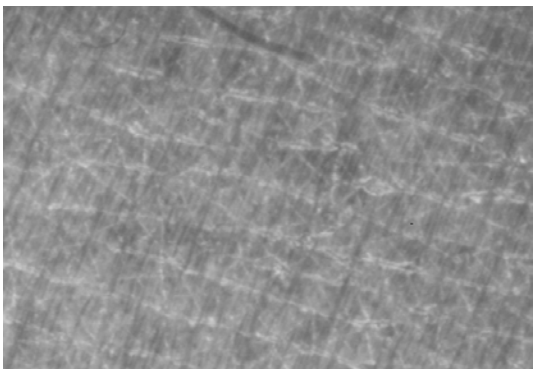


D Mean roughness = 28.75
Circular roughness = 30.25

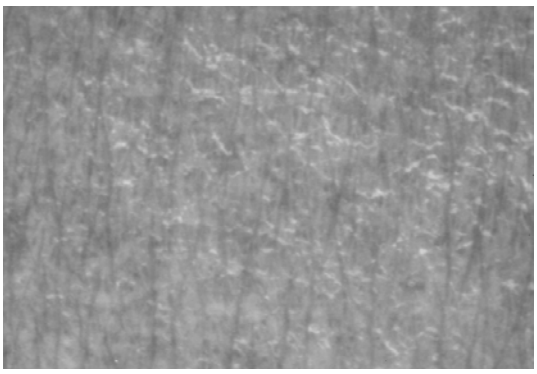


E Mean roughness = 34.50
Circular roughness = 35.75

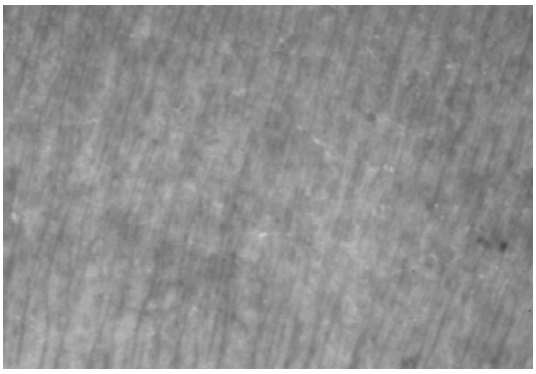
Figure 116 Representative skin surface of subject number 5 after applying the SLN containing Puag-Haad for different times in comparison with the initial or baseline measurement (A = week 0, B = week 2, C = week 4, D = week 6 and E = week 8)



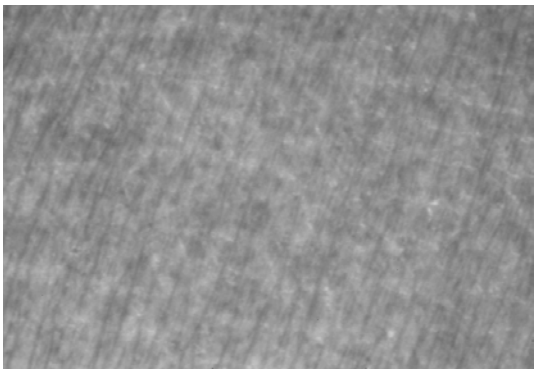
A Mean roughness = 49.50
Circular roughness = 51.50



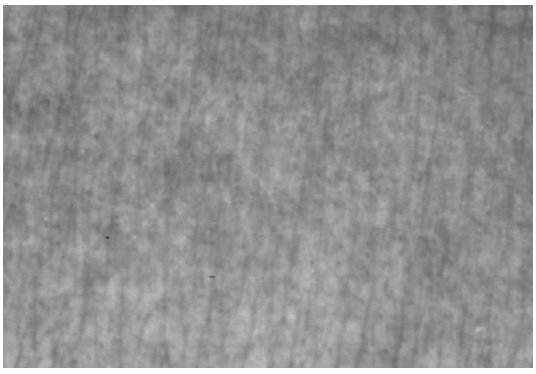
B Mean roughness = 44.00
Circular roughness = 46.75



C Mean roughness = 36.75
Circular roughness = 38.00

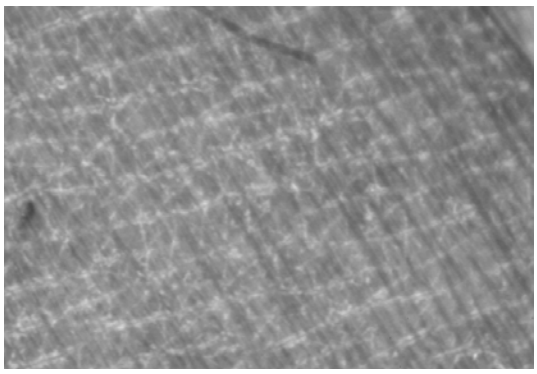


D Mean roughness = 37.75
Circular roughness = 38.50

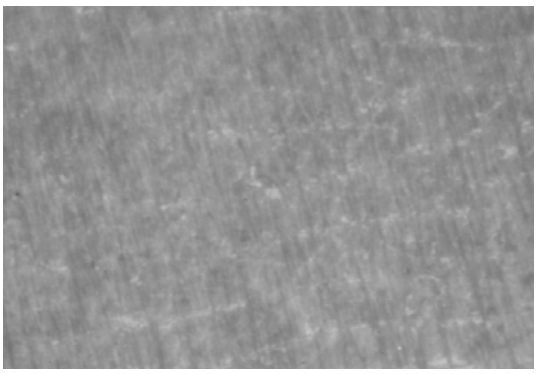


E Mean roughness = 40.25
Circular roughness = 42.50

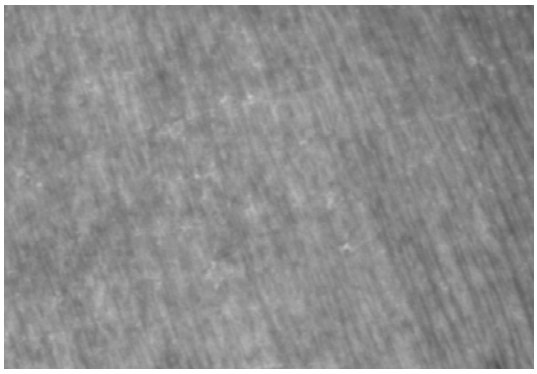
Figure 117 Representative skin surface of subject number 11 after applying the micellar solution of Puag-Haad for different times in comparison with the initial or baseline measurement (A = week 0, B = week 2, C = week 4, D = week 6 and E = week 8)



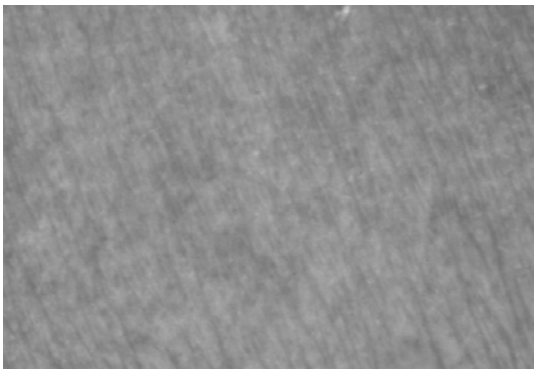
A Mean roughness = 53.00
Circular roughness = 54.75



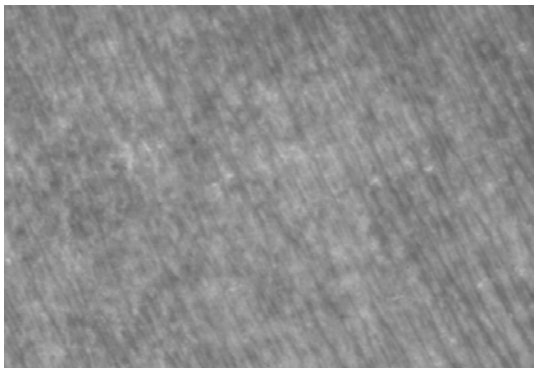
B Mean roughness = 35.75
Circular roughness = 38.50



C Mean roughness = 34.75
Circular roughness = 36.50



D Mean roughness = 32.50
Circular roughness = 33.75



E Mean roughness = 35.50
Circular roughness = 38.00

Figure 118 Representative skin surface of subject number 11 after applying the SLN containing Puag-Haad for different times in comparison with the initial or baseline measurement (A = week 0, B = week 2, C = week 4, D = week 6 and E = week 8)

3. Skin elasticity

The skin elasticity was evaluated as the value of Young's modulus. The Young's modulus value was inversely related to the skin elasticity.

Table 81 shows the Young's modulus values in woman volunteers after applying Puag-Haad formulations for 8 weeks.

Table 81 The Young's modulus values in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks (n = 26 subjects)

Formulation	Young's modulus values				
	Week 0	Week 2	Week 4	Week 6	Week 8
Solution (SD)	9.48 (2.46)	9.16 (2.07)	8.86 (1.85)	9.04 (2.02)	8.93 (2.17)
SLN (SD)	10.01 (2.47)	9.68 (2.42)	8.92# (2.13)	8.14# (2.29)	8.35# (2.79)
P-value (At the same week)	0.379	0.259	0.924	0.030*	0.145

* $P < 0.05$ by paired t-test, compared with solution side at the same week

\# $P < 0.05$ by paired t-test, compared with baseline (week 0) of the same side

The skin elasticity (Young's modulus) values are graphically represented for each formulation in Figure 119.

From Table 81 and Figure 119, it can be seen that application of micellar solution did not have any effect on the Young's modulus values at all time points. No significant difference was found in the case of the solution when compared to the initial value ($P > 0.05$). In contrast, the Young's modulus values of SLN side slightly decreased with time and became significantly different from baseline at week 4 and afterwards. Furthermore, significant difference between the two formulations was detected at week 6 of application. This suggested that the SLN formulation may be more effective in increasing the skin elasticity than the micellar solution.

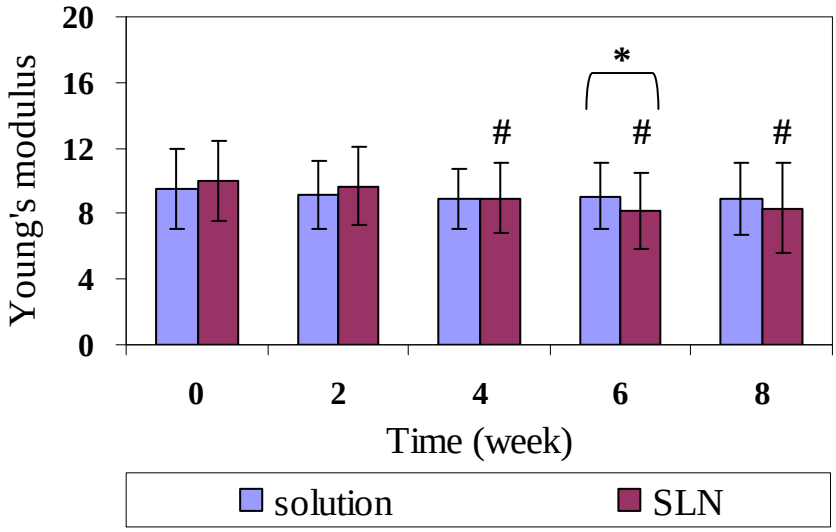


Figure 119 Skin elasticity values (Young's modulus) after applying 0.25% Puag-Haad formulations for different times. Data = mean ± SD (n = 26)

* $P < 0.05$ by paired t-test, compared with solution side at the same week

\# $P < 0.05$ by paired t-test, compared with baseline (week 0) of the same side

In contrast to the previous report of Manatchaya Wanawatanakun (2006), she studied the efficacy of lotion (o/w emulsion) containing Puag-Haad to improve skin elasticity in comparison with lotion base. She found that no significant differences were found in both cases at all weeks measured. Her results indicated that the lotion containing Puag-Haad did not improve the skin elasticity over its lotion base throughout the 8-week study period. However, the sensitivity of the instrument and technique to measure could be the factors contributing to the results. In Manatchaya Wanawatanakun's study, Cutometer® was used to measure skin elasticity. Although the principle was also based on application of vacuum on the skin, Cutometer® needs a well-trained measurement skill as well as more extensive experience to interpret the results. Therefore, in this study, Dermalab® was used instead of Cutometer® to measure skin elasticity. The double sticking rings were used to precisely and reproducibly place the probe onto the subject's skin for each measurement in order to reduce the fluctuation in responses.

The data were also further analyzed by Repeated measures ANOVA after baseline correction. By subtracting the baseline measurement from each observation, the measurements would represent changes from baseline. Since the Young's modulus values generally decreased with application times, the changes from baseline in this case were calculated from the formula: Young's modulus (week 0) – Young's modulus (week 2, 4, 6 or 8) to obtain positive values. Table 82 shows the changes from baseline of Young's modulus values after applying Puag-Haad formulations for 8 weeks.

Table 82 The changes from baseline (week 0) of Young's modulus values over 8 weeks

Formulation	Changes from baseline of Young's modulus values				
	Week 2	Week 4	Week 6	Week 8	Overall
Solution					
Sum	8.32	16.04	11.49	14.28	50.13
Mean	0.32	0.62	0.44	0.55	0.48
(SD)	(1.83)	(2.72)	(1.98)	(2.19)	
SLN					
Sum	8.58	28.42	48.57	43.23	128.80
Mean	0.33	1.09	1.87	1.66	1.24
(SD)	(2.04)	(2.41)	(2.56)	(1.82)	

The final ANOVA Table is shown in Table 83.

Table 83 Repeated measures ANOVA on the Young's modulus values

Source of variance	df	SS	MS	F	P-value
Subjects	50	634.59	12.69		
Weeks	3	22.56	7.52		
Drugs	1	183.68	183.68	14.47	0.0004
Weeks × Drugs	3	15.75	5.25	2.26	0.0837
Error	150	348.29	2.32		
Total	207	1204.87			

The overall average change from baseline for SLN was 1.24 whereas it was 0.48 for solution. The F test for "drugs" difference was 14.47 which was greater than critical $F_{1, 50}$ of 4.03. This difference was significant ($P < 0.05$). The significant result indicated that on the average, the SLN was superior to the solution with regard to lowering the circular roughness values.

The F test for "weeks × drugs" differences was 2.26 which was less than critical $F_{3, 150}$ of 2.66. This non significant result ($P > 0.05$) suggested that the pattern of response was constant for solution and SLN for the entire period of study. The curves of weeks × drugs interaction are shown as in Figure 120.

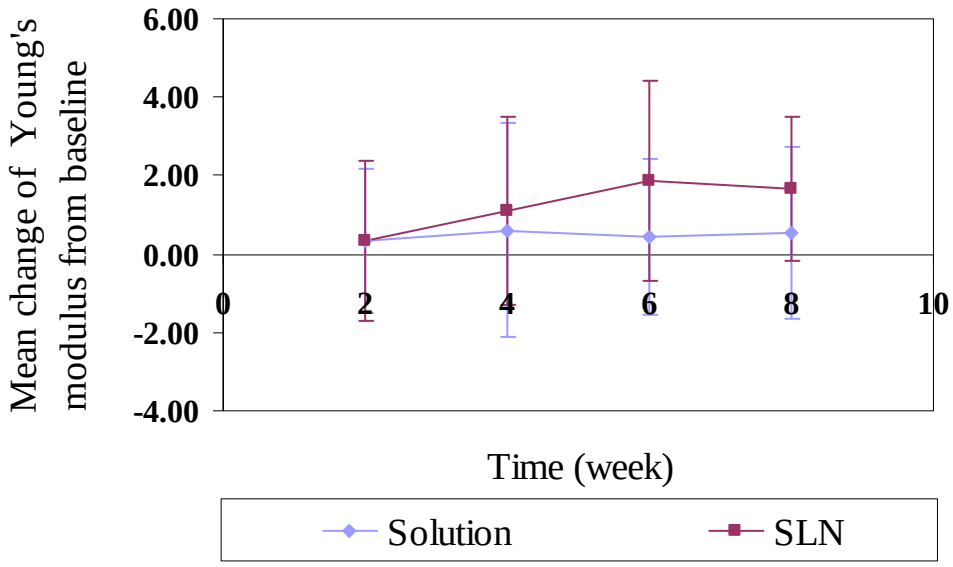


Figure 120 The changes from baseline of Young's modulus values over 8 weeks

From this result above, it was found that the SLN was effective in improving skin elasticity better than the solution. According to Souto and Müller (2008), the aging dermis loses much of its elasticity. The skin is stretched by muscular movement and fails to shrink back to its normal smoothness resulting in wrinkle formation. It is thus possible that the skin hydration properties of lipid in SLN together with the antioxidant activities of Puag-Haad may have a synergistic effect on the skin wrinkle improvement. However, more studies are needed to confirm this hypothesis.

4. Skin hydration

Table 84 shows the skin hydration values in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks.

Table 84 The skin hydration values in woman volunteers after applying 0.25% Puag-Haad formulations for 8 weeks (n = 26 subjects)

Formulation	Skin hydration values				
	Week 0	Week 2	Week 4	Week 6	Week 8
Solution (SD)	23.34 (12.45)	22.41 (12.68)	26.88 (6.95)	25.47 (7.94)	23.76 (6.95)
SLN (SD)	23.38 (12.63)	28.72# (8.34)	40.14# (12.44)	34.05# (8.94)	32.08# (7.21)
P-value At the same week	0.984	0.020*	0.000*	0.000*	0.000*

* P < 0.05 by paired t-test, compared with solution side at the same week

\# P < 0.05 by paired t-test, compared with baseline (week 0) of the same side

The skin hydration values are graphically represented for each formulation in Figure 121.

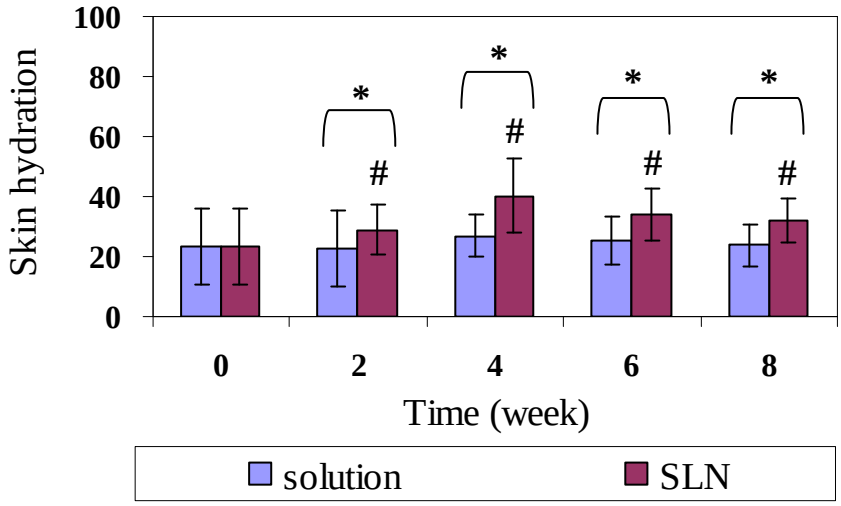


Figure 121 Skin hydration values after applying 0.25% Puag-Haad formulations for different times. Data = mean ± SD (n = 26)

* P < 0.05 by paired t-test, compared with solution side at the same week

\# P < 0.05 by paired t-test, compared with baseline (week 0) of the same side

From Table 84 and Figure 121, it was found that there was no significant difference in the skin hydration values of the solution side from baseline measurement during the entire application period ($P > 0.05$). In contrast, the SLN formulation was able to significantly increase the skin hydration values. There was a significant difference in skin hydration values from baseline ($P < 0.05$) after applying SLN formulation for only 2 weeks and throughout the entire study. Furthermore, the significant difference in skin hydration values between the two formulations was observed at every week after application ($P < 0.05$). It was apparent that the SLN was more effective than the solution. One reason for this was the pronounced occlusive effect of nano-size range lipid particles. The SLN was believed to adhere to the skin surface, giving rise to the tendency to fuse and form a dense film after application. Water evaporation through this film was decreased due to increasing skin occlusion and hydration (Müller, Radtke, and Wissing, 2002).

The data were also further analyzed by Repeated measures ANOVA after baseline correction. By subtracting the baseline measurement from each observation, the measurements would represent changes from baseline. Since the skin hydration values generally increased with application times, the changes from baseline in this case were calculated from the formula: Skin hydration (week 2, 4, 6 or 8) – Skin hydration (week 0) to obtain positive values. Table 85 shows the changes from baseline of Skin hydration values after applying Puag-Haad formulations for 8 weeks.

Table 85 The changes from baseline (week 0) of skin hydration values over 8 weeks

Formulation	Changes from baseline of skin hydration values				
	Week 2	Week 4	Week 6	Week 8	Overall
Solution					
Sum	-24.00	92.00	55.50	11.00	134.50
Mean	-0.92	3.54	2.13	0.42	1.29
(SD)	(15.94)	(10.31)	(11.87)	(11.42)	
SLN					
Sum	139.00	436.00	277.50	226.25	1078.75
Mean	5.35	16.77	10.67	8.70	10.37
(SD)	(12.53)	(14.82)	(11.01)	(10.49)	

The final ANOVA Table is shown in Table 86.

Table 86 Repeated measures ANOVA on the skin hydration values

Source of variance	df	SS	MS	F	P-value
Subjects	50	23195.48	463.91		
Weeks	3	1753.69	584.56		
Drugs	1	11363.38	11363.38	24.49	0.0000
Weeks × Drugs	3	338.84	112.95	2.18	0.0932
Error	150	7785.30	51.90		
Total	207	44436.69			

The overall average change from baseline for SLN was 10.37 whereas it was 1.29 for solution. The F test for “drugs” difference was 24.49 which was greater than critical $F_{1, 50}$ of 4.03. This difference was significant ($P < 0.05$). The significant result indicated that on the average, the SLN was superior to the solution with regard to increase the skin hydration values.

The F test for “weeks × drugs” differences was 2.18 which was less than critical $F_{3,\ 150}$ of 2.66. This non significant result ($P > 0.05$) suggested that the pattern of response was constant for solution and SLN for the entire period of study. The curves of weeks × drugs interaction are shown as in Figure 122.

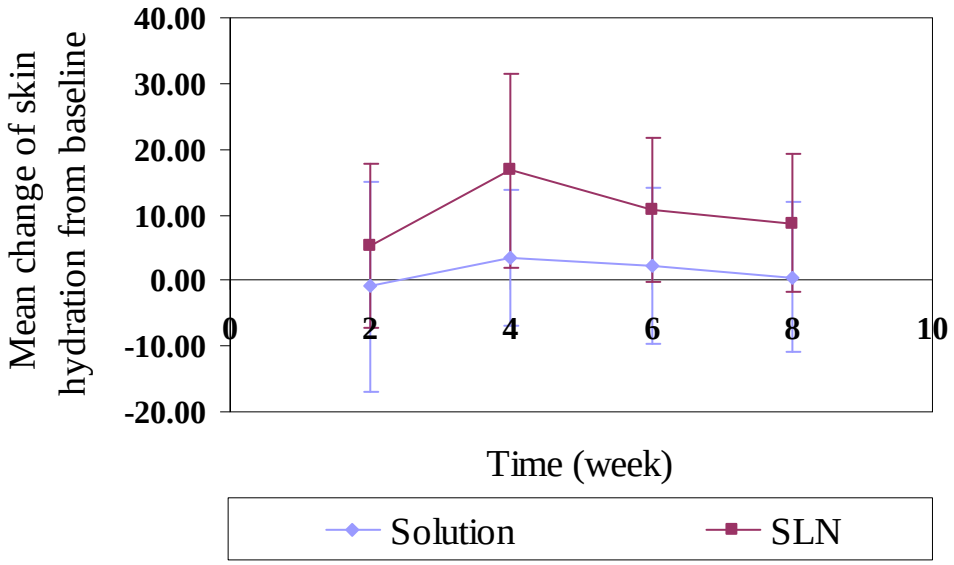


Figure 122 The changes from baseline of skin hydration values over 8 weeks

In conclusion, the results obtained from this part indicated that the SLN containing Puag-Haad gave better anti-wrinkle and skin elasticity improvement than the micellar solution. Furthermore, the skin hydration was obviously improved by the SLN formulation whereas the solution could not. On the other hand, the solution showed slightly greater whitening effect than the SLN formulation.

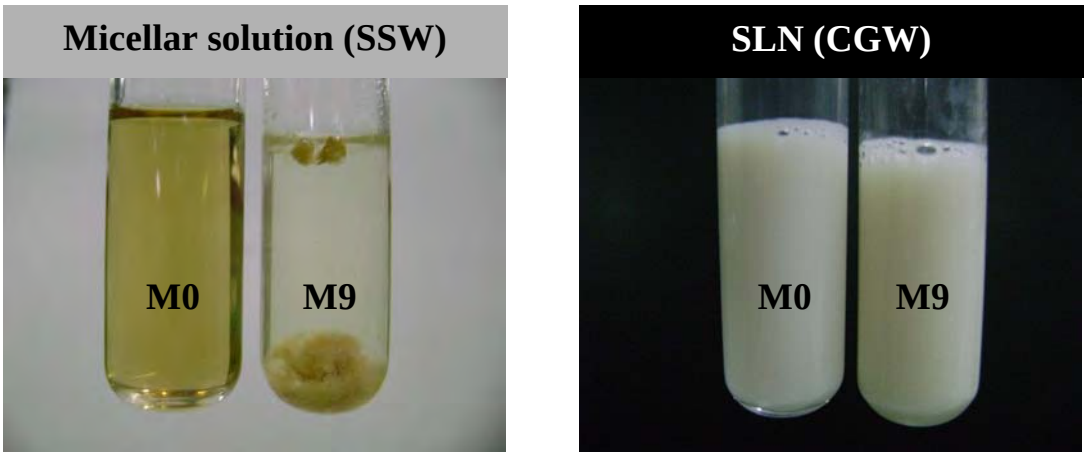
All the subjects completed the study without developing any unwanted side effects or withdrawal. Both formulations appeared to be well tolerated and accepted by the volunteers. At the end of the study, all subjects (n = 26) were also asked to fill the questionnaire to evaluate their satisfaction on the products. It was found that the majority of subjects (73.08%) were satisfied with the SLN. 15.38% of the subjects were satisfied with the micellar solution and 11.54% of the subjects were satisfied with both formulations.

Table 87 Level of product satisfaction evaluated by the subjects (% of total subjects, n = 26)

Formulation	Skin feel	Level of satisfaction		
		Most	Moderate	Least
Solution	Whitening	57.69	38.46	3.85
	Roughness	38.46	53.85	7.69
	Elasticity	23.08	61.54	15.38
	Hydration	15.38	50.00	34.62
SLN	Whitening	61.54	34.61	3.85
	Roughness	57.69	38.46	3.85
	Elasticity	53.84	42.31	3.85
	Hydration	61.54	34.61	3.85

Table 87 shows the results of the subjects' self-evaluation on their skin feel at the end of the study. The skin feel attributes were skin whitening, skin roughness, skin elasticity and skin hydration. From this Table, more than half (>50%) of the subjects were highly satisfied with the SLN formulations in all skin feel attributes. Only one subject (= 3.85%) graded all attributes as least satisfied. Considering the subjects' satisfaction of solution, it was found that the subjects (≥50%) were moderate satisfied with skin roughness, skin elasticity and skin hydration. However, 57.69% of the subjects were highly satisfied with skin whitening.

Furthermore, the SLN formulations remained an acceptable physical appearance even kept at 30±2°C for 9 months. On the other hand, the precipitation occurred as seen in the micellar solution. From Figure 123, it indicated that the SLN showed better physical stability than micellar solution. The good physical appearance of formulation was the advantage that could improve product appearance and reliability as well as its efficacy.



(a) (b)

Figure 123 Physical stability of micellar solution of Puag-Haad, SSW (a) and SLN containing Puag-Haad by cold homogenization method, CGW (b) using water as vehicle at 30±2°C (M0 = month 0, M9 = month 9)

Therefore, the SLN containing Puag-Haad had a very promising potential for further development into a novel and effective skin whitening and anti-wrinkle agent delivery system for cosmetic applications.

CHAPTER V

CONCLUSIONS

The result obtained in this work can be summarized as follows:

Part A. Development and validation of UV-Visible Spectrophotometric and HPLC method for determination of Puag-Haad and oxyresveratrol content

1. UV-Visible Spectrophotometric method for determination of Puag-Haad content
   - The amount of Puag-Haad was determined from its maximum absorption at 327 nm. Methanol was used as solvent in this system.
   - The method showed good specificity, linearity, accuracy and precision and subsequently used for the assay of Puag-Haad content in SLN containing Puag-Haad during the initial development phase.

2. HPLC method for determination of oxyresveratrol content
   - The column was Luna Phenomenex® C18 (2) (5 μm, 250 x 4.6 nm) and the mobile phase was methanol : water (40 : 60) with the flow rate of 1 ml/min. The UV detector was set at 329 nm. The retention times for oxyresveratrol, resveratrol (its related substance), and the internal standard (furazolidone) were 12.49, 21.63 and 5.88 min, respectively.
   - The method showed good specificity, linearity, accuracy and precision and subsequently used for the assay of oxyresveratrol content in SLN containing Puag-Haad during the step of characterization of SLN containing Puag-Haad as well as its stability evaluation.

Part B. Development of SLN containing Puag-Haad

1. Preparation of blank SLN

Preparation of blank SLN by w/o/w double emulsion method

- The best ratio of mixed surfactant (S-40 : F-68) that generated the largest region of w/o microemulsion was 7 : 3, 10 : 0 and 10 : 0 for lipid GMS, compritol® and gelucire®, respectively.
- Blank SLN from GMS and compritol® were successfully prepared by this method when using either S-40 or F-68 as external surfactant. On the other hand, No SLN was formed when using gelucire® as lipid and S-40 as external surfactant and had undesirable appearances when using F-68 as external surfactant. For GMS and compritol®, the lipid concentration at 50% w/w (in w/o microemulsion) showed a good appearance of SLN when freshly prepared, had an appropriate particle size (and size distribution) and showed a good physical stability after stored at ambient temperature for 4 weeks.
- For compritol®, SLN formulations gave larger particle size than GMS. F-68 in external surfactant could generate smaller particle size than S-40. In contrast, when using GMS as lipid, S-40 was more appropriate than F-68 in generating smaller SLN.
- The percentage of lipid and surfactant in the formulations could influence their mean particle sizes. The mean particle size increased when increasing the percentage of lipid. On the contrary, increasing the percentage of surfactant resulted in the decrease in mean particle size.
- Two blank SLN formulations by this method that were selected to load Puag-Haad in the next step were using GMS at 50% w/w (in w/o microemulsion) as lipid with S-40 as external surfactant and compritol® at the same concentration as GMS, with F-68 as external surfactant.

Preparation of blank SLN by cold homogenization method

- Blank SLN from GMS were successfully prepared by this method when using either S-40 or F-68 as external surfactant. On the other hand, no SLN formulations from compritol® could be prepared due to limitations of the

instrument and the process and the SLN from gelucire® showed undesirable appearances when using either S-40 or F-68 as external surfactant.

- The mean particle size by this method was larger with broader size distribution than w/o/w double emulsion method.
- For GMS, S-40 generated smaller particle size and better physical stability after storage at ambient temperature for 4 weeks than F-68 as external surfactant.
- Similar to w/o/w double emulsion method, the percentage of surfactant in the formulations could influence their mean particle sizes. The increase in surfactant concentration resulted in smaller particle size.
- Only one blank SLN formulation by this method that was selected to load Puag-Haad in the next step was using GMS as lipid and S-40 as external surfactant.

2. Preparation of SLN containing Puag-Haad

Preparation of SLN containing Puag-Haad by w/o/w double emulsion method

- The percentage of drug loading and surfactant concentration showed significant influence on particle size of SLN containing Puag-Haad. The incorporation of Puag-Haad in SLN showed the increase in the particle size. Furthermore, the particle size and PI decreased with the increase in the external surfactant concentration. These results were observed when using both lipids (GMS and compritol®).
- The percentage of drug loading and surfactant concentration showed significant influence on entrapment efficiency of SLN containing Puag-Haad especially for GMS. The concentration of incorporated drug increased, the entrapment efficiency decreased. Furthermore, the increase in the surfactant concentration increased in the entrapment efficiency. For compritol®, the decrease in entrapment efficiency was due to the drug loading whereas the surfactant concentration did not seem to influence entrapment efficiency.
- The same trend of Puag-Haad loading and surfactant concentration on particle size and entrapment efficiency was observed when using citrate buffer pH 5.5 as vehicle. SLN containing Puag-Haad in citrate buffer pH 5.5 gave larger

particle size than water system and the SLN formulations when using compritol® as lipid showed larger particle size than GMS. Thus, GMS was selected as a model lipid for the next studies. Higher percentage of surfactant (3% S-40) was also selected for the next experiment.

Preparation of SLN containing Puag-Haad by cold homogenization method

- 1% S-40 was not sufficient to stabilize the SLN containing Puag-Haad to have acceptable physical appearances. Increasing the concentration of S-40 to 3% could form stable SLN containing Puag-Haad with good physical appearance.
- The concentration of drug loading did not appear to influence the particle size and entrapment efficiency as previously observed with the w/o/w double emulsion method. However, the effect of surfactant concentration and vehicle type showed the same trend as the w/o/w double emulsion method. Therefore, GMS and 3% S-40 were also selected as model lipid and surfactant for the cold homogenization method.

Part C. Characterization of SLN containing Puag-Haad

1. Four SLN formulations containing Puag-Haad (MGW, MGB, CGW and CGB) were prepared from GMS and S-40 using both methods and both types of vehicles.
2. The morphological features were investigated using TEM. The nanoparticles exhibited spherical shape and were homogenous in size distribution. The particle sizes were approximately around 200 nm for MGW and MGB and 300 nm for CGW and CGB which correlated with the particle size measurement by PCS.
3. The zeta potential of SLN containing Puag-Haad in water system (MGW and CGW) was negative charge. In contrast, in citrate buffer pH 5.5 system they showed positive charge.
4. All SLN containing Puag-Haad (MGW, MGB, CGW and CGB) showed high entrapment efficiency about 68 – 86% depending on the formulation, with the high percentage of recovery of more than 85%.
5. All SLN containing Puag-Haad (MGW, MGB, CGW and CGB) as well as their blank formulations had reduction in enthalpy and melting point when compared with

bulk lipid. The formation of SLN correlated with less ordered crystal lattice which required less energy than perfect crystalline material.

6. The release profiles of all SLN formulations (MGW, MGB, CGW and CGB) showed sustained release of oxyresveratrol without any burst at all and well fitted the Higuchi equation with $R^2 > 0.98$ which described the diffusion of drug from dispersed, matrix systems.

Part D. Physical and chemical stability study of SLN containing Puag-Haad formulations in comparison with aqueous solutions of Puag-Haad

1. All SLN containing Puag-Haad (MGW, MGB, CGW and CGB) could improve physical and chemical stability of Puag-Haad better than the aqueous solutions of Puag-Haad (both in 20% propylene glycol and 3% S-40) after storage at 30±2°C for 4 months.

2. The presence of S-40 and citrate buffer pH 5.5 had synergistic effect in stabilizing Puag-Haad both physically and chemically.

3. During the storage, the oxyresveratrol was expelled from the increasingly more ordered lipid matrix into the external aqueous phase resulting in the reduction of the entrapment efficiency which agreed with the DSC results that showed the increase in melting point and enthalpy endotherm.

4. The storage temperature of SLN containing Puag-Haad could influence both physical and chemical stability of SLN. Keeping the SLN in the refrigerator (4 – 8°C) resulted in marked improvement in both physical and chemical stability.

Part E. Evaluation of whitening and anti-wrinkle efficacy of SLN containing Puag-Haad in human volunteers

1. The initial values of melanin index, skin roughness, skin elasticity and skin hydration parameters measured at week 0 were similar among two sides of the application area (left and right forearm) when analyzed by paired t-test, indicating the homogenity and balanced distribution of the subjects for the study.

2. When using paired t-test to compare the effect at different times with the initial values, the SLN formulations gave significant improvement in skin whitening, skin roughness, skin hydration and skin elasticity at week 2, 2, 2 and 4, respectively. On the other hand, the micellar solution of Puag-Haad (positive control) showed significant improvement in skin whitening and skin roughness after week 2 and week 4, respectively, whereas it showed no improvement on skin elasticity and skin hydration.

3. When using repeated measures ANOVA, the SLN containing Puag-Haad (0.25%), gave better overall anti-wrinkle, skin elasticity and skin hydration efficacies than the micellar solution of Puag-Haad. On the other hand, the micellar solution showed slightly greater whitening effect than the SLN formulation.

4. All the subjects completed the study without developing any unwanted side effects or withdrawal. Both formulations appeared to be well tolerated and accepted by the volunteers.

5. The majority of subjects (73.08%) were satisfied with the SLN whereas 15.38% and 11.54% of the subjects were satisfied with the micellar solution and both formulations, respectively.

6. More than half of the subjects were highly satisfied with the SLN in all skin feel (skin whitening, skin roughness, skin elasticity and skin hydration) whereas half of the subjects were moderate satisfied with the micellar solution in skin roughness, skin elasticity and skin hydration. However, 57.69% of the subjects were highly satisfied with solution in skin whitening.

In conclusion, the results in this present work have demonstrated that the SLN containing Puag-Haad could improve physical and chemical stability of Puag-Haad and showed the whitening and anti-wrinkle efficacies in woman volunteers. Furthermore, the skin hydration was obviously improved. Its overall effect on the skin was superior to Puag-Haad micellar solution. Therefore, the SLN containing Puag-Haad has a very promising potential for use as a multi-functional skin-whitening and anti-wrinkle formulation in cosmetic applications.

In the future experiment, SLN containing Puag-Haad will develop using different lipids and surfactants as well as the preparation techniques in order to improve their physicochemical stability of SLN formulations.

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APPENDICES

APPENDIX A

Titration results to form microemulsion boundary line within each phase diagram

Table 88 The titration results to form microemulsion boundary line within the phase diagram of GMS, mixed surfactants (S-40 : F-68) and water at 70°C

The ratio of mixed surfactants (S-40 : F-68)	The ratio of lipid and mixed surfactants	Lipid	Mixed surfactant	Water	Total
0 : 10	1:9	7.89	70.98	21.14	100
	2:8	15.81	63.24	20.95	100
	3:7	23.64	55.16	21.20	100
	4:6	31.95	47.92	20.13	100
	5:5	41.25	41.25	17.49	100
	6:4	50.46	33.64	15.90	100
	7:3	63.99	27.42	8.59	100
	8:2	72.99	18.25	8.76	100
	9:1	86.46	9.61	3.94	100
3 : 7	1:9	6.76	60.81	32.43	100
	2:8	15.72	62.89	21.38	100
	3:7	24.02	56.04	19.94	100
	4:6	32.84	49.26	17.90	100
	5:5	41.70	41.70	16.60	100
	6:4	52.04	34.69	13.27	100
	7:3	63.64	27.27	9.09	100
	8:2	74.63	18.66	6.72	100
	9:1	85.07	9.45	5.48	100
5 : 5	1:9	6.20	55.80	38.00	100
	2:8	15.12	60.47	24.41	100
	3:7	23.70	55.29	21.01	100
	4:6	32.55	48.82	18.63	100
	5:5	41.74	41.74	16.53	100
	6:4	51.72	34.48	13.79	100
	7:3	63.01	27.00	9.99	100
	8:2	74.77	18.69	6.54	100
	9:1	85.55	9.51	4.94	100

Table 88 (Cont.) The titration results to form microemulsion boundary line within the phase diagram of GMS, mixed surfactants (S-40 : F-68) and water at 70°C

The ratio of mixed surfactants (S-40 : F-68)	The ratio of lipid and mixed surfactants	Lipid	Mixed surfactant	Water	Total
7 : 3	1:9	5.00	44.96	50.05	100
	2:8	13.00	51.98	35.02	100
	3:7	23.38	54.56	22.06	100
	4:6	32.18	48.27	19.55	100
	5:5	40.29	40.29	19.42	100
	6:4	51.33	34.22	14.46	100
	7:3	61.51	26.36	12.13	100
	8:2	73.46	18.37	8.17	100
	9:1	85.80	9.53	4.67	100
10 : 0	1:9	5.88	52.94	41.18	100
	2:8	13.05	52.22	34.73	100
	3:7	24.00	56.00	20.00	100
	4:6	32.79	49.18	18.03	100
	5:5	43.40	43.40	13.19	100
	6:4	53.57	35.71	10.71	100
	7:3	65.18	27.93	6.89	100
	8:2	78.35	19.59	2.06	100
	9:1	88.32	9.81	1.86	100

Table 89 The titration results to form microemulsion boundary line within the phase diagram of Compritol® 888 ATO, mixed surfactants (S-40 : F-68) and water at 85°C

The ratio of mixed surfactants (S-40 : F-68)	The ratio of lipid and mixed surfactants	Lipid	Mixed surfactant	Water	Total
0 : 10	1:9	8.90	80.14	10.95	100
	2:8	18.45	73.80	7.75	100
	3:7	27.37	63.87	8.76	100
	4:6	36.83	55.25	7.92	100
	5:5	45.70	45.70	8.59	100
	6:4	54.79	36.53	8.68	100
	7:3	63.35	27.15	9.50	100
	8:2	70.61	17.65	11.74	100
	9:1	84.43	9.38	6.19	100
3 : 7	1:9	7.46	67.11	25.43	100
	2:8	16.49	65.95	17.56	100
	3:7	25.62	59.78	14.60	100
	4:6	35.40	53.10	11.50	100
	5:5	45.91	45.91	8.17	100
	6:4	53.81	35.87	10.31	100
	7:3	62.67	26.86	10.47	100
	8:2	71.05	17.76	11.19	100
	9:1	84.35	9.37	6.28	100
5 : 5	1:9	6.51	58.63	34.85	100
	2:8	15.00	60.02	24.98	100
	3:7	24.71	57.66	17.63	100
	4:6	33.70	50.55	15.75	100
	5:5	42.81	42.81	14.38	100
	6:4	50.51	33.67	15.82	100
	7:3	57.42	24.61	17.97	100
	8:2	68.38	17.09	14.53	100
	9:1	83.80	9.31	6.89	100

Table 89 (Cont.) The titration results to form microemulsion boundary line within the phase diagram of Compritol® 888 ATO, mixed surfactants (S-40 : F-68) and water at 85°C

The ratio of mixed surfactants (S-40 : F-68)	The ratio of lipid and mixed surfactants	Lipid	Mixed surfactant	Water	Total
7 : 3	1:9	5.87	52.79	41.35	100
	2:8	13.84	55.36	30.80	100
	3:7	24.02	56.04	19.94	100
	4:6	33.67	50.51	15.82	100
	5:5	42.66	42.66	14.68	100
	6:4	50.46	33.64	15.90	100
	7:3	53.89	23.09	23.02	100
	8:2	66.28	16.57	17.15	100
	9:1	79.09	8.79	12.13	100
10 : 0	1:9	3.87	34.84	61.29	100
	2:8	12.45	49.81	37.73	100
	3:7	23.44	54.69	21.88	100
	4:6	32.60	48.90	18.50	100
	5:5	40.49	40.49	19.03	100
	6:4	49.30	32.87	17.83	100
	7:3	53.52	22.94	23.55	100
	8:2	61.02	15.26	23.72	100
	9:1	74.94	8.33	16.74	100

Table 90 The titration results to form microemulsion boundary line within the phase diagram of Gelucire® 50/13, mixed surfactants (S-40 : F-68) and water at 60°C

The ratio of mixed surfactants (S-40 : F-68)	The ratio of lipid and mixed surfactants	Lipid	Mixed surfactant	Water	Total
0 : 10	1:9	8.29	74.63	17.08	100
	2:8	16.64	66.56	16.81	100
	3:7	23.15	54.01	22.84	100
	4:6	32.10	48.15	19.74	100
	5:5	36.44	36.44	27.11	100
	6:4	44.28	29.52	26.20	100
	7:3	49.93	21.40	28.67	100
	8:2	60.47	15.12	24.41	100
	9:1	71.71	7.97	20.32	100
3 : 7	1:9	7.58	68.18	24.24	100
	2:8	14.16	56.66	29.18	100
	3:7	21.38	49.89	28.72	100
	4:6	26.16	39.24	34.60	100
	5:5	32.64	32.64	34.73	100
	6:4	39.95	26.63	33.42	100
	7:3	45.37	19.44	35.19	100
	8:2	57.35	14.34	28.32	100
	9:1	70.81	7.87	21.32	100
5 : 5	1:9	5.58	50.22	44.20	100
	2:8	11.75	47.00	41.25	100
	3:7	18.15	42.35	39.50	100
	4:6	25.86	38.78	35.36	100
	5:5	30.30	30.30	39.39	100
	6:4	38.51	25.67	35.82	100
	7:3	45.69	19.58	34.73	100
	8:2	54.57	13.64	31.79	100
	9:1	63.42	7.05	29.53	100

Table 90 (Cont.) The titration results to form microemulsion boundary line within the phase diagram of Gelucire® 50/13, mixed surfactants (S-40 : F-68) and water at 60°C

The ratio of mixed surfactants (S-40 : F-68)	The ratio of lipid and mixed surfactants	Lipid	Mixed surfactant	Water	Total
7 : 3	1:9	5.07	45.62	49.32	100
	2:8	11.40	45.61	42.99	100
	3:7	17.55	40.96	41.49	100
	4:6	22.90	34.34	42.76	100
	5:5	28.59	28.59	42.82	100
	6:4	35.93	23.95	40.12	100
	7:3	43.42	18.61	37.97	100
	8:2	52.88	13.22	33.91	100
	9:1	61.60	6.84	31.55	100
10 : 0	1:9	4.80	43.23	51.97	100
	2:8	10.76	43.03	46.21	100
	3:7	16.49	38.48	45.02	100
	4:6	22.36	33.54	44.10	100
	5:5	28.36	28.36	43.28	100
	6:4	35.27	23.52	41.21	100
	7:3	42.66	18.28	39.06	100
	8:2	50.92	12.73	36.35	100
	9:1	65.65	7.29	27.06	100

APPENDIX B

The protocol from Ethics Committee

Protocol Review No. 08-33-จธน. 013

Study Protocol Approval

The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol dated and/ or amended as follows:

Study Title: **Development of solid lipid nanoparticles containing *Artocarpus lakoocha* heartwood extract (Puag-Haad) for whitening and anti-wrinkle effects**

Study Code: -

Centre: CHULALONGKORN UNIVERSITY

Principal Investigator : Miss Suratchawadee Amorndechawat

Protocol Date : **February 27, 2008**

A list of the Ethics Committee members and positions present at the Ethics Committee meeting on the date of approval of this study has been attached.

This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Chairman of Ethics Committee: .....

(Rungpetch Sakulbumrungsil, Ph.D.)

Secretary of Ethics Committee: Suyanee Pongthananikorn .....

(Suyanee Pongthananikorn, Ph.D.)

Date of Approval: **May 20, 2008**

APPENDIX C

Questionnaire

แบบสอบถามซักประวัติอาสาสมัครก่อนเริ่มการทดลอง

1. ชื่อ นาง / นางสาว…….....นามสกุล……ชื่อเล่น……...
   อายุ……..….ปี อาชีพ……..
2. ที่อยู่……...……
3. เบอร์โทรศัพท์ที่ติดต่อได้สะดวก……...
4. ชนิดของผิว ☐ แห้ง ☐ ธรรมดา ☐ ผสม ☐ มัน
5. ประวัติการแพ้เครื่องสำอาง ☐ ไม่มี ☐ มี ระบุ…….....
   อาการที่เกิดจากการแพ้……...
6. ผลิตภัณฑ์ที่ใช้ทำความสะอาดผิวกาย……
7. ปกติท่านใช้ผลิตภัณฑ์บำรุงผิวกาย / ผลิตภัณฑ์กันแดด หรือไม่
   ☐ ไม่ใช้ ☐ ใช้ ระบุ……
8. ขณะนี้ท่านกำลังรับประทานยา / อาหารเสริม หรือไม่
   ☐ ไม่ใช่ ☐ ใช่ ระบุ……
9. ท่านสามารถเดินทางมาวัดสภาพผิวทุก 2 สัปดาห์ติดต่อกันตลอดการทดลองในวัน / เวลาใด
   ……...

แบบสอบถามความพึงพอใจของอาสาสมัครหลังสิ้นสุดการทดลอง

1. ชื่อ นาง / นางสาว…….……นามสกุล……..……...…อายุ…….ปี
2. ชนิดของผิว ☐ แห้ง ☐ ธรรมดา ☐ ผสม ☐ มัน
3. ประวัติการแพ้เครื่องสำอาง ☐ ไม่มี ☐ มี ระบุ…….....
   อาการที่เกิดจากการแพ้……..
4. อาการแพ้ / ระคายเคือง ภายหลังได้รับผลิตภัณฑ์
   สารขุ่นขาว ☐ ไม่มี ☐ มี ระบุ……
   สารละลายใส ☐ ไม่มี ☐ มี ระบุ……
5. ความพึงพอใจภายหลังได้รับผลิตภัณฑ์

	สารขุ่นขาว			สารละลายใส		
การเพิ่มความชุ่มชื้น	☐ มาก	☐ ปานกลาง	☐ น้อย	☐ มาก	☐ ปานกลาง	☐ น้อย
ความเข้มสีผิวลดลง	☐ มาก	☐ ปานกลาง	☐ น้อย	☐ มาก	☐ ปานกลาง	☐ น้อย
ความเด่งตึงเพิ่มขึ้น	☐ มาก	☐ ปานกลาง	☐ น้อย	☐ มาก	☐ ปานกลาง	☐ น้อย
ริ้วรอยลดลง	☐ มาก	☐ ปานกลาง	☐ น้อย	☐ มาก	☐ ปานกลาง	☐ น้อย
ความมัน / เหนอะหนะ	☐ มาก	☐ ปานกลาง	☐ น้อย	☐ มาก	☐ ปานกลาง	☐ น้อย

6. ท่านชอบผลิตภัณฑ์ชนิดไหนมากกว่ากัน
   ☐ สารขุ่นขาว เพราะ……..……
   ☐ สารละลายใส เพราะ……..……

7. ข้อเสนอแนะอื่น ๆ (แนะนำ ติชมได้ทุกเรื่อง)
   ……...…
   ……...….

VITA

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