EFFECTS OF LIPIDS AND SURFACTANTS ON THE CHARACTERISTICS OF ASIATIC ACID-LOADED SOLID LIPID MICROPARTICLES

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ผลของไขมันและสารลดแรงตึงผิวต่อคุณลักษณะของอนุภาคไมโครชนิดไขมันแข็งที่บรรจุกรดเอเชียติก

น.ส.จตุพร วิตารัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	EFFECTS OF LIPIDS AND SURFACTANTS ON THE CHARACTERISTICS OF ASIATIC ACID-LOADED SOLID LIPID MICROPARTICLES
By	Miss Jatuporn Witarat
Field of Study	Cosmetic Science
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จตุพร วิตารัตน์ : ผลของไขมันและสารลดแรงตึงผิวต่อคุณลักษณะของอนุภาคไมโครชนิดไขมันแข็งที่บรรจุกรดเอเชียติก. (EFFECTS OF LIPIDS AND SURFACTANTS ON THE CHARACTERISTICS OF ASIATIC ACID-LOADED SOLID LIPID MICROPARTICLES) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร. รมย์ฉัตร ชูโตประพัฒน์

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาอิทธิพลของไขมันแข็ง 2 ชนิด ได้แก่ บีแวกซ์, เซทิลแอลกอฮอล์ และสารลดแรงตึงผิว 3 ชนิด ได้แก่ ทวีน 80, ซอยบีนเลซิติน และพอลอกซาเมอร์ 188 ต่อคุณลักษณะของอนุภาคไมโครชนิดไขมันแข็งหรือโซลิดลิปิดไมโครพาร์ทิเคิลที่บรรจุกรดเอเชียติก โดยสูตรตำรับโซลิดลิปิดไมโครพาร์ทิเคิลที่บรรจุกรดเอเชียติก (AASLM) ถูกเตรียมโดยวิธีการเตรียมอิมัลชันที่อุณหภูมิสูงด้วยเครื่องผสมแรงเฉือนสูง หลังได้สูตรตำรับในลักษณะอิมัลชันแล้ว จึงนำไปผ่านกระบวนการทำแห้งเยือกแข็งแบบสุญญากาศหรือฟรีซดรายเพื่อเตรียมในรูปแบบผงแห้ง สูตรตำรับทั้ง 12 สูตร (F1-F12) ที่เตรียมขึ้นถูกประเมินคุณสมบัติทางกายภาพและเคมี ได้แก่ ลักษณะทางกายภาพ, ขนาดและการกระจายตัวของอนุภาค, สัณฐานวิทยา , ความเป็นผลึกของอนุภาค, ร้อยละของปริมาณสารสำคัญที่วัดได้ และประสิทธิภาพในการกักเก็บสารสำคัญ จากการตรวจสอบลักษณะทางกายภาพของสูตรตำรับหลังฟรีซดราย พบว่ามี 10 สูตรตำรับที่มีลักษณะทางกายภาพเป็นผงแห้ง แต่สูตร F2 (10% บีแวกซ์ : ซอยบีนเลซิติน) และ F8 (15% บีแวกซ์ : ซอยบีนเลซิติน) ไม่สามารถเตรียมให้มีลักษณะเป็นผงแห้งภายหลังจากฟรีซดรายได้ สูตรตำรับที่ใช้บีแวกซ์ให้ลักษณะเป็นผงหยาบสีขาวเหลืองอ่อน ในขณะที่เตรียมด้วยเซทิลแอลกอฮอล์ทำให้ได้ลักษณะผงที่มีสีขาวและมีสัมผัสที่ละเอียด โดยอนุภาคไมโครชนิดไขมันแข็งที่บรรจุกรดเอเชียติกหลังฟรีซดรายมีขนาดอนุภาคเฉลี่ยอยู่ในช่วง 7.46 ± 0.08 ถึง 38.86 ± 0.34 ไมโครเมตร สัณฐานวิทยาของอิมัลชันก่อนฟรีซดรายและหลังฟรีซดรายตรวจสอบด้วยกล้องจุลทรรศน์แบบใช้แสง พบว่าสูตรตำรับที่ใช้สารลดแรงตึงผิวชนิดพอลอกซาเมอร์ 188 (F3, F6, F9, F12) มีรูปร่างทรงกลม ในขณะที่สูตรตำรับที่ใช้สารลดแรงตึงผิวชนิดทวีน 80 (F1, F4, F5, F7, F10, F11) มีรูปร่างไม่เป็นทรงกลม มีลักษณะเป็นแผ่นเล็กๆ เกาะกลุ่มกัน จากการประเมินปริมาณสารสำคัญกรดเอเชียติกใน AASLM มีค่าเฉลี่ยตั้งแต่ 90.43±0.02% ถึง 95.38±0.02% ซึ่งอยู่ในเกณฑ์ที่ยอมรับได้ ส่วนประสิทธิภาพการเก็บกักสารสำคัญกรดเอเชียติกของอนุภาค AASLM ที่พัฒนาขึ้น มีค่าเฉลี่ยในช่วง 53.75±0.01% ถึง 100.00±0.00% โดยพบว่า สูตรตำรับ F3, F6, F9, F12 ซึ่งเป็น AASLM ที่เตรียมด้วยพอลอกซาเมอร์ 188 สามารถกักเก็บสารสำคัญได้ร้อยละ 100 จึงมีศักยภาพสูงที่จะใช้เป็นระบบนำส่งกรดเอเชียติกเพื่อใช้ทางผิวหนัง อย่างไรก็ตาม ควรมีการศึกษาการนำไปใช้ประโยชน์ของ AASLM ในทางเครื่องสำอางต่อไป

สาขาวิชา	วิทยาศาสตร์เครื่องสำอาง	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Jatuporn Witarat : EFFECTS OF LIPIDS AND SURFACTANTS ON THE CHARACTERISTICS OF ASIATIC ACID-LOADED SOLID LIPID MICROPARTICLES. Advisor: Asst. Prof. Romchat Chutoprapat, Ph.D.

The objective of this study was to evaluate the effects of solid lipids: beeswax and cetyl alcohol, and surfactants: Tween 80®, soybean lecithin and poloxamer 188 on the characteristics of solid lipid microparticles containing asiatic acid (AASLM). The AASLM were prepared from 10% or 15% of beeswax or cetyl alcohol together with 3% of surfactant F1-F12) by melt dispersion cum freeze-drying technique to obtain a solid dosage form. The physicochemical characteristics of the AASLM powders, including appearance, surface morphology, particle size, %labeled amount of asiatic acid (AA) and %entrapment efficiency (EE) were evaluated. The results revealed that AASLM prepared from 10% or 15% beeswax with 3% soybean lecithin (F2 and F8) could not yield a dry powder form, whereas those with Tween 80® (Tw80) or poloxamer 188 (F1, F3, F7 and F9) appeared as white to slightly yellowish coarse powders. AASLM were prepared from 10% or 15% cetyl alcohol with Tw80 or soybean lecithin or poloxamer 188 and appeared as white fine powders (F4, F5, F6, F10, F11, F12). The mean particle sizes of obtaining AASLM powders ranged from 7.46±0.08 to 38.86±0.34 microns. The surface morphology of AASLM powders, was examined by scanning electron microscope, showed a non-spherical shape. The morphology of AASLM dispersions before freeze-drying and after redispersion of the freeze-dried powder were also investigated using the optical microscope. In all the dispersions, the AASLM prepared from beeswax or cetyl alcohol with poloxamer 188 (F3, F6, F9, F12) had spherical shape, whereas those prepared from beeswax with Tw80 or soybean lecithin (F1, F7, F5, F11) and cetyl alcohol with Tw80 (F4, F10) seemed to have irregular shape. The %labeled amount of AASLMs ranged from 90.43±0.02% to 95.38±0.02% which were considered acceptable. The %EE of resultant AASLMs ranged from 53.75±0.01% to 100.00±0.00%. It was found that F3, F6, F9, F12 could entrap Asiatic acid of 100%. Therefore, it could be concluded that AASLM prepared with beeswax or cetyl alcohol with poloxamer 188 has the greatest potential among the test formulations as a topical carrier for AA. Further studies should be conducted to explore its utilization in cosmetics.

Field of Study: Cosmetic Science Student's Signature .....

Academic Year: 2022 Advisor's Signature .....

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Jatuporn Witarat

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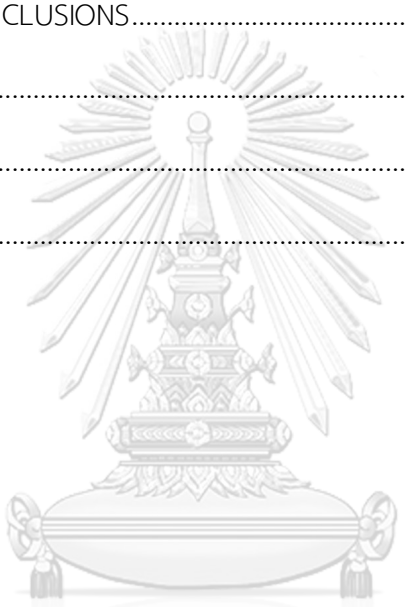

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LIST OF ABBREVIATION

ANOVA	=	analysis of variance
AA	=	asiatic acid
AASLMs	=	asiatic acid loaded solid lipid microparticles
AL	=	active loading
B	=	beeswax
C	=	Cetyl alcohol
° C	=	degree Celsius
conc	=	concentration
DSC	=	differential scanning calorimetry
EE	=	entrapment efficiency
et al.	=	et aliti, 'and others'
g	=	gram
LB	=	labelled amount
mg	=	milligram
min	=	minute
ml	=	milliliter
MW	=	molecular weight
P188	=	poloxamer 188
pH	=	the negative logarithm of the hydrogen ion concentration
R^2	=	coefficient of determination
HPH	=	high pressure homogenizer
HPLC	=	high performance liquid chromatography
hr	=	hour
rpm	=	revolution per minute
SD	=	standard deviation
SL	=	Soybean lecithin
SLM	=	solid lipid microparticle

LIST OF ABBREVIATIONS (CONT.)

Tw80	=	polysorbate 80
µm	=	micrometer
w/v	=	weight by volume
w/w	=	weight by weight
v/v	=	volume by volume
%	=	percentage
<	=	less than
>	=	more than
v/v	=	volume by volume
%	=	percentage
<	=	less than

CHAPTER I

INTRODUCTION

Nowadays, naturally occurring constituents from plants have received much attention as an alternative source of raw materials for dermatological products. Asiatic acid is one of the biological actives containing in the extract of *Centella asiatica* which is a medicinal plant that has been used in oriental medicine for several decades and reported as miracle elixirs of life for more than 2000 years in China (Gohil et al., 2010). Asiatic acid (AA) is an aglycon triterpenoids or pentacyclic triterpenoid found in *Centella asiatica* which is a poorly water-soluble substance. Its water solubility is approximately 10 µg/mL (Borhan et al., 2012; Chen et al., 2020; Lv et al., 2018; Puttarak & Panichayupakaranant, 2012; Rafat et al., 2008). AA has many beneficial effects such as wound healing (Thakor et al., 2017), anti-inflammation, anti-oxidation (Huang et al., 2011; Lv et al., 2017; Yun et al., 2008) and anti-microbial activity (Djoukeng et al., 2005; Liu et al., 2015; Pojanaukij & Kajorncheappunngam, 2010), hence AA is one of the promising active ingredients used in cosmetics and pharmaceutical products.

Solid lipid nanoparticles (SLNs) and solid lipid microparticles (SLMs) are drug delivery systems which demonstrated many benefits comparing to other drug carriers. They can deliver and encapsulate active substance by solid lipid which are safe, biocompatible and biodegradable, and they can control a release of active substance to target site. It was reported that the solid lipid nanoparticles (SLN) loaded with AA can successfully transfer the drug (AA) to nasal route for Alzheimer's prevention and treatment. The obtaining formulas showed good physicochemical characteristics including appearance, pH, particle size, size distribution and drug loading. They were stable with no sign of precipitation under different storage conditions (4 °C, ambient temperature and 45 °C) for 6 months (Khunathum, 2011). Solid lipid microparticles (SLMs) are similar to SLNs in term of a composition. However, the size of SLM (1-1000 µm) is bigger than that of SLN (50-500 nm) (Jaspart et al., 2005; Pardeike et al., 2009; Sznitowska et al., 2017). The incorporation of AA in SLMs has not been reported to date, thus SLMs can be a newly developed carrier systems for the delivery of AA for dermal application. Solid lipid microparticles (SLMs) are lipid-based systems composed of a hydrophobic core made from solid lipid which is stabilized by a surfactant in aqueous medium. SLMs has satisfactory loading capacity of lipophilic compounds and a good affinity for the stratum corneum (Long et al., 2006). They also exhibited

several advantages including controlled release, biocompatibility, biodegradability, low toxicity, avoidance of organic solvents in production, the possibility of large-scale production and protection of active substances from the surrounding environment (Chen et al., 2020; Jaspart et al., 2005). Physiological and biocompatible solid lipids such as fatty acid, glyceride, fatty alcohol and solid wax are usually used in SLMs preparation (Jaspart et al., 2005; Long et al., 2006).

Many research works have shown that the selection of lipids, surfactants, and their composition can affect the physicochemical characteristics (such as the appearance, morphology, particle size, drug loading, % entrapment efficiency) of SLMs (Pietkiewicz & Sznitowska, 2004; Sznitowska et al., 2017; Wolska & Sznitowska, 2013). It has been reported that when using different types of lipids could have different effects on physicochemical characteristics of SLMs. According to Wolska and Sznitowska (2013), Cyclosporin A (Cs) encapsulated in SLMs made of glyceryl behenate (melting point of 69-74 ^{0}C) exhibited a liquid dispersion form and their particles were spherical in shape. For SLMs made of glyceryl palmitostearate, they were in semi-solid (still pourable) form and their particles were in needle shape. SLMs made of glyceryl behenate was more stable (at 4 °C for 18 months) than SLMs composed of glyceryl palmitostearate. In another example, ibuprofen encapsulated in solid lipid microparticles made of glyceryl behenate and cetyl alcohol (melting point of 49.3 °C) showed a spherical shape with average particle size of 4 .67±0.4 2 and 4.24±0.31 μm, respectively, and their entrapment efficiencies were 85.3 and 75.4%, respectively. The result indicated that SLMs made of glyceryl behenate had larger particle size and higher entrapment efficiency than those of SLMs made of cetyl alcohol (Long et al., 2006). Beeswax has a melting point of 62-64 °C which is similar to the melting point of glyceryl behenate. Beeswax consists mainly of esters of long chain fatty acids and alcohols (C24-C36) and small quantities of hydrocarbons, acids and other substances (Bogdanov, 2004; Tulloch, 1980; Tulloch & Hoffman, 1972). It was commonly used in cosmetics because is a safe, low-cost and compatible with skin. SLMs made of beeswax was successfully developed as topical carriers for octyl methoxy cinnamate (OMC). They had a spherical shape with particle size of 100-250 μm and the maximum entrapment efficiencies of OMC of 87.52%. The obtaining SLMs could reduce skin irritation caused by octyl methoxy cinnamate (Yener et al., 2003). In addition, beeswax was used in the formation of solid lipid microparticles loaded with vitamin E by melting-emulsion solidification. The obtaining SLMs exhibited a controlled-release of vitamin E (Souza et al., 2020). In previous research, solid lipid particles made of beeswax was successfully developed as topical

carriers for tacrolimus which had a high drug loading (Dantas et al., 2018), hence beeswax is one of the promising solid lipid used in our study. Besides, cetyl alcohol (melting point of 49.3 °C) is one of the lipids commonly used in cosmetics application due to its properties such as low toxic, low cost, emulsion stabilizer, thickening agent, occlusive emollient. In previous research, SLMs made of cetyl alcohol was successfully developed as topical carriers for Benzophenone-3. They had a spherical shape with particle size of 5-50 μm. They were easy to spread and soft when applied to the skin. The obtaining SLMs could reduce skin irritation caused by benzophenone-3 (Mestres et al., 2010b). Moreover, SLMs made of cetyl alcohol loaded with ibuprofen showed particle size of 125-1000 μm and a sustained-release profile over 10 h (Almeida et al., 2012). Sznitowska et al. (Sznitowska et al., 2017) had prepared the SLMs by using two different types of lipids including glyceryl behenate and glyceryl palmitostearate with three different levels of concentrations (10%, 20%, 30% w/w). The result revealed that the particle size of SLMs made of glyceryl behenate increased when the concentration of lipids increased. Whereas the particle size of SLM made of glyceryl palmitostearate decreased when the concentration of lipid increased. It was found that SLMs with larger particle size exhibited a sustained release over a longer duration (Wolska & Sznitowska, 2013; Yener et al., 2003). Moreover, the concentration of lipid can affect the fluidity of SLMs (Sznitowska et al., 2017; Wolska & Sznitowska, 2013). In 2017, Pietkiewicz and Sznitowska studied the effect of different concentrations (10, 20, 30%w/w) of lipid phase on fluidity of SLMs during a long-term storage. The results indicated that the fluidity of SLMs with lipid concentration of 10% w/w remained the same upon storage. Lipid concentrations of 20 and 30 %w/w increased a viscosity of SLMs formulation significantly (Sznitowska et al., 2017).

There are many different types of surfactants used in SLMs preparation such as nonionic surfactants (e.g., polysorbate (Tween), poloxamer) and amphoteric surfactants (e.g., soya lecithin, egg phosphatidyl choline) (Jaspart et al., 2005). Polysorbate 80 (Tween 80®) is a non-ionic surfactant derived from polyethoxylated sorbitan and oleic acid ($C_{64}H_{124}O_{26}$) with a HLB value of 15. Tween 80® is a viscous yellow liquid (Khunathum, 2011) which is widely used in cosmetics and food products as an emulsifier. Poloxamer 188 (P188) is a nonionic linear copolymer with a HLB value of 29 (Khunathum, 2011). It is a white solid granule and odorless. It is used in a variety of topical formulation owing to its non-toxic and non-irritation. Soybean lecithin is amphoteric surfactants derived from soya. It is a pale yellow waxy solid granule with HLB value of 7. Soybean

lecithin is commonly used in food and cosmetics as an emulsifier and moisturizer. Previous research reported that the type of surfactants also affected the physicochemical characteristics (appearance, morphology, particle size, viscosity) of solid lipid particles (Kheradmandnia et al., 2010; Pietkiewicz & Sznitowska, 2004; Sznitowska et al., 2017; Wolska & Sznitowska, 2013). In 2017, Sznitowska et al. studied the physical characteristic of SLMs composed of lipid (10%w/w) with two different types of surfactants including polysorbate 80 with HLB value of 15 and Tago care 450 (polyglyceryl-3 methylglucose distearate) with HLB value of 12. The results suggested that type of surfactants had an effect on the particle size and viscosity of obtaining SLMs. SLMs containing Tween80 had a smaller particle size (2.77-7 μm) than that of SLMs containing Tago care 450 (38.2-67.6 μm). The obtaining SLMs containing Tween 80® was a liquid dispersion, while obtaining SLMs containing Tago care 450 was semi-solid. A previous study revealed that SLMs containing Tween 80® had a different range of particle size (1-30 μm) compared to that of SLMs containing lecithin (10-30 μm). Moreover, they had a different appearance and polymorphic form. In 2017, Sznitowska et al. studied the physical characteristic of SLMs composed of lipid (10%w/w) and Tween 80® (2 and 3%w/w). The results indicated that the difference of surfactant concentrations used in SLMs preparation had no effect on particle size. However, the particles shape of SLM containing 3%w/w of tween80 had a more spherical than that of SLM containing 2%w/w of tween80 (Sznitowska et al., 2017). According to the work done by Paucar et al. (2016), vitamin D3 loaded in solid lipid microparticles composed of lipid (1%w/w) and soybean lecithin 1%w/w prepared by spray congealing presented the spherical shape and a smooth and continuous surface. Particle size distribution of obtaining SLMs was mainly in the range of 80–100 μm. Besides, SLMs retained 71.8–86.3% of vitamin D3 after 65 days at ambient temperature, compared to the non-immobilized vitamin (60.8%) (Paucar et al., 2016). Chalella Mazzocato, Thomazini, and Favaro-Trindade (2019) reported that vitamin B12 loaded in solid lipid microparticles composed of vegetable fat and soybean lecithin at 2.5 and 5%w/w prepared by spray congealing had a similar spherical shape and smooth surface, and initial mean particle size (15.06±4.38, 14.83±1.72 μm, respectively). Moreover, physicochemical properties including distribution size, morphology and color of both solid lipid microparticles loaded with vitamin B12 had no change during storage for 120 days at 25 °C (Chalella Mazzocato et al., 2019). In 2009, Yadav et al. reported that curcumin loaded in SLMs made of lipid (10%w/w) and poloxamer 188

(0.5%w/w) had the spherical shape, a smooth surface, particle size of 108 ± 0.25 μm and entrapment efficiency of 79.24%. The resulting SLMs exhibited excellent in-vitro release characteristics when compared with pure curcumin (Yadav et al., 2009). In another study, the solid lipid microparticles composed of lipid (5%w/w) and poloxamer 188 at 0.3% and 0.4%w/w had a similar mean particle size of 4 to 5 μm. The resulting particles showed a spherical shape and smooth surface. Moreover, the morphology, size and size distribution of resulting SLMs were not substantially changed after lyophilization and sterilization (Vanna Sanna et al., 2004). According to data from various literatures mentioned above, it suggests that the different types and concentrations of lipids and the different types of surfactants used in SLMs preparation are important factors that influence the physicochemical property of SLMs. Therefore, the aim of this study is to examine the effects of different types (beeswax and cetyl alcohol) and concentrations (10, 15%w/w) of lipids and different types of surfactants (tween80, poloxamer 188, soybean lecithin) on the physicochemical characteristics of asiatic acid loaded in SLMs (AASLMs).

The specific objectives of this investigation were as follows:

To examine the effects of the different types and concentrations of lipids and the different types of surfactants on the physicochemical characteristics of AASLMs.

CHAPTER II

LITERATURE REVIEW

Asiatic acid (AA) is an aglycon triterpenoids or pentacyclic triterpenoid found in *Centella asiatica* which is a poorly water-soluble substance. Its water solubility is approximately 10 µg/mL (Borhan et al., 2012; Chen et al., 2020; Lv et al., 2018; Puttarak & Panichayupakaranant, 2012; Rafat et al., 2008). The molecular formula of AA (2,3,23-trihydroxy-urs-12-ene-28-oic-acid) is $C_{30}H_{48}O_5$ with molecular weight of 488.709 g/mol. Chemical structure of AA is shown in Figure 1. The solubility in phosphate buffer saline (PBS), hydrophilic-lipophilic balance (HLB) value and partition coefficient (log P) value of asiatic acid are 15.0 µg/mL (30.6 µM), 5.5 and 5.7, respectively (Borhan et al., 2012; Mohamed et al., 2018; Rafat et al., 2008).

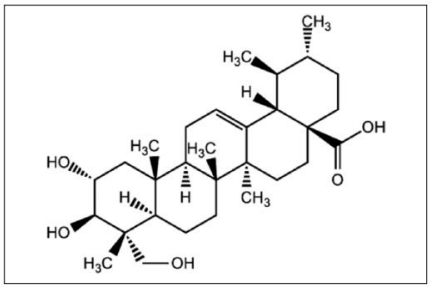


Figure 1 Structural formula of Asiatic acid

AA has many beneficial effects such as wound healing (Thakor et al., 2017), anti-inflammation, anti-oxidation (Huang et al., 2011; Lv et al., 2017; Yun et al., 2008) and anti-microbial activity (Djoukeng et al., 2005; Liu et al., 2015; Pojanaukij & Kajorncheappunngam, 2010). In previous report, asiatic acid at 0.0039 mg/mL could stimulate anti-inflammatory in human skin keratinocyte (HaCaT) by inhibition of IL-1beta, IL-6, TNF-alpha (Yun et al., 2008). Moreover, asiatic acid (0.0192 mg/mL) containing in *Centella asiatica* exhibited inhibitory effects against *Propionibacterium acnes* and *Staphylococcus aureus* (Pojanaukij & Kajorncheappunngam, 2010), hence AA is one of the promising active ingredients used in cosmetics and pharmaceutical products.

Solid lipid Microparticles (SLMs)

Solid lipid microparticles (SLMs) are lipid carriers, generated from a matrix made from bio-compatible lipids (fatty acid, fatty alcohol, glyceride and solid wax) and stabilized by surfactant molecules (Dalpiaz et al., 2008), with particle size in the range of 1 to 1000 um SLMs can be prepared by well-established manufacturing processes. An incorporated drug could be distributed homogenously throughout the lipid matrix , or it could be encapsulated into a surfactant layer (Yang & Alexandridis, 2000).

Biocompatible lipid microparticles have recently been reported as prospective medication delivery systems (V. Sanna et al., 2004). They can be thought of as being physicochemically stable, physiochemically compatible, and allowing a large-scale production at a relatively low production cost. Furthermore, the advantages of Solid Lipid Microparticles (SLMs) for active molecules include possibility of controlled drug release and drug targeting, protection of incorporated labile drug against chemical degradation, and allowing hydrophilic and/or hydrophobic drugs to be incorporated.

SLMs have been already widely used in many topical medication and cosmetic applications, and their use for the topical administration of oily substances has been growing in popularity recently. This is so because numerous substances can be dissolved in these materials. SLMs have incredibly low acute and chronic toxicities. It does not cause irritation to the skin. Applying SLMs topically results in the formation of a monolayered lipid film with smaller interparticle pores, which is linked to higher occlusiveness and, consequently, higher levels of moisture and emolliency in the skin. It has been reported that SLMs could exhibit the UV blocking properties, which also depend on the lipid type and particle size. After applying , they can serve as carriers for the substance and boost the SPF (Souto et al., 2007). Several studies have reported on the application of SLMs with active compounds such as econazole nitrate, octyldimethyl aminobenzoate (Tursilli et al., 2007) and juniper oil (Gavini et al., 2005) for topical administration.

Hot homogenization technique is the foremost technique that has gained widespread acceptance in the preparation of SLMs because it offers numerous advantages compared to the other methods used in SLM preparation. Some of the advantages include avoidance of organic solvents, short production time, and easy scale up. This technique is depicted below.

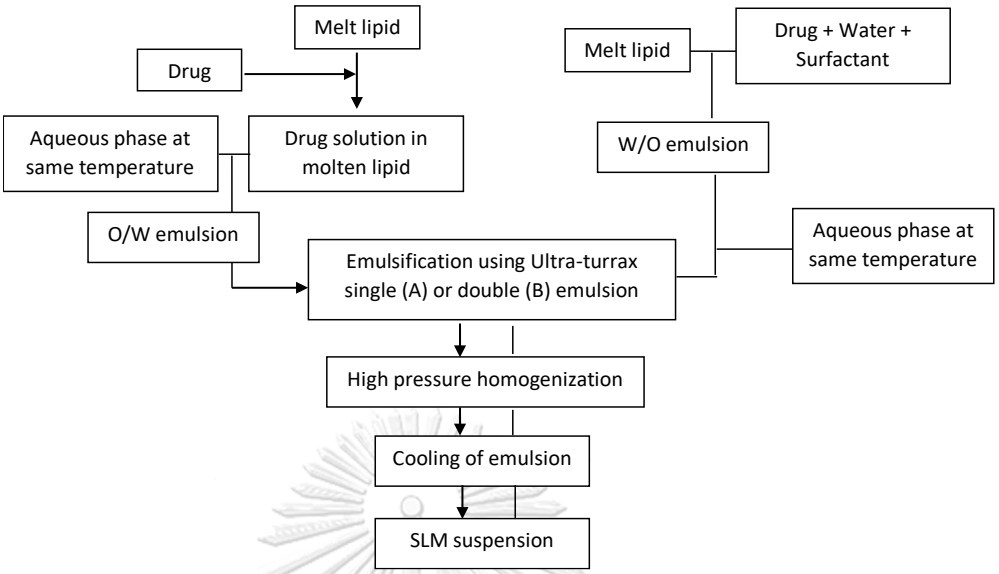


Figure 2 Hot homogenization technique of SLMs preparation

Another popular technique for SLMs preparation is solvent evaporation. This method uses an organic solvent to dissolve lipid and active. It has the advantage of being a non-heat system. Nevertheless, the disadvantage of this technique is large amounts of organic solvents used, which are toxic to the skin if not completely eliminated causing the increased production cost (Jaspart et al., 2005). This technique is depicted below.

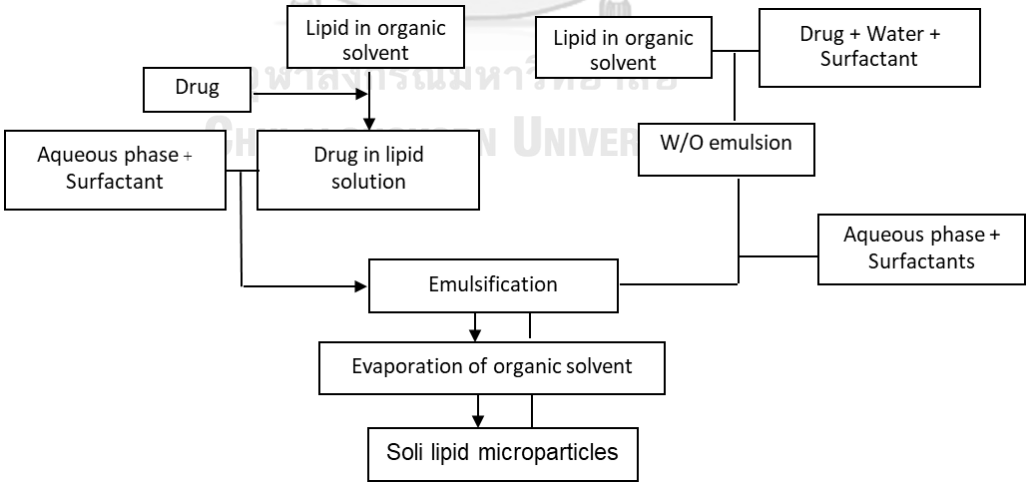


Figure 3 Solvent evaporation method of SLMs preparation

Generally, SLMs are composed of solid lipids such as glyceryl behenate (Compritol 888 ATO), glyceryl tripalmitate, cetyl alcohol, beeswax etc, (Long et al., 2006; Souza et al., 2020;

Sznitowska et al., 2017; Yener et al., 2003). Beeswax consists mainly of esters of long chain fatty acids and alcohols (C24-C36) and small quantities of hydrocarbons, acids and other substances (Bogdanov, 2004; Tulloch, 1980; Tulloch & Hoffman, 1972). The molecular formula of beeswax is $C_{15}H_{31}COOC_{30}H_{61}$. It was commonly used in cosmetics because is safe, low-cost and compatible with skin. SLMs made of beeswax was successfully developed as topical carriers for octyl methoxy cinnamate (OMC). They had a spherical shape with particle size of 100-250 μm and the maximum entrapment efficiencies of OMC of 87.52%. The obtaining SLMs could reduce skin irritation caused by octyl methoxy cinnamate (Yener et al., 2003). In addition, beeswax was used in the formation of solid lipid microparticles loaded with vitamin E by melting-emulsion solidification. The obtaining SLMs exhibited a controlled-release of vitamin E (Souza et al., 2020).

Besides, cetyl alcohol (melting point of 49.3 °C) is one of the lipids commonly used in cosmetics application due to its properties such as low toxic, low cost, emulsion stabilizer, thickening agent, occlusive emollient. In previous research, SLMs made of cetyl alcohol was successfully developed as topical carriers for Benzophenone-3. They had a spherical shape with particle size of 5-50 μm. They were easy to spread and soft when applied to the skin. The obtaining SLMs could reduce skin irritation caused by benzophenone-3 (Mestres et al., 2010b). Moreover, SLMs made of cetyl alcohol loaded with ibuprofen showed particle size of 125-1000 μm and a sustained-release profile over 10 h (Almeida et al., 2012).

Sznitowska et al. (Sznitowska et al., 2017) had prepared the SLMs by using two different types of lipids including glyceryl behenate and glyceryl palmitostearate with three different levels of concentrations (10%, 20%, 30% w/w). The result revealed that the particle size of SLMs made of glyceryl behenate increased when the concentration of lipids increased. Whereas the particle size of SLM made of glyceryl palmitostearate decreased when the concentration of lipid increased. It was found that SLMs with larger particle size exhibited a sustained release over a longer duration (Wolska & Sznitowska, 2013; Yener et al., 2003). Moreover, the concentration of lipid can affect the fluidity of SLMs (Sznitowska et al., 2017; Wolska & Sznitowska, 2013). In 2017, Pietkiewicz and Sznitowska studied the effect of different concentrations (10, 20, 30%w/w) of lipid phase on fluidity of SLMs during a long-term storage. The results indicated that the fluidity of SLMs with lipid concentration of 10% w/w remained the same upon storage. Lipid concentrations of 20 and 30 %w/w increased a viscosity of SLMs formulation significantly (Sznitowska et al., 2017).

Lipid mixtures can produce matrices with high or low crystalline arrangements, each of which has a specific use for drug delivery. An increased crystalline state may result in difficulty in

holding drugs previously incorporated in the molten state. Increasing the drug-holding ability of lipid matrices therefore necessary required some degree of disorder in a crystalline state (Salvi & Pawar, 2019). Binary mixture of lipids and fatty acids that differ by two or more carbon atoms frequently exhibit incongruent melting behavior and partial solid solutions, possibly due to crystal arrangement distortion. Imperfections in the crystal arrangement of a lipid matrix favor drug incorporation because drugs can be found within fatty acids, between fatty acids, within crystals, or within crystal imperfections. When lipids with similar fatty acids are involved, solid solutions form, resulting in an increase in crystallinity and difficulty in holding drugs. While more complex lipids or lipid mixtures containing fatty acids of different chain lengths form less perfect crystals with many imperfections that provide space for drugs to occupy, highly crystalline lipid matrices with a perfect lattice show very sharp and narrow endothermic peaks that cause drug expulsion (Attama et al., 2007).

Polysorbate 80 (Tween 80®) is a non-ionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid. Polysorbate 80 is a viscous, water-soluble yellow liquid. The molecular formula of Tween 80® is $C_{64}H_{124}O_{26}$ and chemical structure of Tween 80®is shown in Figure 4. The hydrophilic-lipophilic balance (HLB) value of Tween 80® is 15. The hydrophilic groups in this compound are polyethers also known as polyoxyethylene groups which are polymers of ethylene oxide. Polysorbate 80 is often used in food and other products as an emulsifier (Khunathum, 2011; *Polysorbate 80*, 2022).

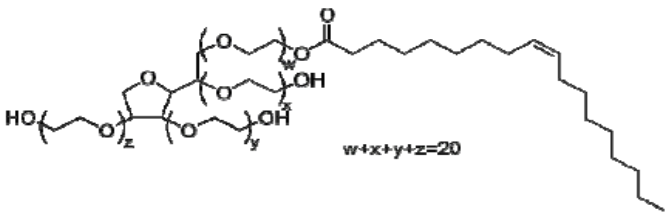


Figure 4 Structural formula of polysorbate 80 (Tween80).

In 2017, Sznitowska et al. studied the physical characteristic of SLMs composed of lipid (10%w/w) with two different types of surfactants including polysorbate 80 (Tween80) with HLB value of 15 and Tago care 450 (polyglyceryl-3 methylglucose distearate) with HLB value of 12. The results suggested that type of surfactants had an affect on the particle size and viscosity of obtaining SLMs. SLMs containing Tween80 had a smaller particle size (2.77-7 μm) than that of

SLMs containing Tago care 450 (38.2-67.6 μm). The obtaining SLMs containing Tween80 was a liquid dispersion, while obtaining SLMs containing Tago care 450 was semi-solid. A previous study revealed that SLMs containing Tw80 had different ranges of particle sizes (1-30 μm) compared to that of SLMs containing lecithin (10-30 μm). Moreover, they had different appearances and polymorphic forms. In 2017, Sznitowska et al. studied the physical characteristic of SLMs composed of lipid (10%w/w) and Tween 80® (2 and 3%w/w). The results indicated that the difference of surfactant concentrations used in SLMs preparation had no effect on particle size. However, the particles shape of SLM containing 3%w/w of tween80 had a more spherical than that of SLM containing 2%w/w of tween80 (Sznitowska et al., 2017).

Soybean lecithin (HLB 7) is amphoteric surfactants derived from soya. It is a pale yellow waxy solid granule with HLB value of 7. Soybean lecithin is commonly used in food and cosmetics as an emulsifier and moisturizer. Previous research reported that the type of surfactants also affected the physicochemical characteristics (appearance, morphology, particle size, viscosity) of solid lipid particles (Kheradmandnia et al., 2010; Pietkiewicz & Sznitowska, 2004; Sznitowska et al., 2017; Wolska & Sznitowska, 2013). According to the work done by Paucar et al. (2016), vitamin D3 loaded in solid lipid microparticles composed of lipid (1%w/w) and soybean lecithin 1%w/w prepared by spray congealing presented the spherical shape and a smooth and continuous surface. Particle size distribution of obtaining SLMs was mainly in the range of 80–100 μm. Besides, SLMs retained 71.8–86.3% of vitamin D3 after 65 days at ambient temperature, compared to the non-immobilized vitamin (60.8%) (Paucar et al., 2016). Chalella Mazzocato, Thomazini, and Favaro-Trindade (2019) reported that vitamin B12 loaded in solid lipid microparticles composed of vegetable fat (TRI CS 48, with melting point around 48 °C) and soybean lecithin at 2.5 and 5%w/w prepared by spray congealing had a similar spherical shape and smooth surface, and initial mean particle size (15.06±4.38, 14.83±1.72 μm, respectively). Moreover, physicochemical properties including distribution size, morphology and color of both solid lipid microparticles loaded with vitamin B12 had no change during storage for 120 days at 25 °C (Chalella Mazzocato et al., 2019).

Poloxamer 188 (P188) is a non-ionic amphiphilic copolymer consisting of a central chain of hydrophobic polyoxypropylene flanked at both ends by hydrophilic polyoxyethylene. The molecular formula of poloxamer 188 is $HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$ (Raymond C. Rowe et al., 2006). The average molecular weight is about 8,500 kD chemical structure of poloxamer 188 is shown in Figure 5 (Emanuele & Balasubramaniam, 2014). It generally occurs as white, waxy, free-flowing 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.

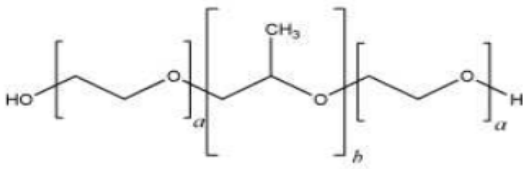


Figure 5 Structural formula of poloxamer 188. With n = 80 and m = 27, P188 has a calculated molecular weight of 8,624 kD

In 2009, Yadav et al. reported that curcumin loaded in SLMs made of lipid (10%w/w) and poloxamer 188 (0.5%w/w) had the spherical shape, a smooth surface, particle size of 108 ± 0.25 μm and entrapment efficiency of 79.24%. The resulting SLMs exhibited excellent in-vitro release characteristics when compared with pure curcumin (Yadav et al., 2009). In another study, the solid lipid microparticles composed of lipid (5%w/w) and poloxamer 188 at 0.3% and 0.4%w/w had a similar mean particle size of 4 to 5 μm. The resulting particles showed a spherical shape and smooth surface. Moreover, the morphology, size and size distribution of resulting SLMs were not substantially changed after lyophilization and sterilization (Vanna Sanna et al., 2004). Moreover, Hariya (2022) studied, SLMs made of 5% glyceryl behenate with 2% poloxamer 188 was successfully developed as carriers for Quercetin which had a spherical morphology, good drug loading, and entrapment efficiency (Hariyadi et al., 2022).

Solid lipid microparticles characterization

Determination of solid lipid microparticle morphology.

The general morphology of SLMs is typically examined using microscopy (optical or scanning electron microscopy, (Emami et al., 2019; V. Sanna et al., 2004). The shape of SLMs can be significantly different from a spherical shape. The surface characteristics of SLMs (smooth or rough, regular or not) can be visualised by microscopy. Their surface morphology has proved to vary depending on the excipients used.

Determination of particle size distribution

One of the methods for analyzing sizes most frequently used is laser diffractometry (LD). The principle that particles of a given size diffract light through a given angle, which increases with decreasing particle size. To determine the size distribution from the light intensity reaching the detectors can be used to two different diffraction theories (Mye and Fraunhofer).

Additionally, the image analysis system is a new technology that has been developed to determine and analyze particle size and shape (0.7 - 2000 m). This technique can be seen as a type of automated microscope that combines the accuracy and sensitivity of a regular microscope with the statistical relevance of the number of particles being analyzed. This can be

done either in real time or within a short period of time (Malvern, 2022). Its capacity to analyze particle shape gives users high-quality, practical information to fully characterize materials (emulsions, suspensions, or powders) (Malvern, 2022). As a result, the image analysis system can be used to better understand the behavior of materials (such as the flowability of powder, for example). The software establishes morphological metrics such as sieve diameter (Malvern, 2022), mean diameter, convexity, roundness, and elongation, among others. Even though the device is still somewhat pricey, this technology is destined to grow in popularity (Washington, 1992). Laser diffractometry is often used to investigate the general size analysis of SLMs, although new image analysis techniques can also be applied (Malvern, 2022).

Differential scanning calorimetry

DSC is an important tool for characterizing raw materials used in lipid-based drug delivery systems, such as lipid nano and microparticles (Kumar et al., 2014; Rahimpour et al., 2016; Silva et al., 2016), providing relevant information on their physical state, crystallinity and differentiation of the samples through thermal behavior (Kumar et al., 2014; Silva et al., 2016). In addition, this technique allows us to assess the drug compatibility with lipid excipients and to choose lipids with adequate thermal stability (Kumar et al., 2014; Rahimpour et al., 2016; Silva et al., 2016). Solid lipids are subjected to recrystallization. Therefore, the evaluation of the crystalline modification is very important in proving the stability of the formulation.

Entrapment efficiency and drug loading determination

In general, drug loading and encapsulation efficiency are determined as follows. SLMs are separated from the aqueous phase first. To separate SLMs from the aqueous phase, the aqueous SLM suspension is filtered, centrifuged, or ultrafiltered (for the smallest microparticles). SLMs are then dissolved in an appropriate solvent or heated with an appropriate aqueous solvent in which the drug is soluble and shaken to extract the drug in the solvent. The drug assay is performed on the obtained solution, typically using a spectrophotometric technique or High Performance Liquid Chromatography (HPLC) (Jaspart et al., 2005).

CHAPTER III

MATERIALS AND METHODS

Materials

Model active

- Asiatic acid (95%) (SEPPIC, France)

Solid lipids

- Beeswax (Lot. No. F1810038-001, Germany)
- Cetyl alcohol (Lot. No. 2319111001, Germany)

Surfactants

- Polysorbate 80 (Tween 80®) (Lot. No.142050.1611, Panreac applichem, Germany)
- Soybean lecithin (Lot. No. 3319813, Merck, Germany)
- Poloxamer 188 (Kolliphor® P188 Geismar) (Lot. No. GNC33321BT, BASF, Germany)

Chemicals

- Acetonitrile for analytical reagent grade (Lot. No. LC1005, Acros Organics, France)
- Dextrose anhydrous AR/ACS (Lot No. B336652006, Loba Chemie PVT. Ltd., India)
- Ethanol for analytical reagent grade (Lot. No. 21020035, RCI Labscan Limited, Thailand)
- Methanol for analytical reagent grade (Lot No. 21030101, RCI Labscan Limited, Thailand)
- Trifluoroacetic acid 99% extra pure (Lot. No. A0403748, Acros Organics, France)

Equipments

- Freeze-dryer (Labconco Lyophilizer, USA)
- High speed homogenizer (Model Ultra-Turrax®, IKA® Works Co. Ltd., Thailand)
- HPLC column (Model MGII 5 µm, Osaka Soda,
- Sonicator (Model Elmasonic E 30 H, Elma, DKSH, Thailand)
- Vortex mixer (Model Votex-Genisc-2™, Scientific Industries, USA)
- Weighing machine (Model PG403-S, Mettler Toledo, Thailand)

Laboratory supplies

- Aluminium bag (MMP Packing, Thailand)
- Aluminium foil (MMP Packing, Thailand)
- Beaker (Pyrex, USA)
- Centrifuge tube (Nunc, Denmark)
- Microcentrifuge tube (Corning, USA)
- Nylon membrane 0.45 µm (Lot. No. H7400491, CNW Technologies, China)
- Parafilm (Bemis, USA)
- Volumetric flask (ISO lab, Germany)

Method

1. Preparation of solid lipid microparticles

1.1 Solubility of asiatic acid (AA) in lipids

The solubility of AA in solid lipids (Beeswax and Cetyl alcohol) was studied qualitatively. Small amounts of pure AA (1 mg) were added to 1 g of melted lipids at 70 ± 1 °C. Solubility of AA in the molten lipids (in a glass tube) was estimated by visual observation (Wolska & Sznitowska, 2013).

1.2 Preparation of AASLMs

AA was dissolved in ethanol, in ratio 0.01 g: 1.5 ml and the solution was then added to the melted lipid phase (the melting temperature depending on the lipid used) and The mixture was stirred for 30 min to evaporate ethanol (Wolska & Sznitowska, 2013). The hot lipid mixture was then emulsified into an aqueous surfactant solution that was heated 70 °C to produce the O/W emulsion. The emulsion, which was obtained by mixing with a high shear device (Ultra-Turrax® [IKA]) at 8000 rpm for 5 min, was finally allowed to cool in an ice bath (Wolska & Sznitowska, 2013). Then, the AASLMs was placed into a freeze-dryer (Labconco Lyophilizer, USA) (Zhang et al., 2008) to make a dry powder. Freeze-drying was equipped with the condenser operating at -50 °C and a chamber with cooled shelves. The freeze-drying was conducted at a pressure of 5.0 Pa and the process lasted for 24 hours to allow a complete solidification. Dextrose anhydrous 5% w/w was used as the cryoprotectants for the freeze-drying process of AASLMs (Zhang et al., 2008). The formulations of asiatic acid loaded SLMs were shown in Table 1.

Table 1 The asiatic acid loaded in SLMs formulations.

Formulations	Asiatic acid (g)	Absolute Ethanol (g)	Lipids (g)		Surfactants (g)		
			B	C	Tw80	L	P188
F1	0.01	1.5	10	-	3	-	-
F2	0.01	1.5	10	-	-	3	-
F3	0.01	1.5	10	-	-	-	3
F4	0.01	1.5	-	10	3	-	-
F5	0.01	1.5	-	10	-	3	-
F6	0.01	1.5	-	10	-	-	3
F7	0.01	1.5	15	-	3	-	-
F8	0.01	1.5	15	-	-	3	-
F9	0.01	1.5	15	-	-	-	3
F10	0.01	1.5	-	15	3	-	-
F11	0.01	1.5	-	15	-	3	-
F12	0.01	1.5	-	15	-	-	3

B = Beeswax, C = Cetyl alcohol, Tw80 = Tween80, L = Lecithin, P188 = Poloxamer188

2. Characterization of solid lipid microparticles

2.1 Physical appearance

The physical appearance of AASLMs powder was assessed by visual observation.

2.2 Morphological analysis

2.2.1 Optical microscope

The shapes of AASLMs before freeze-drying and after re-dispersion were inspected by using the optical microscope (Nikon Coolpix 5400, Nikon Corporation, Japan).

2.2.2 Scanning Electron Microscope (SEM)

The surface morphology of AASLMs was examined by scanning electron microscope. The samples were prepared by using cotton swabs to scoop and distribute the AASLMs powder onto the specimen stub fixed with double-sided tape. A rubber ball was used to blow away dust or non-stick particles. The specimen was then photographed under the SEM (JEOL JSM-6610LV, Tokyo, Japan) (Rahimpour et al., 2016).

2.3 Particle size and size distribution

The particle size and size distribution were measured by static automated imaging technique (Morphologi 4, Malvern Panalytical, Germany). The AASLMs powder was put in a dispersed chamber and then placed inside the instrument, using a pressure of 4 bar for dispersion on a quartz glass slide. All measurements were done in triplicate and data were expressed as means ± SD. The size was expressed by the circle equivalent (CE) diameter and size distribution was described by the cumulative distribution which is the span (d90 – d10) and the relative span is a common calculation to quantify distribution width;

$$\text{Relative span} = \frac{D90 - D10}{D50} \quad (1)$$

where D90, D50, and D10 refer to the particle sizes when cumulative total distributions are 90%, 50%, and 10%, respectively (Rahimpour et al., 2016).

2.4 Differential Scanning Calorimetry (DSC)

The analysis of lipid polymorphism (thermal behavior) of AASLMs was studied using differential scanning calorimeter (DSC). Each sample was heated in temperature range of 25 to 350 °C at the rate of 20 °C/min, under nitrogen purge (50 ml/min). A standard aluminum sample pan (40 µl) was used. About 10 mg AASLMs powder was taken for analysis. An empty pan was used as a reference. A physical mixture of AA and lipids, freeze-dry solid lipid microparticles without AA, the pure lipids and pure AA were used as controls. All controls were prepared at the same weight ratios in SLMs formulation and tested with the same thermal cycles (Khunathum, 2011).

2.5 Entrapment efficiency and active loading

The entrapment efficiency of AA was determined by measuring the concentration of free AA in the dispersion medium. 8 ml of AASLMs were centrifuged at 65,000 rpm, 4 °C for 1.5 h by using the ultra-centrifuge device (Hitachi CP100NX ultracentrifugation, Japan). 1 ml of supernatant (free active) was filtrated through 0.45 µm syringe filter and assayed by HPLC at 210 nm. The sediment was extracted by using methanol and adjusted the final volume to 5 ml. 1 ml of obtaining mixture was then filtrated through 0.45 µm syringe filter and subjected to HPLC analysis. The percentage of entrapment efficiency and active loading were calculated by the following equations:

$$\% \text{ Entrapment efficiency (EE)} = \frac{(W_{\text{total active}} - W_{\text{free active}}) \times 100}{W_{\text{total active}}} \quad (2)$$

$$\% \text{ Active loading (Al)} = \frac{(W_{\text{total active}} - W_{\text{free active}}) \times 100}{\text{total weight of SLM powder}} \quad (3)$$

where $W_{\text{total active}}$ is total amount of AA added to the system, $W_{\text{free active}}$ is the analyzed amount of free active. The values were averaged on three determinations (Jaspart et al., 2005; Rahimpour et al., 2016; Rosita et al., 2019).

%Labeled amount of AA in SLMs is calculated by equations below:

$$\%\text{Labeled amount} = \frac{(\text{Amount of AA in the sediment} + \text{Amount of free AA in the supernatant}) \times 100}{(\text{Known added amount of AA})} \quad (4)$$

Note: acceptable standard range is 85-115% for topical drugs (United States Pharmacopeial, 2004)

3. Stability studies

The AASLMs formulations were kept in aluminum foil bag (with sealing) in three conditions: 4 °C, ambient temperature and 45 °C for 3 months. Formulations at regular intervals (initial, 1, 2 and 3 months) were tested for physical changes, particle size and size distribution, and entrapment efficiency.

4. Validation of HPLC method

The HPLC condition was validated four topics were studied i.e. linearity, specificity, accuracy and precision (Khunathum, 2011).

Linearity: Three sets of six standard solutions were prepared and analyzed. Linear regression analysis of the peak area compared with their concentrations is performed. The linearity was determined from the coefficient of determination (R^2). Acceptance criteria: The coefficient of determination should be more than 0.9990.

Specificity: Under the chromatographic conditions used, the reagents were added and stored in conditions as described below for determining the decomposed products from AA. The peak of AA must be completely separated when compared with the standard peak and not be interfered by the peaks of other components in the sample. Reagents and conditions used are a) 1 ml of 0.1 N HCl and then heat 80 °C for 3 hours, b) 0.5 ml 30% v/v H_2O_2 and then heat 80 °C for 3 hours and c) 1 ml of water and then heat 80 °C for 3 hours and light for 6 hours. Acceptance criteria: The resolution value should be more than 1.5 when compare with standard.

Accuracy: The accuracy of the analysis method was expressed by the approximation between the values obtained from the analyzes compared to the actual or accepted reference values. This value was determined from the percentage of the analytical recovery. Three sets of three concentrations of AA at low, medium and high (with AA-free SLMs) to cover the concentrations were determined. The percentage of recovery of each concentration was calculated from the ratio of analyzed concentration to known added concentration multiplied by 100. Acceptance criteria: The percentage of analytical recovery should be within 98.0 – 102.0 % of each nominal concentration.

Precision:

a) Within-run precision

The within-run precision was determined by analyzing five sets of three concentrations of standard solutions of 0.025, 0.1 and 1.0 mg/mL in the same analytical run. The percentage of coefficient of variation (%CV) of each concentration was then determined.

b) Between-run precision

The between-run precision was determined by comparing three concentrations of standard solutions of AA at 0.025, 0.1 and 1.0 mg/mL on three repeated concentrations of analytical running, done on different days, for a total of three days. The percentage of coefficient of variation (%CV) of each concentration was determined.

Acceptance criteria: The present coefficient of variation (%CV) for both within-run and between-run precision should be less than 2 %.

Limit of detection (LOD) Limit of quantitation (LOQ)

Limit of detection (LOD) means the lowest concentration of a standard substance that can be detected or measured. LOD calculated from the standard deviation of the signal (**σ**) and the slope of the standard curve (s) as the following equation: LOD = 3.3**σ**/s. The acceptable minimum test concentration (limit of quantitation; LOQ), or quantitative measurement limit, is the lowest concentration of a standard substance that can be quantified with accuracy which is acceptable. Therefore, the quantitative measurement limit is a property of methods that demonstrate the ability to report results at the lowest concentrations with a certain level of confidence. LOQ calculated from the standard deviation of the signal (**σ**) and the slope of the standard curve (s) as the following equation: LOQ = 10**σ**/s.

5. Determination of AA by HPLC method

5.1 HPLC condition

The mobile phase consisted of 0.05 % Trifluoroacetic acid (TFA) in acetonitrile: 0.05 % Trifluoroacetic acid (TFA) in water (45: 55) was used. The solvent was filtrated through 0.45 μm membrane filter and the degassed for 45 minutes prior use.

Column: Osaka soda C18 (5 μm, 150 x 4.6 mm)

Injection volume: 20 μL

Flow rate: 1.2 mL/minutes

Detector: UV detector at 210 nm

Temperature: ambient

Run time: 16 minutes

5.2 Standard solution of HPLC analysis

A standard stock solution of AA was prepared by accurately weighing 100 mg of AA into a 100 mL volumetric flask. The methanol was then added into a volumetric flask to get the final concentration of stock solution of 100 μg/mL. 90, 250, 500, 1000 and 10000 μL of standard stock solution were pipetted into 10 mL volumetric flask and adjusted the final volume with methanol to make 0.009, 0.025, 0.05, 0.1, 0.5, 1 mg/mL of AA solutions, respectively.

5.3 Preparation of sample

Purified water was added into the dried AASLMs to make the final volume of 10 ml. Then, the sample was sonicated at ambient temperature for 5 min to ensure complete dispersion of AASLMs.

6. Statistical analysis

All experiments were performed in triplicates (n = 3) for validity of statistical analysis. Results were expressed as mean ± SD. The statistical variance was calculated by One-way ANOVA, comparison of each group by Tukey HSD test and differences were considered significant for p value ≤ 0.05.

CHAPTER IV

RESULTS AND DISCUSSIONS

1. Preparation of solid lipid microparticles

1.1 Solubility of asiatic acid (AA) in lipids

The solubility of AA in two types of solid lipids, namely beeswax and cetyl alcohol, was studied. One gram of beeswax was melted at 70 °C and 1 mg of AA was added into it (Pietkiewicz et al., 2006; Wolska & Sznitowska, 2013). A clear, pale yellow liquid without sediment was obtained as shown in Figure 6a. One gram of cetyl alcohol was also melted at 70 °C and 1 mg of AA was added into the melted lipid. A clear liquid without sediment was obtained as shown in Figure 6b. These results indicated that 1 mg of AA was highly soluble in beeswax and cetyl alcohol. This method is generally used to determine the solubility of active substances in solid lipids.

AASLMs were then fabricated by the melt dispersion technique (Chalella Mazzocato et al., 2019; Sznitowska et al., 2017; Wolska & Sznitowska, 2013; Yadav et al., 2009; Zhang et al., 2008) in conjunction with freeze-drying, in order to obtain water-free solid particles. 0.01 g of Asiatic acid was used as active substance. Two types of solid lipids, including beeswax and cetyl alcohol, were chosen and used at different concentrations of 10% and 15% as lipid component for AASLM preparation Tw80, soybean lecithin, and poloxamer 188 were used at 3% as emulsifier or stabilizer.

(a) (b)

Figure 6 Physical appearance of (a) 1 g of beeswax with 1 mg of AA
(b) 1 g of cetyl alcohol with 1 mg of AA.

2. Characterization of solid lipid microparticles

2.1 Physical appearance

According to the result shown in Table 2 and 3, the AASLM prepared from 10% or 15% beeswax with 3% soybean lecithin (F2 and F8) could not yield a dry powder form, whereas the rest of the formulations could yield a dry powder form. AASLMs prepared from beeswax (F1, F3, F7, F9, F12) were white to light yellow powders, whereas AASLMs prepared from cetyl alcohol (F4, F5, F6, F10, F11, F12) were fine white powders. This may because pure beeswax has white to light yellow color but pure cetyl alcohol has white color (Bogdanov, 2004; Cetyl alcohol 2022). Thus, the different types of lipids used could affect on physical characteristics of resultant powder. The different concentrations of solid lipid used was found to have no effect on physical characteristics of AASLMs. This finding was in agreement with previous study using different concentration of solid lipid (10% or 20% glyceryl behenate) to prepare SLM incorporated cyclosporine A for ocular application (Wolska & Sznitowska, 2013). The results also indicated that the color of AASLMs was not significantly affected by the type of surfactant used. This was in agreement with previous study reported by Sznitowska et al. (2017). Moreover, the physical appearances of the obtaining AASLM dispersions (crystallization) and powders (coarse) prepared from beeswax could be attributed to the unique characteristics of beeswax (Bogdanov, 2004).

In addition, the pH values of all AASLM formulations ranged from 5.0-5.5 which were compatible with human skin (pH 4-6) (Klee et al., 2009; Lambers et al., 2006).

Table 2 The characteristics (appearance and pH) of asiatic acid loaded in SLMs formulations.

Formulations	Lipids (g)		Surfactants (g)			pH	Appearance after freeze-dry
	B	C	Tw80	L	P188		
F1	10	-	3	-	-	5	White to light yellow powder, coarse
F2	10	-	-	3	-	5	light yellow, sticky lump
F3	10	-	-	-	3	5.5	White to light yellow powder, coarse
F4	-	10	3	-	-	5	White powder, fine
F5	-	10	-	3	-	5	White powder, fine
F6	-	10	-	-	3	5	White powder, fine
F7	15	-	3	-	-	5	White to light yellow powder, coarse
F8	15	-	-	3	-	5	light yellow, sticky lump
F9	15	-	-	-	3	5.5	White to light yellow powder, coarse
F10	-	15	3	-	-	5	White powder, fine
F11	-	15	-	3	-	5	White powder, fine
F12	-	15	-	-	3	5.5	White powder, fine

B = Beeswax, C = Cetyl alcohol, Tw80 = Tween80, L = Lecithin, P188 = Poloxamer188

Table 3 The physical appearance of AASLMs formulations. (initial)

Formulations	Physical appearance	
	Initial	
F1 (10% B-Tw80)	White to light yellow powder, coarse	
F3 (10% B-P188)	White to light yellow powder, coarse	
F4 (10% C-Tw80)	White powder, fine	
F5 (10% C-SL)	White powder, fine	
F6 (10% C-P188)	White powder, fine	
F7 (15% B-Tw80)	White to light yellow powder, coarse	
F9 (15% B-P188)	White to light yellow powder, coarse	
F10 (15% C-Tw80)	White powder, fine	
F11 (15% C-SL)	White powder, fin	
F12 (15% C-P188)	White powder, fine	

2.2 Morphological analysis

2.2.1 Optical microscope

The morphology of AASLM dispersions before freeze-drying and after redispersion of the freeze-dried powder observed under the optical microscope with a magnification of 100x was shown in Table 4.

Table 4 The morphology of AASLM dispersions before freeze-drying and after redispersion of the freeze-dried powder were also investigated using the optical microscope (100x).

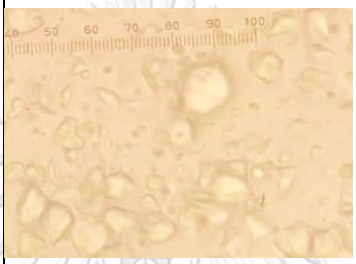
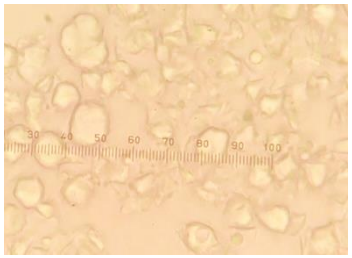
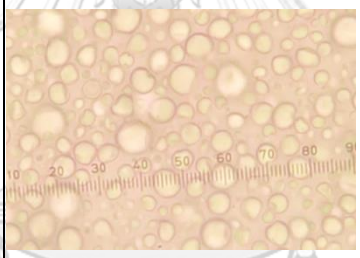
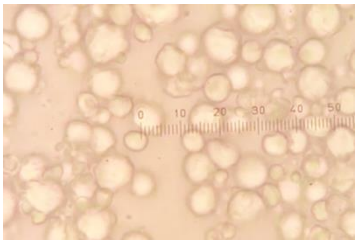
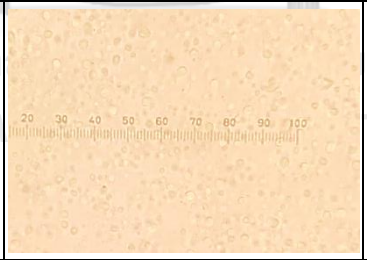
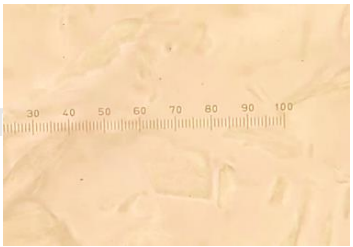
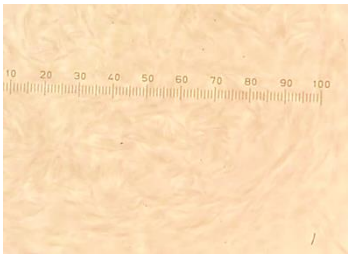
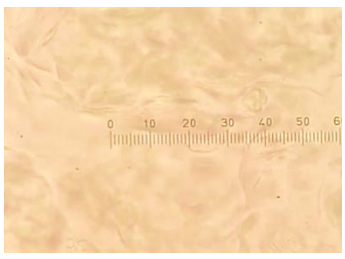
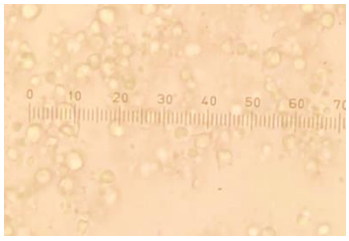
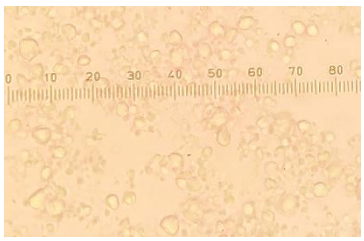
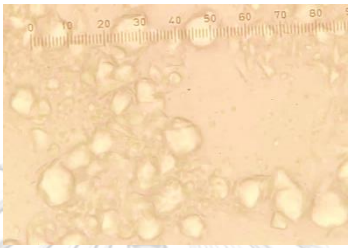
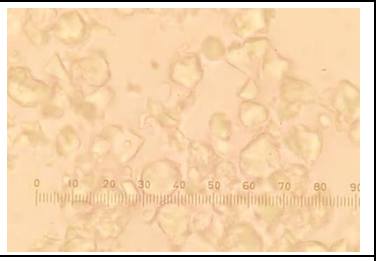
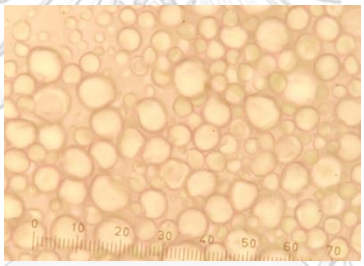
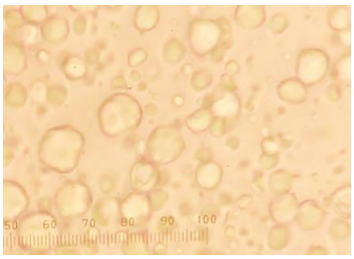
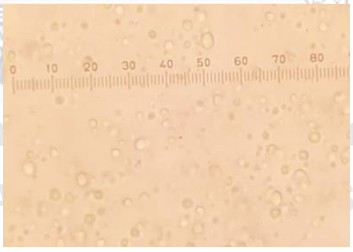
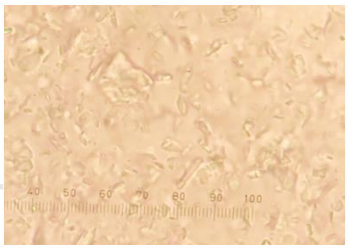
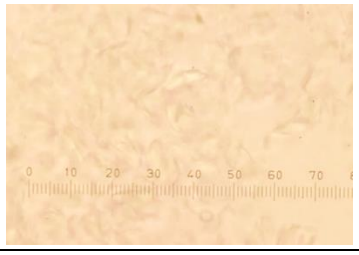
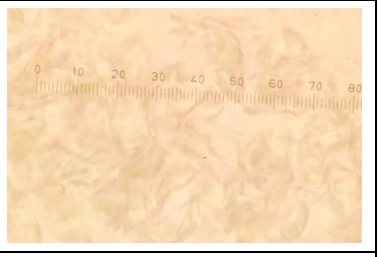
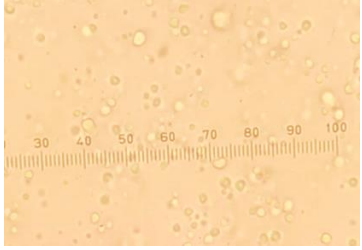
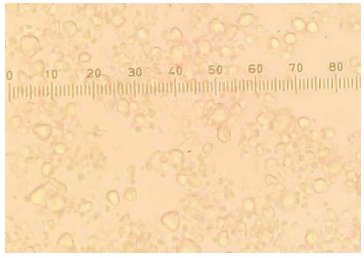
Powder appearance	After formulation	After freeze-dry
F1 (10% beeswax-Tw80)		
F3 (10% beeswax-P188)		
F4 (10% cetyl alcohol-Tw80)		
F5 (10% cetyl alcohol-SL)		

Table 4 The morphology of AASLM dispersions before freeze-drying and after redispersion of the freeze-dried powder were also investigated using the optical microscope (100x) (continuous).

Powder appearance	After formulation	After freeze-dry
F6 (10% cetyl alcohol-P188)		
F7 (15% beeswax-Tw80)		
F9 (15% beeswax-P188)		
F10 (15% cetyl alcohol-Tw80)		
F11 (15% cetyl alcohol-SL)		
F12 (15% cetyl alcohol-P188)		

In all the dispersions, the AASLM prepared from beeswax with poloxamer 188 (F3, F9) and cetyl alcohol with poloxamer 188 (F6, F12) had the spherical particle shape. AASLM prepared from beeswax with Tw80 (F1, F7) seemed to have irregular particle shape, while AASLM prepared from cetyl alcohol with Tw80 (F4, F10) had spherical particle shape. However, the morphology after redispersion of the freeze-dried F4, F10 powder seemed to have irregular particle shape. These results indicated that the type of solid lipid (beeswax and cetyl alcohol) had no effect on the morphology of AASLM stabilized by poloxamer 188. In contrast, type of lipid used had an effect on the morphology of AASLM stabilized by Tw80. The different concentrations of solid lipid used was found to have no effect on morphology of AASLMs before freeze-drying and after redispersion of the freeze-dried powder. The type of surfactant used could affect the particle shape of the AASLMs dispersions. AASLM stabilized by poloxamer 188 had spherical shape, but AASLM stabilized by Tw80 had non-spherical shape. This may be because poloxamer 188 can effectively reduce the surface tension between the lipid phase and the aqueous phase during emulsification, resulting in a spherical particle shape. Moreover, the steric effect of poloxamer 188 could prevent a droplet agglomeration (Hariyadi et al., 2022; Rosita et al., 2019), hence the AASLMs stabilized by poloxamer 188 showed comparable particle shape before and after freeze-drying. Our results were consistent with the study of Hariya (2022) which demonstrated that SLMs made of glyceryl behenate with poloxamer 188, to deliver quercetin, had a spherical morphology (Hariyadi et al., 2022). In 2009, Yadav et al. reported that curcumin loaded in SLMs made of lipid (10%w/w) and poloxamer 188 (0.5%w/w) had the spherical shape (Yadav et al., 2009). In another study, the solid lipid microparticles composed of lipid (5%w/w) and poloxamer 188 at 0.3% and 0.4%w/w were found to have a spherical shape and smooth surface (V. Sanna et al., 2004). On the contrary, Tw80 may not be able to effectively reduce the surface tension between the aqueous phase and the lipid phase during emulsification, resulting in irregular particle shape of AASLMs stabilized by Tw80. This finding was not in agreement with previous studies. Yenner et al. (2003) reported that SLMs made of 0.2% Tw80 with 6% beeswax developed as topical carriers for octyl methoxy cinnamate (OMC), were found to have a spherical shape (Yener et al., 2003). However, the amount of beeswax and Tw80 used in previous study was different from our study, thus the result could be different. In addition, AASLMs stabilized by soybean lecithin was not be able to form a spherical shape. This may be because the HLB values of poloxamer 188 (HLB 29) and Tw80 (HLB 15) are suitable for oil in water emulsion used for preparation of SLN or SLM (Diniz et al., 2018), while HLB of soybean lecithin (HLB 7) is not suitable. Our finding was in agreement with many previous studies which indicated that the use of different type of surfactants affected the morphology of the SLM (Hariyadi et al., 2022; Mestres et al., 2010a; V.

Sanna et al., 2004; Sznitowska et al., 2017; Yadav et al., 2009; Yener et al., 2003). Therefore, the formulas F3, F9, F6 and F12 were selected for further study.

2.2.2 Scanning Electron Microscope (SEM)

The surface morphology of AASLM powders (F3, F6, F9, F12) examined by scanning electron microscope (SEM) with a magnification of 50x and 1000x was shown in Figure 7 and Figure 8, respectively. AASLM powders prepare from both beeswax (Figure 7: F3a, F3b, F9a, F9b) and cetyl alcohol (Figure 7: F6a, F6b, F12a, F12b) with poloxamer showed a non-spherical shape and agglomerated tiny plates. This finding was in agreement with previous studies which reported that the SLM powder produced by freeze-drying process had non-spherical and agglomerated morphology (Alihosseini et al., 2015; Mudrić et al., 2021; Owuor et al., 2017). Moreover, the lipid concentrations had no impact on the surface morphology of SLM (Rosita et al., 2022). The particle agglomeration has a positive effect on flow characteristic of particles and decreasing dust formation (Barbosa-Cánovas et al., 2005; Paucar et al., 2016). Moreover, particle size of SLM observed by SEM was different from those measured by Morphologi. This may be attributed to a larger number of sample sizes used in Morphologi (n ≥ 20,000 particles) in comparing to SEM, which could be a representative sample for particle size analysis.

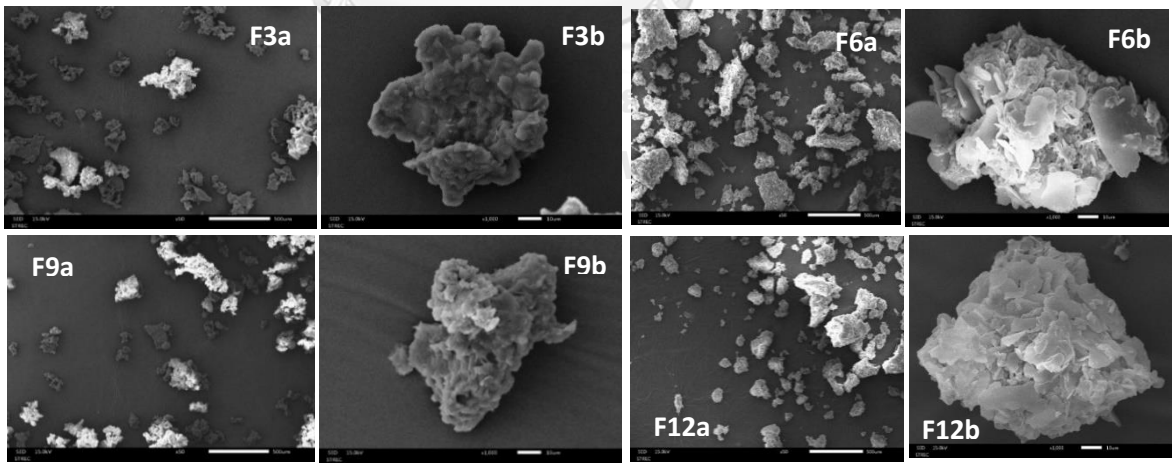


Figure 7 The surface morphology of AASLM powders examined by SEM with a magnification of 50x; (F3a) 10% B-P188 (F9a) 15% B-P188, (F6a) 10% C-P188 and (F12a) 15% C-P188. AASLM powders examined by SEM with a magnification of 1000x (F3b) 10% B-P188 (F9b) 15% B-P188, (F6b) 10% C-P188, (F12b) 15% C-P188, respectively.

2.3 Particle size and size distribution

Particle sizes ($d_{0.9}$) of AASLM dispersions, before freeze drying, prepared from beeswax 10% (F3) and 15% (F9), and cetyl alcohol 10% (F6) and 15% (F12) stabilized by poloxamer 188 determined by Master sizer were 49.17 ± 0.18, 53.43 ± 0.28,43.87 ± 0.05 and 28.87 ± 0.18 μm, respectively (Table 5). The particle sizes of the AASLMs redispersion after freeze-drying were slightly increased in comparing to before freeze-drying.

The particle size of the AASLMs powder prepared from beeswax (F3, F9) was significantly larger than those prepared from cetyl alcohol (F6, F12). This may be because beeswax is a solid fatty acid composed of long-chain fatty acids and alcohols (C24-C36), whereas cetyl alcohol is a solid fatty alcohol (C16) composed of fatty alcohols chain (C16), which has a shorter chain length than beeswax resulting in smaller particle size. The results were similar to previous studies which reported that SLM-loaded ibuprofen made of cetyl alcohol showed smaller average particle size than that made of glyceryl behenate (Long et al., 2006).

Table 5 The average particle size of AASLM prepared from 10% or 15% beeswax (F3, F9) and 10% or 15% cetyl alcohol (F6, F12) with 3% poloxamer 188 before freeze-dry and AASLM redispersion after freeze-dry.

Formulas	Initial mean particle size (μm) ± SD							
	Before freeze-dry				Redispersion after freeze-dry			
	$d_{0.1}$	$d_{0.5}$	$d_{0.9}$	Span	$d_{0.1}$	$d_{0.5}$	$d_{0.9}$	Span
F3	12.07 ± 0.18	29.53 ± 0.05	49.17 ± 0.18	1.26 ± 0.01	12.27 ± 0.05	31.40 ± 0.01	61.20 ± 0.15	1.56 ± 0.01
F6	0.45 ± 0.01	19.83 ± 0.05	43.87 ± 0.05	2.19 ± 0.02	1.72 ± 0.01	18.80 ± 0.01	46.27 ± 0.10	2.37 ± 0.02
F9	12.67 ± 0.80	32.87 ± 0.05	53.43 ± 0.28	1.24 ± 0.03	11.80 ± 0.02	32.30 ± 0.09	69.40 ± 0.48	1.78 ± 0.01
F12	0.70 ± 0.01	13.93 ± 0.05	28.87 ± 0.18	2.03 ± 0.01	0.77 ± 0.01	13.87 ± 0.67	28.97 ± 0.18	2.04 ± 0.01

Moreover, it was observed that the formulation with 10% beeswax (F3) had smaller particle size than that with 15% beeswax (F9), but the formulation with 10% cetyl alcohol (F6) had larger particle size than that with 15% cetyl alcohol (F12). In general, increasing the concentration of lipids used will result in an increase in particle size of solid lipid particles (Bertoni et al., 2020; Jaspart et al., 2005). A smaller particle size with increasing concentration of cetyl alcohol possibly because cetyl alcohol acts like co-surfactants. As the asiatic acid expressed hydrophobicity property, the incorporation of co-surfactants could lead to enhancing AA

solubility in dispersion system which results in decreasing of mean particle size (Artiga-Artigas et al., 2017; Gupta et al., 2016; Khunathum, 2011). This was in agreement with previous studies indicated by Sznitowska et al. (Sznitowska et al., 2017). It was reported that the SLMs prepared by two different types of lipids including glyceryl behenate and glyceryl palmitostearate with three different levels of concentrations (10%, 20%, 30% w/w) exhibited different particle size. The result revealed that the particle size of SLMs made of glyceryl palmitostearate), which can act as co-surfactant, increased when the concentration of lipids increased. It was found that SLMs with larger particle size exhibited a sustained release over a longer duration (Wolska & Sznitowska, 2013; Yener et al., 2003).

According to the results presented in Table 6, the mean particle sizes of AASLM powders ranged from 7.46 ± 0.08 to 38.86±0.34 microns. It was found that the sizes of all AASLM powders were significantly different at the 0.05 level. However, they were still in the range of particle size for topical application (Mestres et al., 2010a; Üner & Karaman, 2013). Üner & Karaman (2013) successfully formulated solid lipid microparticles (SLM) powder entrapped with loratadine (LRT) for the treatment of allergic reactions. Their particle sizes were between 86 ± 5.63 μm and 184 ± 13.21 μm, while the droplet size of their O/W emulsion was 76 ± 3.45 μm. They have also recommended that the size of the obtaining SLM was appropriate and could potentially be used for topical application (Üner & Karaman, 2013). Type of lipids used could also affect the particle size of obtaining powders. AASLM powder prepared from cetyl alcohol showed smaller particle size than that prepared from beeswax (Table 6). This could be due to surface tension reducing activity of cetyl alcohol (Cetyl alcohol 2022; Karewicz, 2014). Our result indicated that the amount of lipid used had an impact on the particle size of AASLM powder.

Table 6 The average particle size of AASLMs powders (F1-F12) determined by Morphologi

Formulations	Initial
	Mean particle size (μm) ± SD
F1 (10% B-Tw80)	38.86 ± 0.34
F3 (10% B-P188)	14.15 ± 0.11
F4 (10% C-Tw80)	25.72 ± 0.28
F5 (10% C-SL)	23.61 ± 0.22
F6 (10% C-P188)	7.49 ± 0.19
F7 (15% B-Tw80)	30.67 ± 0.27
F9 (15% B-P188)	16.87 ± 0.15
F10 (15% C-Tw80)	28.54 ± 0.12
F11 (15% C-SL)	17.92 ± 0.24
F12 (15% C-P188)	7.46 ± 0.08

In addition, it was observed that the AASLM stabilized by Tw80 (F4, F10) was significantly larger than those stabilized by poloxamer 188 (F6, F12) (Table 6). These results clearly indicated that changing the surfactant type from poloxamer 188 to Tw80 could affect the size of AASLM powder. This may be because the higher hydrophilic-lipophilic balance (HLB) values of poloxamer 188 (HLB 29) compared to that of Tw80 (HLB = 15) may sufficient to cover the surface of SLM dispersion and prevent agglomeration of the resultant powders. (Sato, 2001; Sznitowska et al., 2017).

2.4 Differential Scanning Calorimetry (DSC)

DSC was a tool to investigate the melting and recrystallization behavior of crystallinity material like SLM. The melting and recrystallization of the lipids can lead to the occurrence of transitions between multiple polymorphic forms such as unstable (α), metastable (β') and stable (β). Generally, solid lipid particle system was rearranged into a more unstable polymorphic form, an intermediate state between (β) and (β') form, which had good drug incorporation. This form was stable during storage (Attama et al., 2006; Diniz et al., 2018; Souto et al., 2011). DSC thermograms of AA, beeswax, cetyl alcohol, physical mixture of asiatic acid with beeswax and cetyl alcohol, freeze drying of SLM from beeswax and cetyl alcohol (without asiatic acid) and physical mixture of asiatic acid with freeze drying of SLM from beeswax and cetyl alcohol (without asiatic acid) were shown in Figure 8-11. The results revealed that the melting process for the asiatic acid alone took place at 342 °C.

The thermograms of AASLM composed of 10% or 15% beeswax with poloxamer (F3, F9) and 10% or 15% cetyl alcohol with poloxamer (F6, F12) did no show the melting peak of the asiatic acid around 342 °C. The melting peak of the asiatic acid was not observed in all of physical mixtures and freeze drying SLM formulation. This may be due to the concentration of AA was too low that could not be detected by DSC.

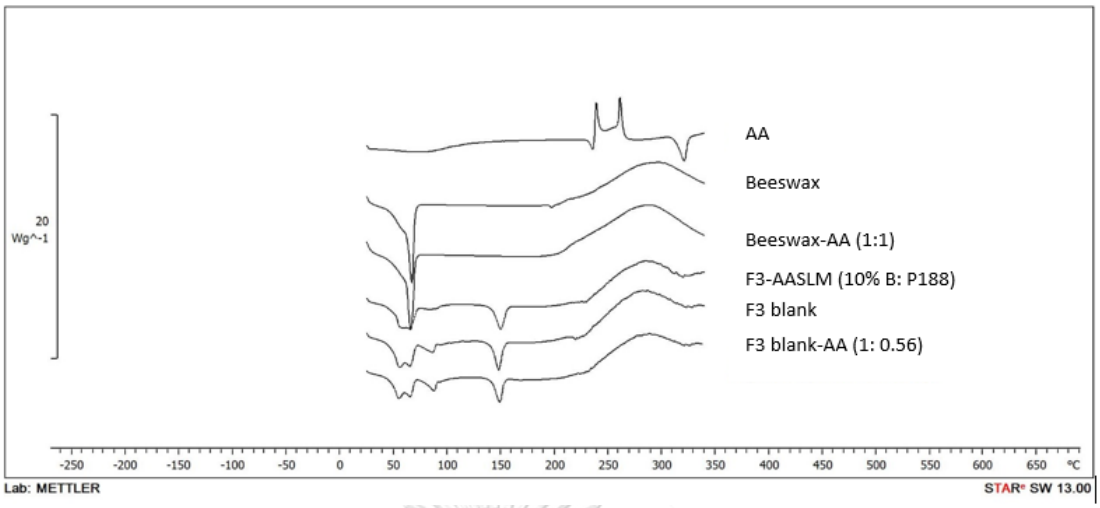


Figure 8 DSC thermograms of AASLM prepared from 10% beeswax with poloxamer 188: AA, beeswax, physical mixture of asiatic acid with beeswax, freeze drying of SLM from beeswax (without asiatic acid) and physical mixture of asiatic acid with freeze drying of SLM from beeswax (without asiatic acid), respectively.

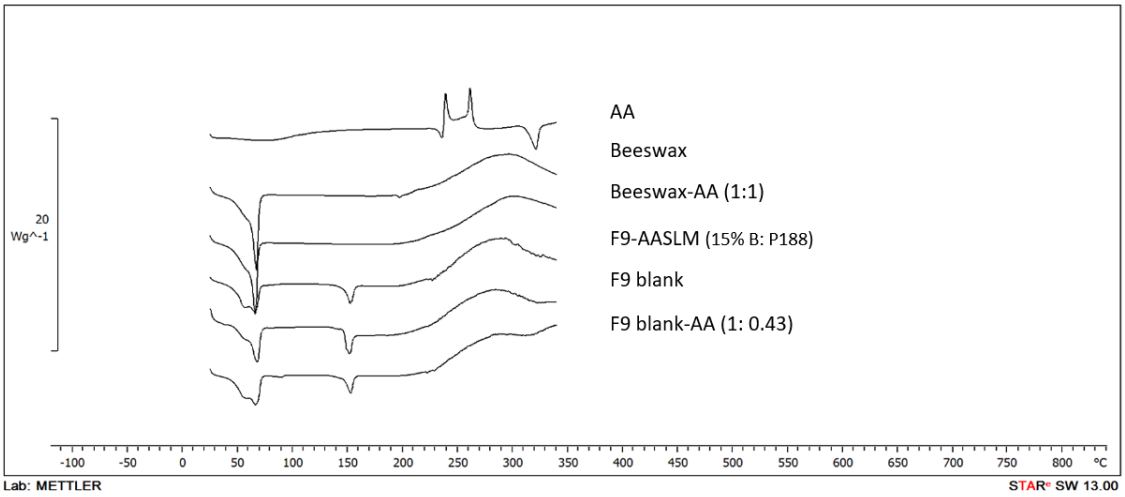


Figure 9 DSC thermograms of AASLM prepared from 15% beeswax with poloxamer 188: AA, beeswax, physical mixture of asiatic acid with beeswax, freeze drying of SLM from beeswax (without asiatic acid) and physical mixture of asiatic acid with freeze drying of SLM from beeswax (without asiatic acid), respectively.

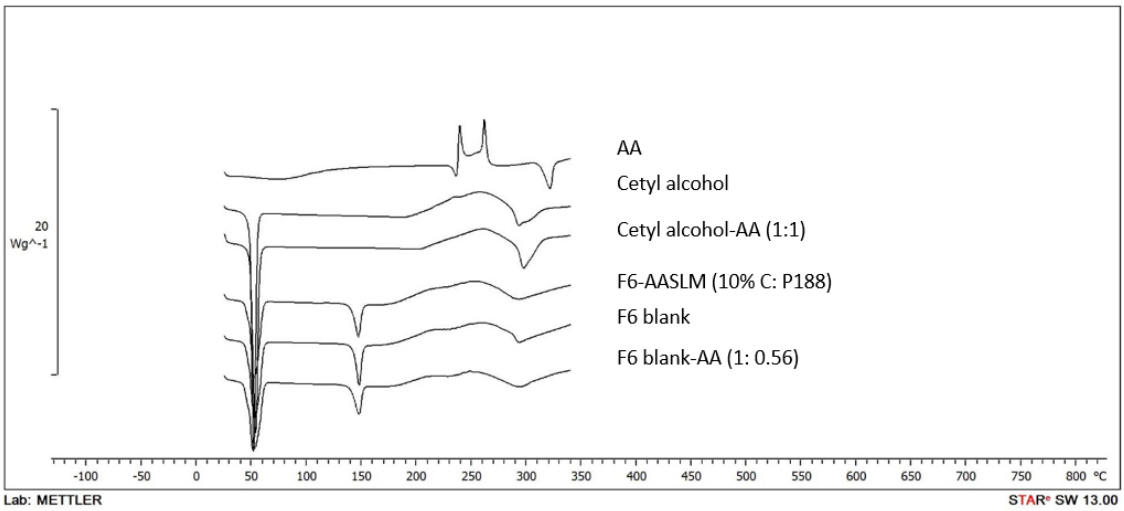


Figure 10 DSC thermograms of AASLM prepared from 10% cetyl alcohol with poloxamer 188: AA, cetyl alcohol, physical mixture of asiatic acid with cetyl alcohol, freeze drying of SLM from cetyl alcohol (without asiatic acid) and physical mixture of asiatic acid with freeze drying of SLM from cetyl alcohol (without asiatic acid), respectively.

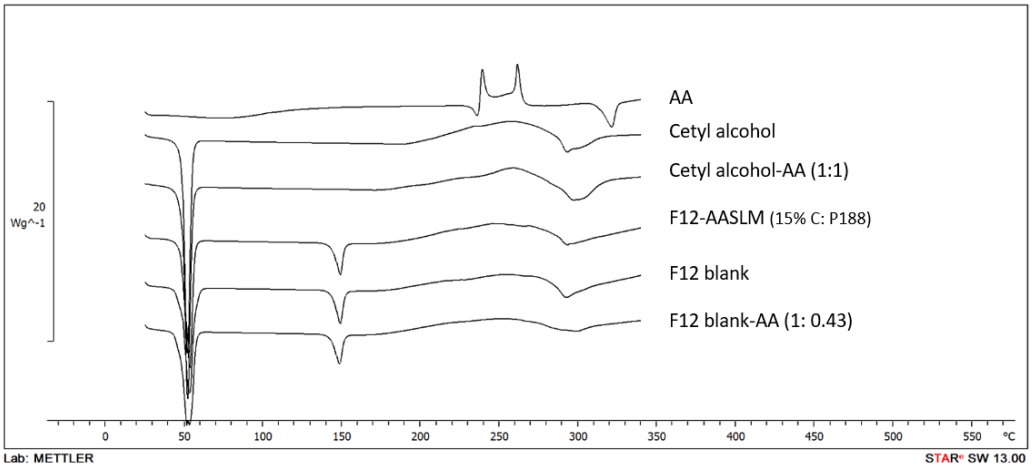


Figure 11 DSC thermograms of AASLM prepared from 15% cetyl alcohol with poloxamer 188: AA, cetyl alcohol, physical mixture of asiatic acid with cetyl alcohol, freeze drying of SLM from cetyl alcohol (without asiatic acid) and physical mixture of asiatic acid with freeze drying of SLM from cetyl alcohol (without asiatic acid), respectively.

2.5 Entrapment efficiency and active loading

The entrapment efficiency (EE) of resultant AASLM powders were shown in Table 7, Figure 15 and 16. AASLMs prepared from beeswax (F1, F7) gave higher %entrapment efficiency (%EE) than the formulas prepared from cetyl alcohol (F4, F10) ($p < 0.05$). The spherical shape of F1 and F7 may attribute to the higher drug incorporation in comparing to F4 and F10 which had irregular shape. This finding was in agreement with previous studies which reported that the SLM powder made of beeswax had a spherical shape with the high entrapment efficiencies of octyl methoxy cinnamate (OMC) (Yener et al., 2003). Also, the highest %EE was observed in formulas F3, F6, F9 and F12 which were spherical in shape (Table 4).

Furthermore, AASLMs prepared from 10% beeswax (F1) gave higher %entrapment efficiency (%EE) than the formula prepared from 15% beeswax (F7) ($p < 0.05$). It is possible that asiatic acid (AA) may be expulsed from the SLM containing high amount of beeswax. AASLMs stabilized by P188 (F6, F12) gave higher %EE than those stabilized by soybean lecithin (F5, F11) and Tw80 (F1, F7) ($p < 0.05$) (Figure 16). Moreover, the results revealed that SLM formulations prepared from beeswax or cetyl alcohol with poloxamer 188 (F3, F9, F6, F12) gave the highest entrapment efficiency (no significant between 4 formulations). This may be because P188 could immobilize the solid lipid system and improve solubility of poorly water soluble drug resulting in lower drug expulsion (Shah & Serajuddin, 2012). Previous study had also demonstrated that the solid lipid microparticle stabilized by poloxamer 188 had high entrapment efficiency of poorly water soluble substance (Hariyadi et al., 2022). Our study confirmed that the surfactant type had an influence on the %EE of AASLMs. A previous study observed that the type of surfactant had an effect on polymorphic lipids because of varying surfactant packing patterns on the lipid matrix surface. Polymorphism is a phenomenon that can be demonstrated in lipids after the fusion process, recrystallization or in the production process (El-Salamouni et al., 2015) which influence the encapsulation efficiency and drug loading (Severino et al., 2012).

Table 7 The average entrapment efficiency (%) and active loading (%) and labeled amount (%) of AASLMs powders (F1-F12)

Formulations	Initial		
	Mean entrapment efficiency (%) ± SD	Mean active loading (%) ± SD	Mean labeled amount (%) ± SD
F1 (10% B-Tw80)	53.75 ± 0.01	0.0272 ± 0.0010	94.23 ± 0.04
F3 (10% B-P188)	100.00 ± 0.00	0.0482 ± 0.0009	90.43 ± 0.02
F4 (10% C-Tw80)	31.56 ± 0.02	0.0167 ± 0.0020	92.15 ± 0.02
F5 (10% C-SL)	58.80 ± 0.02	0.0299 ± 0.0017	90.70 ± 0.02
F6 (10% C-P188)	100.00 ± 0.00	0.0494 ± 0.0008	92.99 ± 0.01
F7 (15% B-Tw80)	51.56 ± 0.03	0.0171 ± 0.0011	95.26 ± 0.02
F9 (15% B-P188)	100.00 ± 0.00	0.0315 ± 0.0008	90.69 ± 0.02
F10 (15% C-Tw80)	42.77 ± 0.03	0.0140 ± 0.0010	94.15 ± 0.05
F11 (15% C-SL)	60.26 ± 0.02	0.0196 ± 0.0015	91.22 ± 0.03
F12 (15% C-P188)	100.00 ± 0.00	0.0332 ± 0.0009	95.38 ± 0.02

In addition, the %labeled amount of all AASLMs powders (Table 7) were in the range of 90.43±0.02 to 95.38±0.02%, which was considered acceptable for topical products (United States Pharmacopeial, 2004).

When considering the %active loading (%AL) of resultant AASLM powders, AASLMs prepared from beeswax (F1, F7) stabilized by Tw80 gave higher %AL than the formula prepared from cetyl alcohol stabilized by Tw80 (F4, F10) ($p < 0.05$) (Table 7). The crystallinity of lipids may influence the drug incorporation in SLM (Jaspart et al., 2005). Matrices with highly ordered and tightly packed lipid structures have limited space for drug embedding, whereas diverse lipid molecules in the mixture are less-ordered packed, more space for drug molecules was offered there upon (Müller et al., 2000; Westesen et al., 1997). In addition, the reason for the decrease of active loading in microparticles could be a polymorphic transition of the lipid leading to expulsion/ leaking/ leaching of the AA from the SLMs during the progression of freeze drying (Liu et al., 2014). Interestingly, AASLMs prepared from cetyl alcohol stabilized by poloxamer 188 (F6, F12) gave higher %active loading (%AL) than the formula prepared from beeswax stabilized by poloxamer 188 (F3, F9) insignificantly ($p > 0.05$) (Table 7). Moreover, AASLMs stabilized by poloxamer 188 (F6) gave higher %AL than those stabilized by soybean lecithin (F5) and Tw80 (F4) ($p < 0.05$) (Table 7). This may be because P188 could immobilize the solid lipid system and

improve packing patterns on the lipid matrix surface which influence drug loading (Severino et al., 2012). In addition, it was observed that F5 had a higher active loading than F12. The larger particle size of F5 in comparing to that of F12 may cause a higher active loading (Jaspart et al., 2005; Passerini et al., 2002).

3. Stability studies

3.1 Physical appearance

The physical appearances of AASLMs formulations after kept at 4 °C, ambient temperature (AT) and 45 °C for 90 days were reported in Table 8. There were no changes in the physical appearance of AASLMs powder prepared from beeswax stabilized with poloxamer (P188) (F3, F9) or cetyl alcohol stabilized with P188 (F6, F12) after storage at 4 °C, RT and 45 °C for 90 days. A previous study observed that SLM with spherical shape were **β or β'** type crystals, which contributed to a good stability (El-Salamouni et al., 2015; Liu et al., 2017; Paucar et al., 2016). AASLM prepared from beeswax stabilized by Tween 80®(Tw80) (F1, F17) was stable under 4 °C, but were clumped together and collapsed when storage at RT and 45 °C. AASLM prepared from cetyl alcohol stabilized by Tw80 (F4, F10) or soybean lecithin (L) (F5, F11) were stable under 4 °C and RT conditions. However, these formulations became more humid and agglomerate powder under 45 ℃. This may be because Tw80 and lecithin have the ability to absorb moisture from the atmosphere. Furthermore, they were easily oxidized (agency, 2018). Our finding was in agreement with previous studied which suggested that the temperature at 4 ℃ was the most favorable storage temperature for solid lipid particles(Freitas & Müller, 1998).

Table 8 The physical appearance of AASLMs formulations at initial and after stored at 4 °C, ambient temperature and 45 °C for 3 months (90 days)

Formulas	Physical appearance at initial (T0)	Physical appearance								
		4 °C			Ambient temperature			45 °C		
		T30	T60	T90	T30	T60	T90	T30	T60	T90
F1	White to light yellow powder, coarse	+	+	+	-	-	-	-	-	-
F3	White to light yellow powder, coarse	+	+	+	+	+	+	+	+	+
F4	White powder, fine	+	+	+	+	+	+	-	-	-
F5	White powder, fine	+	+	+	+	+	+	-	-	-
F6	White powder, fine	+	+	+	+	+	+	+	+	+
F7	White to light yellow powder, coarse	+	+	+	-	-	-	-	-	-
F9	White to light yellow powder, coarse	+	+	+	+	+	+	+	+	+
F10	White powder, fine	+	+	+	+	+	+	-	-	-
F11	White powder, fine	+	+	+	+	+	+	-	-	-
F12	White powder, fine	+	+	+	+	+	+	+	+	+

T0 = initial time, T30 = kept at 30 days, T60 = kept at 60 days, T90 = kept at 90 days, + = acceptable, - = not acceptable

3.2 Particle size and size distribution

The particle size and size distribution of AASLMs formulations after kept at 4 °C, ambient temperature (RT) and 45 °C for 90 days were presented in Table 9. The particle size of all AASLMs formulation stored at 4 °C, RT and 45 °C tended to increase (Figure 19, 20, 21) significantly ($p < 0.05$) and size distribution (span) tended to reduce.

Table 9 The average of particle size and size distribution of AASLMs formulations at initial and after stored in 4 °C, ambient temperature and 45 °C for 90 days.

Formulas	The average particle size and span **Initial**		The average particle size and span **after storage 90 days**					
			4 °C		**Ambient temperature**		**45 °C**	
	Size (µm) ± SD	**Span ± SD**	**Size (µm) ± SD**	**Span ± SD**	**Size (µm) ± SD**	**Span ± SD**	**Size (µm) ± SD**	**Span ± SD**
F1 (10% B-Tw80)	38.86±0.34	3.62±0.03	70.16 ± 0.39	1.67 ± 0.01	61.55±0.24	1.90±0.01	N/A	N/A
F3 (10% B-P188)	14.15±0.11	12.89±0.26	16.42 ± 0.25	3.90 ± 0.03	14.78±0.22	3.22±0.04	17.86±0.24	4.97±0.06
F4 (10% C-Tw80)	25.72±0.28	5.18±0.05	46.23 ± 0.32	4.33 ± 0.02	39.64±0.25	3.44±0.02	36.10±0.29	3.97±0.04
F5 (10% C-SL)	23.61±0.22	4.84±0.04	37.19 ± 0.24	7.30 ± 0.06	35.76±0.16	6.62±0.07	31.90±0.18	5.99±0.04
F6 (10% C-P188)	7.49±0.19	3.68±0.04	15.01 ± 0.16	2.79 ± 0.02	8.73±0.11	3.75±0.06	11.95±0.12	2.77±0.02
F7 (15% B-Tw80)	30.67±0.27	4.29±0.05	68.24 ± 0.34	1.70 ± 0.01	50.89±0.39	1.48±0.01	N/A	N/A
F9 (15% B-P188)	16.87±0.15	14.64±0.42	18.26 ± 0.28	4.62 ± 0.04	18.10±0.19	3.41±0.02	18.96±0.26	4.69±0.04
F10 (15% C-Tw80)	28.54±0.12	4.89±0.04	50.41 ± 0.25	3.88 ± 0.02	47.49±0.16	3.24±0.02	41.53±0.11	4.21±0.03
F11 (15% C-SL)	17.92±0.24	6.91±0.08	29.23 ± 0.27	7.67 ± 0.08	21.19±0.17	8.88±0.14	28.84±0.25	6.96±0.11
F12 (15% C-P188)	7.46±0.08	3.49±0.07	12.67 ± 0.11	3.57 ± 0.02	8.23±0.14	3.66±0.07	11.57±0.17	3.53±0.03

N/A is not applicable.

3.3 Entrapment efficiency and active loading

The entrapment efficiency (%) and active loading (%) of AASLMs formulations after kept at 4 °C, ambient temperature and 45 °C after 90 days were presented in Table 10. After 90 days, formulas F3, F6, F9, F12 stored at 4 °C, RT and 45 °C retained the good %EE and %AL. This may be due to the stabilizing effect of P188. Previous study had also demonstrated that the solid lipid microparticle stabilized by poloxamer 188 had high entrapment efficiency of poorly water soluble substance (Hariyadi et al., 2022). In addition, P188 may be able to intercalate into the lipid matrix which improved polymorphic of lipids. Polymorphism is a phenomenon that can be demonstrated in lipids after the fusion or recrystallization process (El-Salamouni et al., 2015) which influence the encapsulation efficiency, drug loading and stability (Severino et al., 2012). Whereas, %EE and %AL of F1, F4, F7, F10 tended to decrease ($p < 0.05$) after storage at 4 °C, RT and 45 °C for 90 days. This may be because irregular shape of F1, F7, F4 and F10 causes asiatic acid to leak from solid lipid microparticles.

Table 10 The average of %entrapment efficiency (EE) and %active loading (AL) of AASLMs formulations compared with initial and after stored in 4 °C, ambient temperature and 45 °C for 90 days.

Formulas	The average of %EE and %AL Initial		The average of %EE and %AL after **storage 90 days**					
			4 °C		Ambient temperature		45 °C	
	%EE ± SD	**%AL ± SD**	**%EE ± SD**	**%AL ± SD**	**%EE ± SD**	**%AL ± SD**	**%EE ± SD**	**%AL ± SD**
F1	53.75±0.01	0.0272±0.001	40.75±0.04	0.018±0.0027	46.90±0.03	0.0207±0.0018	26.47±0.04	0.0096±0.0014
F3	100.00±0.00	0.0482±0.0009	100.00±0.00	0.0475±0.0019	100.00±0.00	0.0472±0.0020	100.00±0.00	0.0470±0.0028
F4	31.56±0.02	0.0167±0.0020	22.11±0.03	0.0087±0.0015	23.36±0.04	0.0103±0.0017	11.76±0.03	0.0043±0.0010
F5	58.80±0.02	0.0299±0.0017	49.69±0.05	0.0263±0.0038	48.73±0.08	0.0260±0.0043	46.71±0.09	0.0249±0.0046
F6	100.00±0.00	0.0494±0.0008	100.00±0.00	0.0489±0.0023	100.00±0.00	0.0488±0.0020	100.00±0.00	0.0484±0.0022
F7	51.56±0.03	0.0171±0.0011	46.87±0.06	0.0145±0.0009	45.24±0.05	0.0140±0.0018	26.36±0.02	0.0078±0.0007
F9	100.00±0.00	0.0315±0.0008	100.00±0.00	0.0312±0.0017	100.00±0.00	0.0307±0.0025	100.00±0.00	0.0305±0.0011
F10	42.77±0.03	0.0140±0.0010	28.10±0.06	0.0083±0.0018	27.25±0.03	0.0085±0.0008	27.32±0.03	0.0079±0.0005
F11	60.26±0.02	0.0196±0.0016	56.83±0.05	0.0177±0.0015	56.91±0.10	0.0180±0.0007	53.27±0.07	0.0167±0.0020
F12	100.00±0.00	0.0332±0.0008	100.00±0.00	0.0326±0.0017	100.00±0.00	0.0326±0.0014	100.00±0.00	0.0324±0.0017

4. Validation of HPLC method

The developed HPLC technique was used to determine the amount of asiatic acid in SLM formulations. The following topic validated the procedure. The process of validating an analytical method establishes that the method's performance characteristics satisfy the criteria for the intended analytical application. In terms of analytical parameters, the performance characteristics are expressed. Linearity, specificity, accuracy, and precision are some of these for HPLC assay validation.

Linearity

The ability to produce test results that are directly proportional to the concentration of the analyzer in the samples within a specified range, or that are proportionate to it through a clearly stated mathematical transformation, is referred to as the linearity of the analytical method. Table 18-19 displays the asiatic acid standard solutions representative calibration curve data. This line's coefficient of determination (R^2) was 0.9997. These findings suggested that the HPLC approach could accurately measure asiatic acid within the examined range.

Specificity

An analytical method's specificity is its capacity to measure the analysis precisely and specifically even when there are other components in the sample. The mobile phase consisted of 0.05% trifluoroacetic acid (TFA) in acetonitrile and 0.05% TFA in water (45:55). After the reagents were introduced and stored in each condition, it was discovered that there was no interference from additional components (Figure 19). When compared to the nearby peak, each peak's resolution value is more than 1.5. (standard) (Table 18). Therefore, the specificity of the HPLC approach was acceptable. Figure 19. displays the usual chromatograms of the asiatic acid standard solution and the physical mixture of blank SLM with AA. Each chromatogram is displayed with the identical attenuation and scale.

Accuracy

Examining three sets of three asiatic acid concentrations at low, medium, and high (0.009, 0.050, and 1.000 mg/ml) in SLM formulations, the accuracy of the measurements was determined. The inversely calculated concentrations and analytical recovery percentage were in the range of 98.87-100.99% (within range 98-102%), indicating that this approach could be utilized for asiatic acid analysis at all concentrations investigated with good accuracy. Table 21-24 displays the accuracy data.

Precision

When the procedure is repeatedly used to multiple sampling of the homogenous samples, the precision of an analytical method is measured by the degree of agreement among individual test findings. The standard division or relative standard division (also known as the coefficient of variation, or %CV of a sequence of data) is typically used to express the precision of an analytical procedure. The data of the within-run precision and between-run precision of asiatic acid in SLM formulation are shown in Tables 25-26, respectively. The ranges for each coefficient of variation were 0.001-0.056 and 0.002-0.070%. Respectively. An analytical method's coefficient of variation should typically be less than 2%. The quantitative analysis of asiatic acid in the range under study was therefore accurate using the HPLC method.

Limit of detection (LOD) Limit of quantitation (LOQ)

The results showed that the limits of detection (LOD) was found to be equal to 0.00058 mg/ml. and the limit of quantification (LOQ) was found to be equal to 0.0018 mg/ml.

In conclusion, the analysis of asiatic acid content in SLM formulations by HPLC method developed in this study showed good linearity, accuracy, specificity, precision and the limits of detection (LOD) and quantification (LOQ) were both determined and found to be equal 0.00058 and 0.0018 mg/ml, respectively. Thus, this method was used for determination of the content of asiatic acid in SLM formulation to evaluate its stability.

CHAPTER V

CONCLUSIONS

The objective of this study was to evaluate the effects of solid lipid (beeswax and cetyl alcohol) and surfactant namely Tween 80®, soybean lecithin, and poloxamer 188 on the characteristics of solid lipid microparticles containing asiatic acid (AASLM). The AASLM were prepared from 10% or 15% of beeswax together with 3% of surfactants by melt dispersion cum freeze-drying technique to obtain a solid dosage form. Our findings indicated that the difference type of lipids used in AASLMs preparation could significantly affect particle size, entrapment efficiency and active loading of the AASLMs, whereas the amount of solid lipid used could influence the entrapment efficiency (EE) and active loading (AL) of the AASLMs. Moreover, the type of surfactant used in AASLMs preparation could significantly affect the physicochemical properties including morphology, particle size, %EE and %AL of the resultant AASLMs. The AASLMs prepared from beeswax or cetyl alcohol stabilized by poloxamer 188 (F3, F6, F9, F12) provided the highest entrapment efficiency. These formulations were stable under 4 °C, ambient temperature and 45 °C for 90 days. It could be concluded that the AASLM stabilized by poloxamer 188 has the greatest potential among the test formulations as a topical carrier for AA. Further studies should be conducted to explore its utilization in cosmetics.

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APPENDICES

APPENDIX A

Table 11 The average of particle size and size distribution of AASLMs formulations compared with initial and after stored in 4 °C, ambient temperature and 45 °C for 30 days.

Formulas	The average particle size and span **Initial**		The average particle size and span **after storage 30 days**					
			4 °C		**RT**		**45 °C**	
	Size (μm) ± SD	**Span ± SD**	**Size (μm) ± SD**	**Span ± SD**	**Size (μm) ± SD**	**Span ± SD**	**Size (μm) ± SD**	**Span ± SD**
F1 (10% B-Tw80)	38.86±0.34	3.62±0.03	56.60±0.19	2.57 ± 0.01	42.60±0.28	2.57± 0.02	N/A	N/A
F3 (10% B-P188)	14.15±0.11	12.89±0.26	15.10±0.22	4.12 ± 0.06	14.39±0.15	2.66 ±0.03	16.17±0.25	3.02 ± 0.04
F4 (10% C-Tw80)	25.72±0.28	5.18±0.05	39.41±0.15	3.96 ± 0.03	33.88±0.20	2.82 ± 0.02	33.78±0.19	3.84 ± 0.03
F5 (10% C-SL)	23.61±0.22	4.84±0.04	28.17±0.11	5.13 ± 0.04	25.27±0.18	4.82 ± 0.04	27.37±0.18	4.94 ± 0.05
F6 (10% C-P188)	7.49±0.19	3.68±0.04	14.05±0.09	2.70 ± 0.02	8.01±0.10	3.83 ± 0.07	11.26±0.07	2.83 ± 0.03
F7 (15% B-Tw80)	30.67±0.27	4.29±0.05	50.27±0.34	2.69 ± 0.02	39.26±0.32	1.39 ± 0.01	N/A	N/A
F9 (15% B-P188)	16.87±0.15	14.64±0.42	17.69±0.15	4.55 ± 0.05	17.51± 0.24	3.14 ± 0.02	17.84±0.18	3.97 ± 0.04
F10 (15% C-Tw80)	28.54±0.12	4.89±0.04	41.76±0.17	3.85 ± 0.04	37.50± 0.27	2.37 ± 0.01	35.84±0.26	4.50 ± 0.04
F11 (15% C-SL)	17.92±0.24	6.91±0.08	25.90±0.26	8.37 ± 0.10	18.38 ±0.19	6.91 ± 0.07	22.47±0.14	5.82 ± 0.08
F12 (15% C-P188)	7.46±0.08	3.49±0.07	11.75±0.10	2.16 ± 0.02	7.70 ± 0.12	3.33 ± 0.08	10.36±0.15	4.09 ± 0.06

N/A is not applicable.

Table 12 The average of particle size and size distribution of AASLMs formulations compared with initial and after stored in 4 °C, ambient temperature and 45 °C for 60 days.

Formulas	The average particle size and span **Initial**		The average particle size and span **after storage 60 days**					
			4 °C		**RT**		**45 °C**	
	Size (μm) ± SD	**Span ± SD**	**Size (μm) ± SD**	**Span ± SD**	**Size (μm) ± SD**	**Span ± SD**	**Size (μm) ± SD**	**Span ± SD**
F1 (10% B-Tw80)	38.86±0.34	3.62±0.03	68.66 ± 0.34	2.54 ± 0.01	56.12 ±0.29	2.22 ± 0.01	N/A	N/A
F3 (10% B-P188)	14.15±0.11	12.89±0.26	15.76 ± 0.20	3.70 ± 0.05	14.57 ±0.17	2.68 ± 0.04	16.97±0.26	4.28 ± 0.03
F4 (10% C-Tw80)	25.72±0.28	5.18±0.05	43.81 ± 0.27	4.38 ± 0.03	36.26 ±0.22	2.97 ± 0.01	35.46 ±0.15	3.8 ± 0.02
F5 (10% C-SL)	23.61±0.22	4.84±0.04	30.69 ± 0.23	7.23 ± 0.07	28.05 ±0.15	4.70 ± 0.05	29.63 ±0.17	5.4 ± 0.05
F6 (10% C-P188)	7.49±0.19	3.68±0.04	14.89 ± 0.10	2.31 ± 0.02	8.44 ± 0.12	3.55 ± 0.08	11.63±0.09	3.32 ± 0.05
F7 (15% B-Tw80)	30.67±0.27	4.29±0.05	64.67 ± 0.25	2.57 ± 0.02	44.80 ±0.24	1.54 ± 0.01	N/A	N/A
F9 (15% B-P188)	16.87±0.15	14.64±0.42	17.95 ± 0.27	4.61 ± 0.04	17.73 ±0.21	3.54 ± 0.03	18.15±0.25	4.51 ± 0.03
F10 (15% C-Tw80)	28.54±0.12	4.89±0.04	45.27 ± 0.20	3.78 ± 0.03	42.88 ±0.15	2.69 ± 0.02	38.01±0.23	4.4 ± 0.03
F11 (15% C-SL)	17.92±0.24	6.91±0.08	27.78 ± 0.15	6.56 ± 0.07	19.39 ±0.23	8.24 ± 0.19	27.66±0.25	5.69 ± 0.07
F12 (15% C-P188)	7.46±0.08	3.49±0.07	12.21 ± 0.14	2.43 ± 0.02	7.88 ± 0.06	3.44 ± 0.06	10.52±0.12	4.01 ± 0.04

N/A is not applicable.

Table 13 The average of particle size and size distribution of AASLMs formulations compared with initial and after stored in 4 °C, ambient temperature and 45 °C for 90 days.

Formulas	The average particle size and span **Initial**		The average particle size and span **after storage 90 days**					
			4 °C		**RT**		**45 °C**	
	Size (µm) ± SD	**Span ± SD**	**Size (µm) ± SD**	**Span ± SD**	**Size (µm) ± SD**	**Span ± SD**	**Size (µm) ± SD**	**Span ± SD**
F1 (10% B-Tw80)	38.86±0.34	3.62±0.03	70.16 ± 0.39	1.67 ± 0.01	61.55±0.24	1.90±0.01	N/A	N/A
F3 (10% B-P188)	14.15±0.11	12.89±0.26	16.42 ± 0.25	3.90 ± 0.03	14.78±0.22	3.22±0.04	17.86±0.24	4.97±0.06
F4 (10% C-Tw80)	25.72±0.28	5.18±0.05	46.23 ± 0.32	4.33 ± 0.02	39.64±0.25	3.44±0.02	36.10±0.29	3.97±0.04
F5 (10% C-SL)	23.61±0.22	4.84±0.04	37.19 ± 0.24	7.30 ± 0.06	35.76±0.16	6.62±0.07	31.90±0.18	5.99±0.04
F6 (10% C-P188)	7.49±0.19	3.68±0.04	15.01 ± 0.16	2.79 ± 0.02	8.73±0.11	3.75±0.06	11.95±0.12	2.77±0.02
F7 (15% B-Tw80)	30.67±0.27	4.29±0.05	68.24 ± 0.34	1.70 ± 0.01	50.89±0.39	1.48±0.01	N/A	N/A
F9 (15% B-P188)	16.87±0.15	14.64±0.42	18.26 ± 0.28	4.62 ± 0.04	18.10±0.19	3.41±0.02	18.96±0.26	4.69±0.04
F10 (15% C-Tw80)	28.54±0.12	4.89±0.04	50.41 ± 0.25	3.88 ± 0.02	47.49±0.16	3.24±0.02	41.53±0.11	4.21±0.03
F11 (15% C-SL)	17.92±0.24	6.91±0.08	29.23 ± 0.27	7.67 ± 0.08	21.19±0.17	8.88±0.14	28.84±0.25	6.96±0.11
F12 (15% C-P188)	7.46±0.08	3.49±0.07	12.67 ± 0.11	3.57 ± 0.02	8.23±0.14	3.66±0.07	11.57±0.17	3.53±0.03

N/A is not applicable.

Table 14 The average of **% entrapment efficiency** of AASLMs formulations compared with initial and after stored in 4 °C, ambient temperature and 45 °C for 30 days, 60 days and 90 days.

Formulation	The average of % entrapment efficiency									
	Initial	4 °C			Ambient temperature			45 °C		
		30 days	60 days	90 days	30 days	60 days	90 days	30 days	60 days	90 days
F1	53.75 ± 0.01	45.21 ± 0.05	42.26 ± 0.06	40.75 ± 0.04	52.74 ± 0.02	48.27 ± 0.04	46.9 ± 0.03	43.45 ± 0.04	27.18 ± 0.04	26.47 ± 0.04
F3	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
F4	31.56 ± 0.02	24.57 ± 0.03	22.50 ± 0.05	22.11 ± 0.03	27.28 ± 0.02	26.18 ± 0.02	23.36 ± 0.04	27.52 ± 0.03	12.40 ± 0.05	11.76 ± 0.03
F5	58.80 ± 0.02	57.88 ± 0.06	56.65 ± 0.03	49.69 ± 0.05	55.65 ± 0.08	54.41 ± 0.04	48.73 ± 0.08	54.81 ± 0.07	50.52 ± 0.06	46.71 ± 0.09
F6	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
F7	51.56 ± 0.03	51.15 ± 0.03	47.7 ± 0.07	46.87 ± 0.06	50.72 ± 0.04	47.45 ± 0.07	45.24 ± 0.05	50.38 ± 0.03	28.6 ± 0.02	26.36 ± 0.02
F9	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
F10	42.77 ± 0.03	31.38 ± 0.04	27.84 ± 0.05	28.10 ± 0.06	31.75 ± 0.03	28.2 ± 0.04	27.25 ± 0.03	35.92 ± 0.04	29.27 ± 0.05	27.32 ± 0.03
F11	60.26 ± 0.02	59.28 ± 0.03	58.19 ± 0.03	56.83 ± 0.17	58.76 ± 0.04	57.96 ± 0.17	56.91 ± 0.10	56.74 ± 0.05	56.40 ± 0.04	53.27 ± 0.07
F12	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Table 15 The average of **% active loading** of AASLMs formulations compared with initial and after stored in 4 ℃, ambient temperature and 45 ℃ for 30 days, 60 days and 90 days.

Formulation	The average of % Active loading									
	Initial	4 ℃			Ambient temperature			45 ℃		
		30 days	60 days	90 days	30 days	60 days	90 days	30 days	60 days	90 days
F1	0.0272±0.001	0.0224±0.0027	0.0197±0.0036	0.0180±0.0027	0.0262±0.0018	0.0223±0.0028	0.0207±0.0018	0.0214±0.0023	0.0115±0.0019	0.0096±0.0014
F3	0.0482±0.0009	0.0481±0.0010	0.0480±0.0015	0.0475±0.0019	0.0482±0.0012	0.0479±0.0016	0.0472±0.0020	0.0480±0.0010	0.0478±0.0012	0.0470±0.0028
F4	0.0167±0.0020	0.0116±0.0015	0.0093±0.0023	0.0087±0.0015	0.0133±0.0008	0.0126±0.001	0.0103±0.0017	0.0124±0.0017	0.0050±0.0021	0.0043±0.0010
F5	0.0299±0.0017	0.0272±0.0043	0.0275±0.0043	0.0263±0.0038	0.0267±0.0021	0.0262±0.0027	0.0260±0.0043	0.0267±0.0044	0.0256±0.0047	0.0249±0.0046
F6	0.0494±0.0008	0.0492±0.0011	0.0492±0.0012	0.0489±0.0023	0.0492±0.0011	0.0491±0.0012	0.0488±0.0020	0.0492±0.0013	0.0491±0.0009	0.0484±0.0022
F7	0.0171±0.0011	0.0169±0.0009	0.0155±0.0022	0.0145±0.0009	0.0165±0.0013	0.0152±0.0028	0.0140±0.0018	0.0165±0.0008	0.0091±0.0007	0.0078±0.0007
F9	0.0315±0.0008	0.0315±0.0013	0.0314±0.0022	0.0312±0.0017	0.0315±0.0013	0.0313±0.0018	0.0307±0.0025	0.0314±0.0013	0.0312±0.0016	0.0305±0.0011
F10	0.0140±0.0010	0.0099±0.0012	0.0087±0.0020	0.0083±0.0018	0.0102±0.0012	0.0090±0.0015	0.0085±0.0008	0.0114±0.0009	0.0089±0.0015	0.0079±0.0005
F11	0.0196±0.0016	0.0195±0.0012	0.0194±0.0018	0.0177±0.0015	0.0194±0.0008	0.0194±0.0014	0.0180±0.0007	0.0178±0.0009	0.0178±0.0017	0.0167±0.0020
F12	0.0332±0.0008	0.0330±0.0008	0.0327±0.0013	0.0326±0.0017	0.0332±0.0008	0.0328±0.0023	0.0326±0.0014	0.0330±0.0011	0.0327±0.0010	0.0324±0.0017

Table 16 The average of **% labelled amount** of AASLMs formulations compared with initial and after stored in 4 °C, ambient temperature and 45 °C for 30 days, 60 days and 90 days.

Formulation	The average of % labelled amount									
	Initial	4 °C			Ambient temperature			45 °C		
		30 days	60 days	90 days	30 days	60 days	90 days	30 days	60 days	90 days
F1	94.23 ± 0.04	93.12 ± 0.05	86.79 ± 0.06	82.51 ± 0.07	93.07 ± 0.05	86.32 ± 0.06	82.89 ± 0.05	92.84 ± 0.09	78.95 ± 0.05	68.76 ± 0.09
F3	90.43 ± 0.02	90.25 ± 0.02	90.11 ± 0.03	89.15 ± 0.03	90.36 ± 0.02	89.76 ± 0.03	88.55 ± 0.04	89.95 ± 0.02	89.7 ± 0.02	88.17 ± 0.05
F4	92.15 ± 0.02	88.04 ± 0.04	76.9 ± 0.08	74.36 ± 0.09	91.58 ± 0.03	90.53 ± 0.03	82.55 ± 0.06	84.46 ± 0.07	76.13 ± 0.07	69.98 ± 0.11
F5	90.70 ± 0.02	57.88 ± 0.06	56.65 ± 0.03	49.69 ± 0.05	79.41 ± 0.06	54.41 ± 0.04	48.73 ± 0.08	54.81 ± 0.07	52.30 ± 0.04	46.71 ± 0.09
F6	92.99 ± 0.01	92.34 ± 0.02	92.23 ± 0.02	91.68 ± 0.04	92.31 ± 0.02	92.02 ± 0.02	91.56 ± 0.04	92.18 ± 0.02	91.98 ± 0.02	91.43 ± 0.04
F7	95.26 ± 0.02	95.16 ± 0.03	93.44 ± 0.05	89.73 ± 0.07	93.33 ± 0.02	91.83 ± 0.1	88.72 ± 0.06	94.3 ± 0.02	91.75 ± 0.05	85.41 ± 0.09
F9	90.69 ± 0.02	90.42 ± 0.04	90.16 ± 0.06	89.56 ± 0.05	90.62 ± 0.04	90.03 ± 0.05	89.27 ± 0.05	90.14 ± 0.04	89.8 ± 0.05	88.65 ± 0.03
F10	94.15 ± 0.05	90.42 ± 0.05	88.71 ± 0.05	86.91 ± 0.07	92.39 ± 0.03	91.97 ± 0.07	89.68 ± 0.03	91.63 ± 0.06	88.26 ± 0.12	83.49 ± 0.08
F11	91.22 ± 0.03	59.28 ± 0.03	58.19 ± 0.03	56.83 ± 0.17	58.76 ± 0.04	57.96 ± 0.17	56.91 ± 0.1	56.74 ± 0.05	56.40 ± 0.04	53.27 ± 0.07
F12	95.38 ± 0.02	94.91 ± 0.02	94.38 ± 0.03	93.8 ± 0.05	95.10 ± 0.02	94.27 ± 0.07	93.64 ± 0.04	94.82 ± 0.03	94.11 ± 0.03	93.47 ± 0.04

APPENDIX B

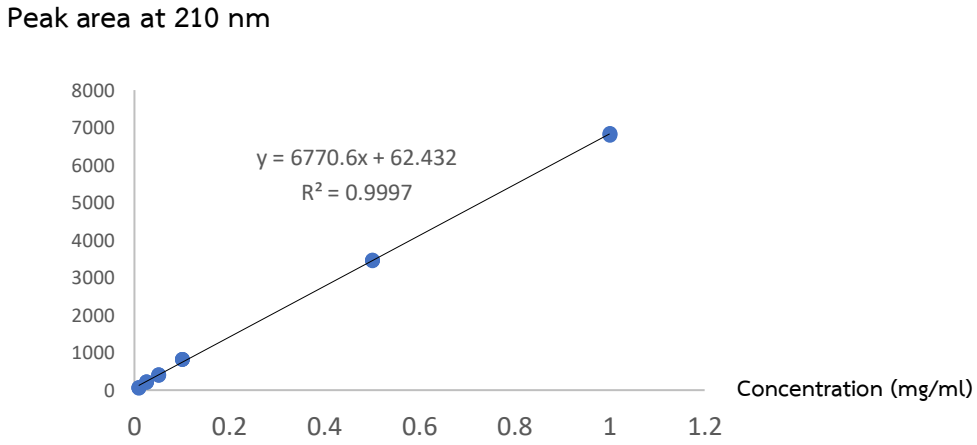


Figure 12 Calibration curve of asiatic acid by HPLC method

Table 17 Data for calibration curve of asiatic acid predicted concentration by HPLC method.

Theorical concentration (mg/ml)	Peak area 210 nm			Predicted concentration (mg/ml)			% Recovery				SD	%CV
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	Mean		
0.009	60.8026	62.0117	60.5976	0.0089	0.0090	0.0088	98.5708	100.5310	98.2384	99.1134	1.2389	1.2499
0.025	165.3123	169.3899	169.3929	0.0241	0.0247	0.0247	96.4792	98.8590	98.8607	98.0663	1.3745	1.4016
0.050	334.8729	345.2622	339.9277	0.0489	0.0504	0.0496	97.7189	100.7506	99.1939	99.2211	1.5160	1.5279
0.100	658.4766	679.9277	680.5054	0.0961	0.0992	0.0993	96.0747	99.2045	99.2888	98.1893	1.8318	1.8656
0.500	3459.3198	3454.9959	3460.7627	0.5047	0.5041	0.5049	100.9460	100.8199	100.9881	100.9180	0.0876	0.0868
1.000	6793.7637	6842.8784	6825.7822	0.9912	0.9984	0.9959	99.1240	99.8406	99.5912	99.5186	0.3638	0.3655

Table 18 Data for calibration curve of asiatic acid by HPLC method

Concentration (mg/ml)	Peak area 210 nm			Mean	SD	%CV
	1st	2nd	3rd			
0.009	60.8026	62.0117	60.5976	61.1373	0.7642	1.2499
0.025	165.3123	169.3899	169.3929	168.0317	2.3551	1.4016
0.050	334.8729	345.2622	339.9277	340.0209	5.1953	1.5279
0.100	658.4766	679.9277	680.5054	672.9699	12.5549	1.8656
0.500	3459.3198	3454.9959	3460.7627	3458.3595	3.0010	0.0868
1.000	6793.7637	6842.8784	6825.7822	6820.8081	24.9323	0.3655
R^2	0.9997	0.9997	0.9997	0.9997	-	-
Intercept	66.9194	61.4851	58.8910	62.4318	-	-
Slope	6743.5707	6788.4432	6779.7490	6770.5876	-	-

Table 19 Data for specificity validation after the reagents were add for HPLC method

Conditions	Retention time	Peak area	Resolution
Standard AA	9.349	328.897	28.223
0.1 HCL	9.126	309.622	23.607
30% H2O2	9.123	324.736	20.151
Heat	9.286	316.174	25.460
Water	9.138	320.561	23.420
Light	9.243	322.551	24.340

Table 20 The percentages of analytical recovery of low, medium and high concentration of asiatic acid with blank SLM formulations (F3) by HPLC method.

Formulations	Concentration (mg/ml)	Estimated concentration (mg/ml)	% Recovery	Mean ± SD
F3	0.009	0.00885	98.33	
		0.00898	99.78	
		0.00889	98.78	98.87 ± 0.57
		0.00886	98.44	
		0.00891	99.00	
	0.050	0.04997	99.94	
		0.04935	98.70	
		0.04934	98.69	99.14 ± 0.54
		0.04980	99.60	
		0.04937	98.74	
	1.000	0.99177	99.18	
		0.98982	98.98	
		0.99157	99.16	99.04 ± 0.56
		0.99629	99.63	
		0.98274	98.27	

Table 21 The percentages of analytical recovery of low, medium and high concentration of asiatic acid with blank SLM formulations (F6) by HPLC method.

Formulations	Concentration (mg/ml)	Estimated concentration (mg/ml)	% Recovery	Mean ± SD
F6	0.009	0.00904	100.44	
		0.00905	100.56	
		0.00898	99.78	100.22 ± 0.53
		0.00896	99.56	
		0.00907	100.78	
	0.050	0.05023	100.46	
		0.05063	101.26	
		0.04951	99.02	100.31 ± 0.89
		0.05047	100.94	
		0.04993	99.86	
	1.000	1.00994	100.99	
		1.00183	100.18	
		1.00352	100.35	100.54 ± 0.35
		1.00611	100.61	
		1.00539	100.54	

Table 22 The percentages of analytical recovery of low, medium and high concentration of asiatic acid with blank SLM formulations (F9) by HPLC method.

Formulations	Concentration (mg/ml)	Estimated concentration (mg/ml)	% Recovery	Mean ± SD
F9	0.009	0.00890	98.84	
		0.00894	99.28	
		0.00906	100.67	99.40 ± 0.82
		0.00897	99.67	
		0.00887	98.56	
	0.050	0.05021	100.42	
		0.04935	98.70	
		0.04989	99.78	99.67 ± 0.84
		0.05031	100.62	
		0.04968	99.36	
	1.000	0.99110	99.11	
		0.98632	98.63	
		0.99729	99.73	99.70 ± 0.97
		1.00329	100.33	
		1.00682	100.68	

Table 23 The percentages of analytical recovery of low, medium and high concentration of asiatic acid with blank SLM formulations (F12) by HPLC method.

Formulations	Concentration (mg/ml)	Estimated concentration (mg/ml)	% Recovery	Mean ± SD
F12	0.009	0.00909	101.00	
		0.00902	100.22	
		0.00911	101.22	100.73 ± 0.41
		0.00904	100.44	
		0.00907	100.78	
	0.050	0.05048	100.96	
		0.05071	101.42	
		0.05051	101.02	100.80 ± 0.55
		0.04997	99.94	
		0.05033	100.66	
	1.000	1.00572	100.57	
		1.00986	100.99	
		1.01085	101.09	100.99 ± 0.44
		1.00657	100.66	
		1.01643	101.64	

Table 24 Data of within-run precision by HPLC method.

Concentration (mg/ml)	Estimated concentration (mg/ml)					Mean	SD	%CV
	1	2	3	4	5			
0.009	0.0092	0.009	0.0094	0.0091	0.0093	0.0092	0.00016	1.7186
0.05	0.0591	0.0595	0.0589	0.0583	0.0582	0.0588	0.00055	0.9315
1.00	1.0074	1.0061	1.0054	1.0057	1.0082	1.0066	0.00119	0.1185

Table 25 Data of between-run precision by HPLC method.

Concentration (mg/ml)	Estimated concentration (mg/ml)					Mean	SD	%CV
	1	2	3	4	5			
0.009	0.0091	0.0089	0.0088	0.0092	0.0091	0.0090	0.00016	1.8217
0.05	0.0574	0.0593	0.0594	0.0596	0.0584	0.0588	0.00092	1.5600
1.00	1.0083	1.0043	1.0036	1.00521	1.0053	1.0053	0.00180	0.1786

APPENDIX C

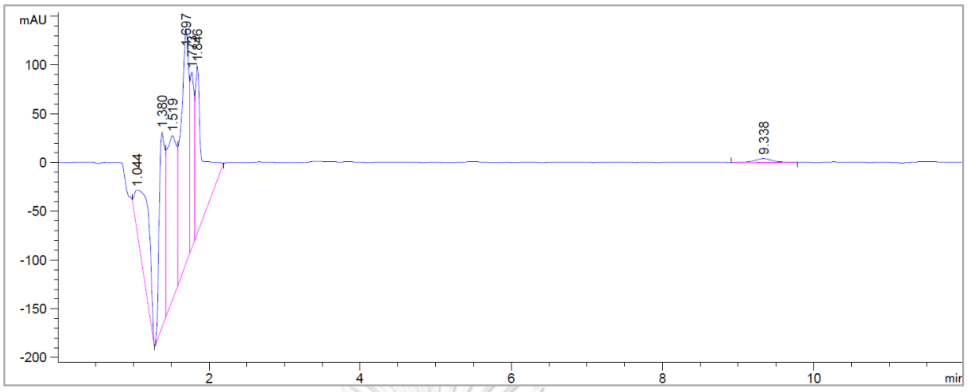


Figure 13 HPLC chromatogram of standard solution o asiatic acid (0.009 mg/ml)

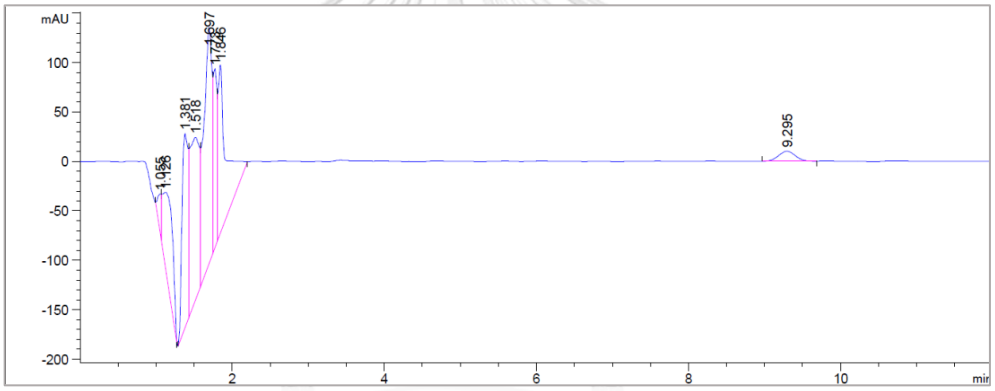


Figure 14 HPLC chromatogram of standard solution o asiatic acid (0.025 mg/ml)

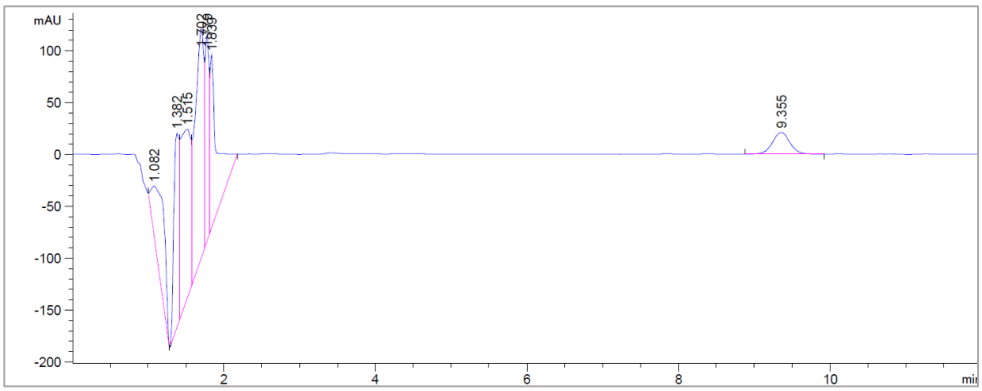


Figure 15 HPLC chromatogram of standard solution o asiatic acid (0.050 mg/ml)

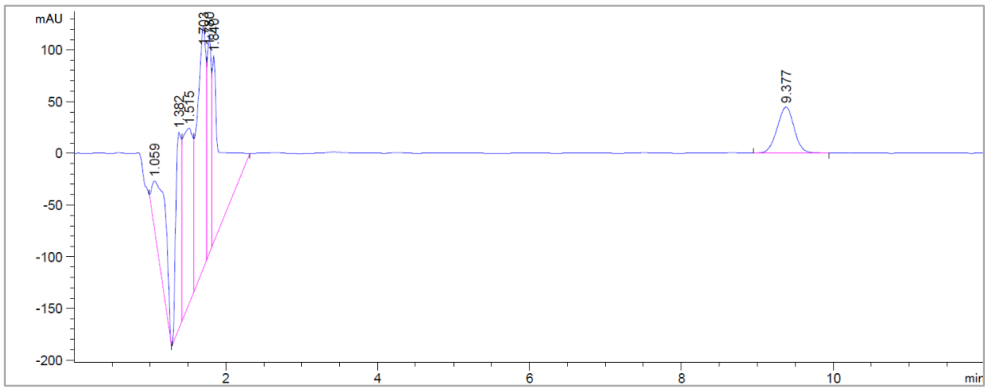


Figure 16 HPLC chromatogram of standard solution o asiatic acid (0.100 mg/ml)

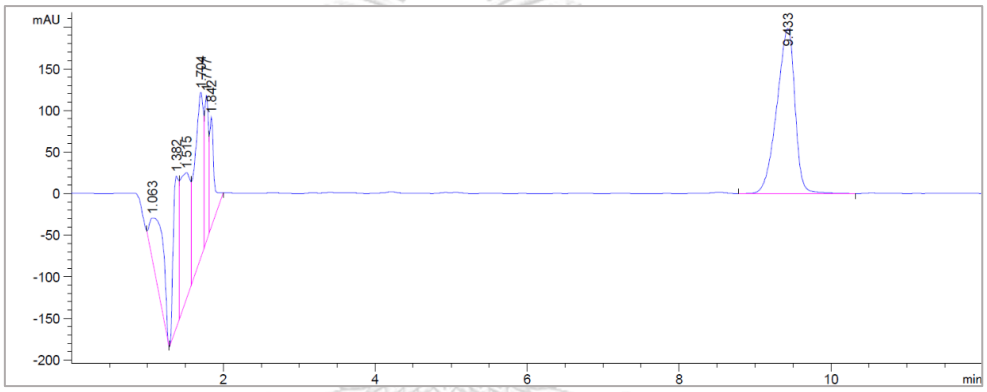


Figure 17 HPLC chromatogram of standard solution o asiatic acid (0.500 mg/ml)

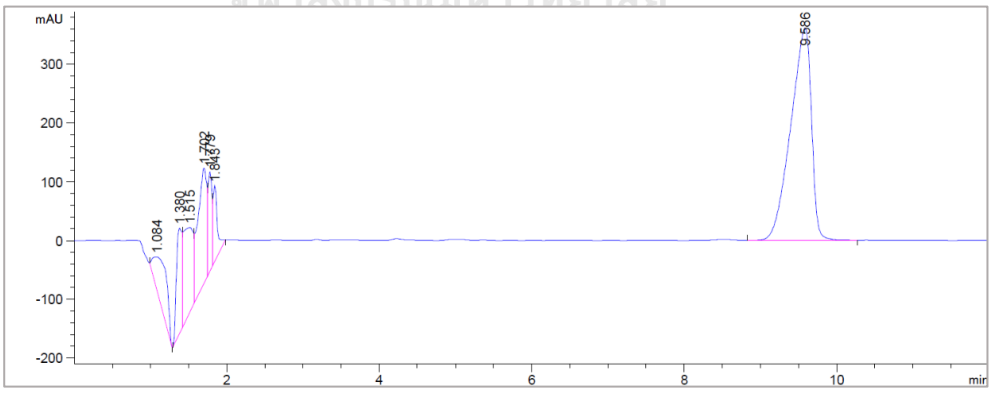
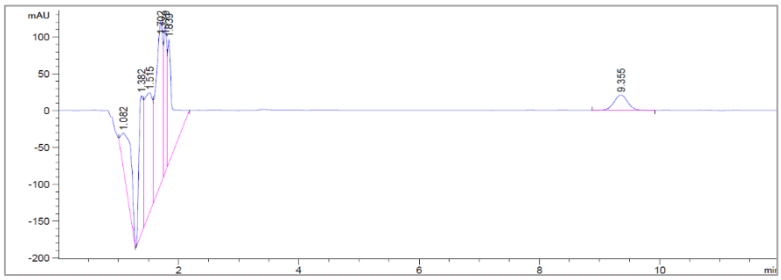
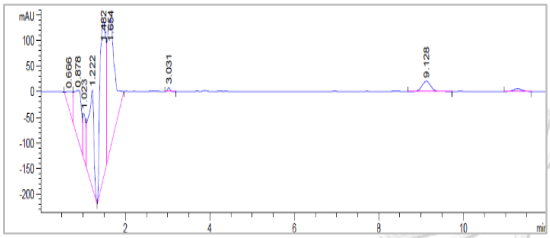


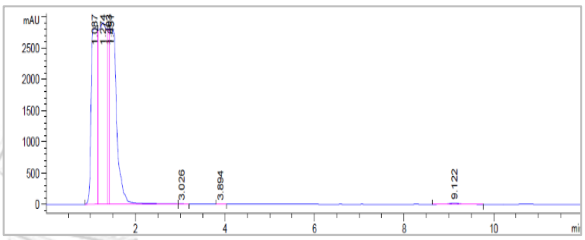
Figure 18 HPLC chromatogram of standard solution o asiatic acid (1.000 mg/ml)



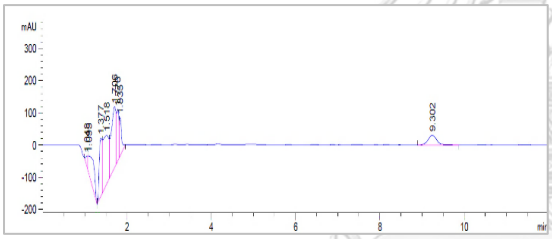
(A) Standard solution of asiatic acid (0.050 mg/ml)



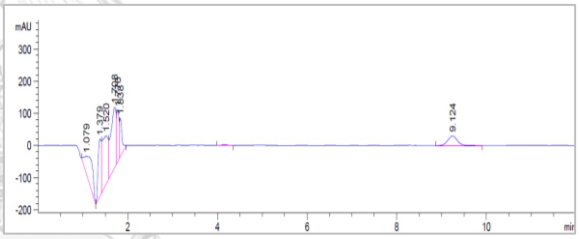
(B) 0.1 N HCl with AA 0.05 mg/ml



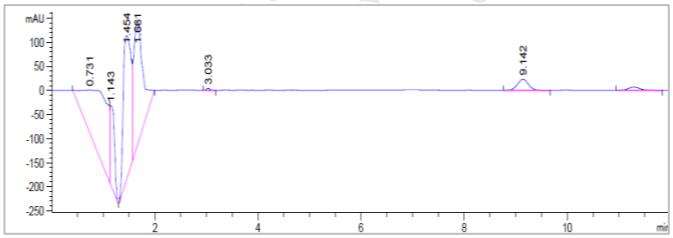
(C) 30% v/v H2O2 with AA 0.05 mg/ml



(D) heat 80 ℃ with AA 0.05 mg/ml



(E) light with AA 0.05 mg/ml



(F) water with AA 0.05 mg/ml

Figure 19 HPLC chromatogram of AA were stored in each condition; (A) Standard solution of asiatic acid (0.050 mg/ml), (B) 0.1 N HCl with AA 0.05 mg/ml, (C) 30% v/v H2O2 with AA 0.05 mg/ml, (D) heat 80 ℃ with AA 0.05 mg/ml, (E) light with AA 0.05 mg/ml, (F) water with AA 0.05 mg/ml, respectively.

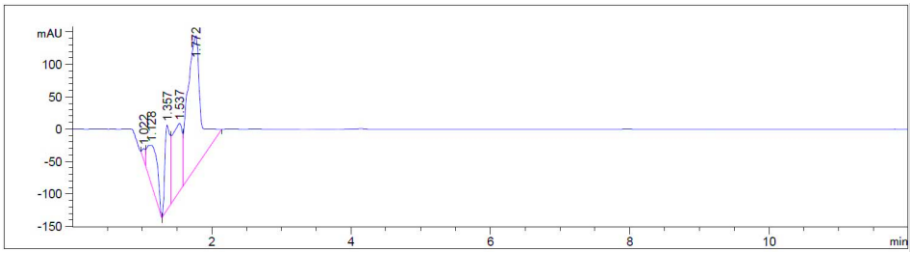


Figure 20 HPLC chromatogram of blank 10% beeswax with 3% P188

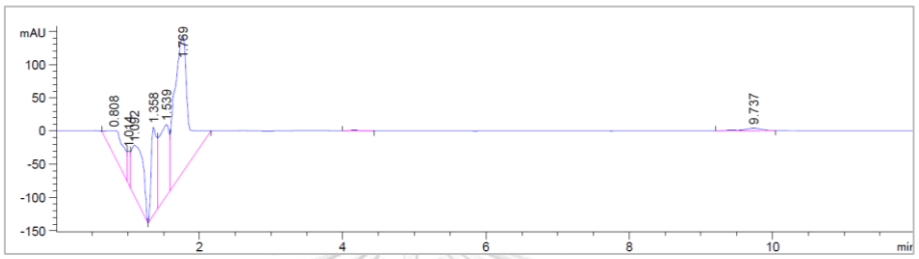


Figure 21 HPLC chromatogram of physical mixture of blank 10% beeswax with 3% P188: AA 0.009 mg/ml

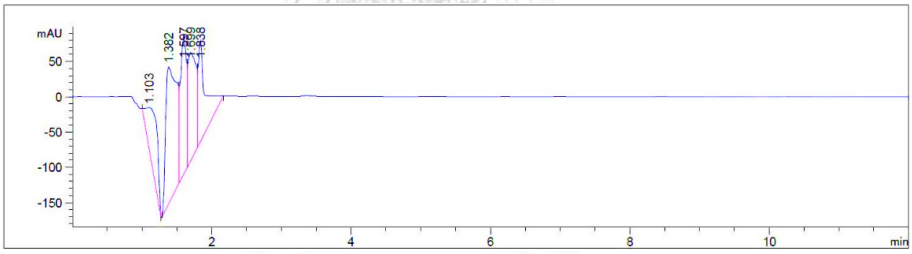


Figure 22 HPLC chromatogram of blank 10% cetyl alcohol with 3% P188

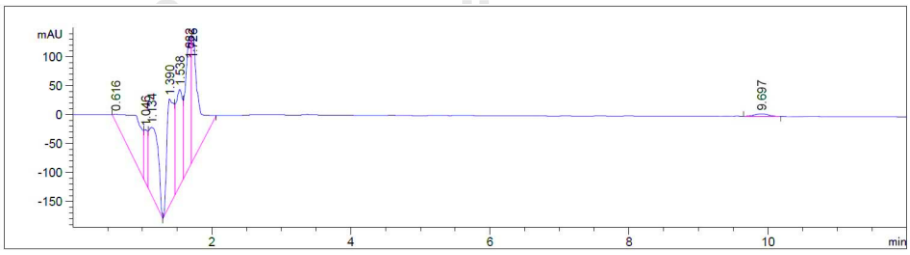


Figure 23 HPLC chromatogram of physical mixture of blank 10% cetyl alcohol with 3% P188: AA 0.009 mg/ml

APPENDIX D

Table 26 Various of AASLMs for particle size by ANOVA test.

Formulation	Value Label
F1	10% B-Tw80
F3	10% B-P188
F4	10% C-Tw80
F5	10% C-SL
F6	10% C-P188
F7	15% B-Tw80
F9	15% B-P188
F10	15% C-Tw80
F11	15% C-SL
F12	15% C-P188

Table 27 Test of homogeneity of variances of particle size in AASLMs (data specified in Table 6, page 29)

Test of homogeneity of variance

Dependent Variable: Size

F	df1	df2	Sig.
7.437	9	80	0.001

Result: Heterogeneity of variance ($p = 0.001 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 28 Multiple comparison of variances of particle size in AASLMs (data specified in Table 6, page 29)

Multiple Comparisons

Dependent Variable: Size

Dunnett T3

Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F1	F3	24.7078*	.11974	p < 0.05	24.1955	25.2200
	F4	13.1433*	.14840	p < 0.05	12.5697	13.7170
	F5	15.2489*	.13512	p < 0.05	14.7138	15.7840
	F6	31.3700*	.12999	p < 0.05	30.8459	31.8941
	F7	8.1867*	.14514	p < 0.05	7.6235	8.7498
	F9	21.9911*	.12421	p < 0.05	21.4757	22.5066
	F10	10.3156*	.12136	p < 0.05	9.8026	10.8286
	F11	20.9389*	.13915	p < 0.05	20.3934	21.4844
	F12	31.3989*	.11764	p < 0.05	30.8868	31.9110
F3	F1	-24.7078*	.11974	p < 0.05	-25.2200	-24.1955
	F4	-11.5644*	.10096	p < 0.05	-11.9889	-11.1400
	F5	-9.4589*	.08018	p < 0.05	-9.7858	-9.1320
	F6	6.6622*	.07120	p < 0.05	6.3772	6.9472
	F7	-16.5211*	.09612	p < 0.05	-16.9228	-16.1194
	F9	-2.7167*	.06001	p < 0.05	-2.9507	-2.4826
	F10	-14.3922*	.05385	p < 0.05	-14.5998	-14.1846
	F11	-3.7689*	.08680	p < 0.05	-4.1268	-3.4109
	F12	6.6911*	.04485	p < 0.05	6.5170	6.8652
F4	F1	-13.1433*	.14840	p < 0.05	-13.7170	-12.5697
	F3	11.5644*	.10096	p < 0.05	11.1400	11.9889
	F5	2.1056*	.11880	p < 0.05	1.6437	2.5674
	F6	18.2267*	.11293	p < 0.05	17.7813	18.6721
	F7	-4.9567*	.13009	p < 0.05	-5.4569	-4.4564
	F9	8.8478*	.10623	p < 0.05	8.4167	9.2788
	F10	-2.8278*	.10288	p < 0.05	-3.2541	-2.4015
	F11	7.7956*	.12336	p < 0.05	7.3191	8.2720
	F12	18.2556*	.09847	p < 0.05	17.8322	18.6789

Table 28 Multiple comparison of variances of particle size in AASLMs (continuous) (data specified in Table 6, page 29)

Multiple Comparisons

Dependent Variable: Size

Dunnett T3

Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F5	F1	-15.2489*	.13512	p < 0.05	-15.7840	-14.7138
	F3	9.4589*	.08018	p < 0.05	9.1320	9.7858
	F4	-2.1056*	.11880	p < 0.05	-2.5674	-1.6437
	F6	16.1211*	.09480	p < 0.05	15.7554	16.4868
	F7	-7.0622*	.11470	p < 0.05	-7.5063	-6.6181
	F9	6.7422*	.08671	p < 0.05	6.4013	7.0831
	F10	-4.9333*	.08257	p < 0.05	-5.2645	-4.6021
	F11	5.6900*	.10702	p < 0.05	5.2781	6.1019
	F12	16.1500*	.07701	p < 0.05	15.8269	16.4731
F6	F1	-31.3700*	.12999	p < 0.05	-31.8941	-30.8459
	F3	-6.6622*	.07120	p < 0.05	-6.9472	-6.3772
	F4	-18.2267*	.11293	p < 0.05	-18.6721	-17.7813
	F5	-16.1211*	.09480	p < 0.05	-16.4868	-15.7554
	F7	-23.1833*	.10862	p < 0.05	-23.6092	-22.7574
	F9	-9.3789*	.07848	p < 0.05	-9.6833	-9.0745
	F10	-21.0544*	.07388	p < 0.05	-21.3457	-20.7632
	F11	-10.4311*	.10046	p < 0.05	-10.8210	-10.0413
	F12	.0289	.06761	1.000	-.2500	.3078
F7	F1	-8.1867*	.14514	p < 0.05	-8.7498	-7.6235
	F3	16.5211*	.09612	p < 0.05	16.1194	16.9228
	F4	4.9567*	.13009	p < 0.05	4.4564	5.4569
	F5	7.0622*	.11470	p < 0.05	6.6181	7.5063
	F6	23.1833*	.10862	p < 0.05	22.7574	23.6092
	F9	13.8044*	.10163	p < 0.05	13.3949	14.2140
	F10	2.1289*	.09812	p < 0.05	1.7249	2.5328
	F11	12.7522*	.11943	p < 0.05	12.2921	13.2123
	F12	23.2122*	.09349	p < 0.05	22.8121	23.6124

Table 28 Multiple comparison of variances of particle size in AASLMs (continuous) (data specified in Table 6, page 29)

Multiple Comparisons

Dependent Variable: Size

Dunnett T3

Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F9	F1	-21.9911*	.12421	p < 0.05	-22.5066	-21.4757
	F3	2.7167*	.06001	p < 0.05	2.4826	2.9507
	F4	-8.8478*	.10623	p < 0.05	-9.2788	-8.4167
	F5	-6.7422*	.08671	p < 0.05	-7.0831	-6.4013
	F6	9.3789*	.07848	p < 0.05	9.0745	9.6833
	F7	-13.8044*	.10163	p < 0.05	-14.2140	-13.3949
	F10	-11.6756*	.06317	p < 0.05	-11.9196	-11.4316
	F11	-1.0522*	.09287	p < 0.05	-1.4212	-.6832
	F12	9.4078*	.05570	p < 0.05	9.1846	9.6310
F10	F1	-10.3156*	.12136	p < 0.05	-10.8286	-9.8026
	F3	14.3922*	.05385	p < 0.05	14.1846	14.5998
	F4	2.8278*	.10288	p < 0.05	2.4015	3.2541
	F5	4.9333*	.08257	p < 0.05	4.6021	5.2645
	F6	21.0544*	.07388	p < 0.05	20.7632	21.3457
	F7	-2.1289*	.09812	p < 0.05	-2.5328	-1.7249
	F9	11.6756*	.06317	p < 0.05	11.4316	11.9196
	F11	10.6233*	.08902	p < 0.05	10.2621	10.9846
	F12	21.0833*	.04901	p < 0.05	20.8908	21.2759
F11	F1	-20.9389*	.13915	p < 0.05	-21.4844	-20.3934
	F3	3.7689*	.08680	p < 0.05	3.4109	4.1268
	F4	-7.7956*	.12336	p < 0.05	-8.2720	-7.3191
	F5	-5.6900*	.10702	p < 0.05	-6.1019	-5.2781
	F6	10.4311*	.10046	p < 0.05	10.0413	10.8210
	F7	-12.7522*	.11943	p < 0.05	-13.2123	-12.2921
	F9	1.0522*	.09287	p < 0.05	.6832	1.4212
	F10	-10.6233*	.08902	p < 0.05	-10.9846	-10.2621
	F12	10.4600*	.08388	p < 0.05	10.1047	10.8153
F12	F1	-31.3989*	.11764	p < 0.05	-31.9110	-30.8868
	F3	-6.6911*	.04485	p < 0.05	-6.8652	-6.5170
	F4	-18.2556*	.09847	p < 0.05	-18.6789	-17.8322
	F5	-16.1500*	.07701	p < 0.05	-16.4731	-15.8269
	F6	-.0289	.06761	1.000	-.3078	.2500
	F7	-23.2122*	.09349	p < 0.05	-23.6124	-22.8121
	F9	-9.4078*	.05570	p < 0.05	-9.6310	-9.1846
	F10	-21.0833*	.04901	p < 0.05	-21.2759	-20.8908
	F11	-10.4600*	.08388	p < 0.05	-10.8153	-10.1047

*. The mean difference is significant at the .05 level.

Table 29 Various of AASLMs for entrapment efficiency (%) by ANOVA test.

Formulation	Value Label
F1	10% B-Tw80
F3	10% B-P188
F4	10% C-Tw80
F5	10% C-SL
F6	10% C-P188
F7	15% B-Tw80
F9	15% B-P188
F10	15% C-Tw80
F11	15% C-SL
F12	15% C-P188

Table 30 Test of homogeneity of variances of entrapment efficiency (%) in AASLMs (data specified in Table 7, page 34)

Test of homogeneity of variance

Dependent Variable: Size

F	df1	df2	Sig.
12.009	9	80	0.001

Result: Heterogeneity of variance ($p = 0.001 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 31 Multiple comparison of entrapment efficiency (%) in AASLMs (data specified in Table 7, page 34)

Multiple Comparisons

Dependent Variable: Entrapment efficiency

Dunnett T3

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F1	F3	-46.2544*	.35343	p < 0.05	-47.8426	-44.6663
	F4	22.1889*	.66921	p < 0.05	19.5352	24.8426
	F5	-4.4367*	.61361	p < 0.05	-6.8383	-2.0351
	F6	-46.2544*	.35343	p < 0.05	-47.8426	-44.6663
	F7	2.1856	1.08599	0.005	-2.4179	6.7890
	F9	-46.2544*	.35343	p < 0.05	-47.8426	-44.6663
	F10	10.9800*	.98741	p < 0.05	6.8384	15.1216
	F11	-20.4789*	.66350	p < 0.05	-23.1064	-17.8514
	F12	-46.2544*	.35343	p < 0.05	-47.8426	-44.6663
F3	F1	46.2544*	.35343	p < 0.05	44.6663	47.8426
	F4	68.4433*	.56827	p < 0.05	65.8898	70.9968
	F5	41.8178*	.50161	p < 0.05	39.5638	44.0717
	F6	0.0000	0.00000	1.000	0.0000	0.0000
	F7	48.4400*	1.02687	p < 0.05	43.8258	53.0542
	F9	0.0000	0.00000	1.000	0.0000	0.0000
	F10	57.2344*	.92199	p < 0.05	53.0915	61.3774
	F11	25.7756*	.56153	p < 0.05	23.2524	28.2988
	F12	0.0000	0.00000	1.000	0.0000	0.0000
F4	F1	-22.1889*	.66921	p < 0.05	-24.8426	-19.5352
	F3	-68.4433*	.56827	p < 0.05	-70.9968	-65.8898
	F5	-26.6256*	.75799	p < 0.05	-29.5462	-23.7049
	F6	-68.4433*	.56827	p < 0.05	-70.9968	-65.8898
	F7	-20.0033*	1.17363	p < 0.05	-24.7213	-15.2853
	F9	-68.4433*	.56827	p < 0.05	-70.9968	-65.8898
	F10	-11.2089*	1.08305	p < 0.05	-15.5080	-6.9098
	F11	-42.6678*	.79891	p < 0.05	-45.7385	-39.5970
	F12	-68.4433*	.56827	p < 0.05	-70.9968	-65.8898

Table 31 Multiple comparison of entrapment efficiency (%) in AASLMs (continuous) (data specified in Table 7, page 34)

Multiple Comparisons

Dependent Variable: Entrapment efficiency
Dunnett T3

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F5	F1	4.4367*	.61361	p < 0.05	2.0351	6.8383
	F3	-41.8178*	.50161	p < 0.05	-44.0717	-39.5638
	F4	26.6256*	.75799	p < 0.05	23.7049	29.5462
	F6	-41.8178*	.50161	p < 0.05	-44.0717	-39.5638
	F7	6.6222*	1.14284	p < 0.05	1.9582	11.2862
	F9	-41.8178*	.50161	p < 0.05	-44.0717	-39.5638
	F10	15.4167*	1.04961	p < 0.05	11.1886	19.6448
	F11	-16.0422*	.75294	p < 0.05	-18.9421	-13.1423
	F12	-41.8178*	.50161	p < 0.05	-44.0717	-39.5638
F6	F1	46.2544*	.35343	p < 0.05	44.6663	47.8426
	F3	0.0000	0.00000	1.000	0.0000	0.0000
	F4	68.4433*	.56827	p < 0.05	65.8898	70.9968
	F5	41.8178*	.50161	p < 0.05	39.5638	44.0717
	F7	48.4400*	1.02687	p < 0.05	43.8258	53.0542
	F9	0.0000	0.00000	1.000	0.0000	0.0000
	F10	57.2344*	.92199	p < 0.05	53.0915	61.3774
	F11	25.7756*	.56153	p < 0.05	23.2524	28.2988
	F12	0.0000	0.00000	1.000	0.0000	0.0000
F7	F1	-2.1856	1.08599	0.005	-6.7890	2.4179
	F3	-48.4400*	1.02687	p < 0.05	-53.0542	-43.8258
	F4	20.0033*	1.17363	p < 0.05	15.2853	24.7213
	F5	-6.6222*	1.14284	0.003	-11.2862	-1.9582
	F6	-48.4400*	1.02687	p < 0.05	-53.0542	-43.8258
	F9	-48.4400*	1.02687	p < 0.05	-53.0542	-43.8258
	F10	8.7944*	1.38005	p < 0.05	3.4803	14.1086
	F11	-22.6644*	1.17038	p < 0.05	-27.3761	-17.9527
	F12	-48.4400*	1.02687	p < 0.05	-53.0542	-43.8258
F9	F1	46.2544*	.35343	p < 0.05	44.6663	47.8426
	F3	0.0000	0.00000	1.000	0.0000	0.0000
	F4	68.4433*	.56827	p < 0.05	65.8898	70.9968
	F5	41.8178*	.50161	p < 0.05	39.5638	44.0717
	F6	0.0000	0.00000	1.000	0.0000	0.0000
	F7	48.4400*	1.02687	p < 0.05	43.8258	53.0542
	F10	57.2344*	.92199	p < 0.05	53.0915	61.3774
	F11	25.7756*	.56153	p < 0.05	23.2524	28.2988
	F12	0.0000	0.00000	1.000	0.0000	0.0000

Table 31 Multiple comparison of entrapment efficiency (%) in AASLMs (continuous) (data specified in Table 7, page 34)

Multiple Comparisons

Dependent Variable: Entrapment efficiency

Dunnett T3

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F10	F1	-10.9800*	.98741	p < 0.05	-15.1216	-6.8384
	F3	-57.2344*	.92199	p < 0.05	-61.3774	-53.0915
	F4	11.2089*	1.08305	p < 0.05	6.9098	15.5080
	F5	-15.4167*	1.04961	p < 0.05	-19.6448	-11.1886
	F6	-57.2344*	.92199	p < 0.05	-61.3774	-53.0915
	F7	-8.7944*	1.38005	p < 0.05	-14.1086	-3.4803
	F9	-57.2344*	.92199	p < 0.05	-61.3774	-53.0915
	F11	-31.4589*	1.07953	p < 0.05	-35.7498	-27.1680
	F12	-57.2344*	.92199	p < 0.05	-61.3774	-53.0915
F11	F1	20.4789*	.66350	p < 0.05	17.8514	23.1064
	F3	-25.7756*	.56153	p < 0.05	-28.2988	-23.2524
	F4	42.6678*	.79891	p < 0.05	39.5970	45.7385
	F5	16.0422*	.75294	p < 0.05	13.1423	18.9421
	F6	-25.7756*	.56153	p < 0.05	-28.2988	-23.2524
	F7	22.6644*	1.17038	p < 0.05	17.9527	27.3761
	F9	-25.7756*	.56153	p < 0.05	-28.2988	-23.2524
	F10	31.4589*	1.07953	p < 0.05	27.1680	35.7498
	F12	-25.7756*	.56153	p < 0.05	-28.2988	-23.2524
F12	F1	46.2544*	.35343	p < 0.05	44.6663	47.8426
	F3	0.0000	0.00000	1.000	0.0000	0.0000
	F4	68.4433*	.56827	p < 0.05	65.8898	70.9968
	F5	41.8178*	.50161	p < 0.05	39.5638	44.0717
	F6	0.0000	0.00000	1.000	0.0000	0.0000
	F7	48.4400*	1.02687	p < 0.05	43.8258	53.0542
	F9	0.0000	0.00000	1.000	0.0000	0.0000
	F10	57.2344*	.92199	p < 0.05	53.0915	61.3774
	F11	25.7756*	.56153	p < 0.05	23.2524	28.2988

*. The mean difference is significant at the .05 level.

Table 32 Various of active loading (%) in AASLMs for by ANOVA test.

Formulation	Value Label
F1	10% B-Tw80
F3	10% B-P188
F4	10% C-Tw80
F5	10% C-SL
F6	10% C-P188
F7	15% B-Tw80
F9	15% B-P188
F10	15% C-Tw80
F11	15% C-SL
F12	15% C-P188

Table 33 Test of homogeneity of variances of active loading (%) in AASLMs (data specified in Table 7, page 34)

Levene's Test of Equality of Error Variances[a]

Dependent Variable: AL

F	df1	df2	Sig.
1.785	9	80	0.084

Result: Homogeneity of variance (p = 0.0.84 > α = 0.05) implying that variances are equal and homogeneity of variance assumption of ANOVA has been showed. Then ANOVA could test in Turkey's test.

Table 34 Multiple comparison of active loading (%) in AASLMs (data specified in Table 7, page 34)

Multiple Comparisons

Dependent Variable: Active loading (Tukey HSD)

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F1	F3	-.021233*	.0005475	p < 0.05	-.023015	-.019451
	F4	.011489*	.0005475	p < 0.05	.009707	.013271
	F5	-.001911*	.0005475	p < 0.05	-.003693	-.000129
	F6	-.022367*	.0005475	p < 0.05	-.024149	-.020585
	F7	.009922*	.0005475	p < 0.05	.008140	.011704
	F9	-.004533*	.0005475	p < 0.05	-.006315	-.002751
	F10	.013000*	.0005475	p < 0.05	.011218	.014782
	F11	.010678*	.0005475	p < 0.05	-.005104	.012460
	F12	-.006167*	.0005475	p < 0.05	-.007949	-.004385
F3	F1	.021233*	.0005475	p < 0.05	.019451	.023015
	F4	.032722*	.0005475	p < 0.05	.030940	.034504
	F5	.019322*	.0005475	p < 0.05	.017540	.021104
	F6	-.001133	.0005475	0.554	-.002915	.000649
	F7	.031156*	.0005475	p < 0.05	.029374	.032937
	F9	.016700*	.0005475	p < 0.05	.014918	.018482
	F10	.034233*	.0005475	p < 0.05	.032451	.036015
	F11	.021911*	.0005475	p < 0.05	.020129	.023693
	F12	.015067*	.0005475	p < 0.05	.013285	.016849
F4	F1	-.011489*	.0005475	p < 0.05	-.013271	-.009707
	F3	-.032722*	.0005475	p < 0.05	-.034504	-.030940
	F5	-.013400*	.0005475	p < 0.05	-.015182	-.011618
	F6	-.033856*	.0005475	p < 0.05	-.035637	-.032074
	F7	-.015667*	.0005475	p < 0.05	-.013349	-.021522
	F9	-.016022*	.0005475	p < 0.05	-.017804	-.014240
	F10	-.000241*	.0005475	p < 0.05	.000271	.004375
	F11	-.010811*	.0005475	p < 0.05	-.012593	-.009029
	F12	-.017656*	.0005475	p < 0.05	-.019437	-.015874
F5	F1	.001911*	.0005475	p < 0.05	.000129	.003693
	F3	-.019322*	.0005475	p < 0.05	-.021104	-.017540
	F4	.013400*	.0005475	p < 0.05	.011618	.015182
	F6	-.020456*	.0005475	p < 0.05	-.022237	-.018674
	F7	.011833*	.0005475	p < 0.05	.010051	.013615
	F9	-.002622*	.0005475	p < 0.05	-.004404	-.000840
	F10	.014911*	.0005475	p < 0.05	.013129	.016693
	F11	.002589*	.0005475	p < 0.05	.000807	.004371
	F12	-.004256*	.0005475	p < 0.05	-.006037	-.002474

Table 34 Multiple comparison of active loading (%) in AASLMs (continuous) (data specified in Table 7, page 34)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F6	F1	.022367*	.0005475	p < 0.05	.020585	.024149
	F3	.001133	.0005475	0.554	-.000649	.002915
	F4	.033856*	.0005475	p < 0.05	.032074	.035637
	F5	.020456*	.0005475	p < 0.05	.018674	.022237
	F7	.032289*	.0005475	p < 0.05	.030507	.034071
	F9	.017833*	.0005475	p < 0.05	.016051	.019615
	F10	.035367*	.0005475	p < 0.05	.033585	.037149
	F11	.023044*	.0005475	p < 0.05	.021263	.024826
	F12	.016200*	.0005475	p < 0.05	.014418	.017982
F7	F1	-.009922*	.0005475	p < 0.05	-.011704	-.008140
	F3	-.031156*	.0005475	p < 0.05	-.032937	-.029374
	F4	-.015667	.0005475	p < 0.05	-.013349	-.021522
	F5	-.011833*	.0005475	p < 0.05	-.013615	-.010051
	F6	-.032289*	.0005475	p < 0.05	-.034071	-.030507
	F9	-.014456*	.0005475	p < 0.05	-.016237	-.012674
	F10	.003078*	.0005475	p < 0.05	.001296	.004860
	F11	-.009244*	.0005475	p < 0.05	-.011026	-.007463
	F12	-.016089*	.0005475	p < 0.05	-.017871	-.014307
F9	F1	.004533*	.0005475	p < 0.05	.002751	.006315
	F3	-.016700*	.0005475	p < 0.05	-.018482	-.014918
	F4	.016022*	.0005475	p < 0.05	.014240	.017804
	F5	.002622*	.0005475	p < 0.05	.000840	.004404
	F6	-.017833*	.0005475	p < 0.05	-.019615	-.016051
	F7	.014456*	.0005475	p < 0.05	.012674	.016237
	F10	.017533*	.0005475	p < 0.05	.015751	.019315
	F11	.005211*	.0005475	p < 0.05	.003429	.006993
	F12	-.001633	.0005475	0.101	-.003415	.000149

Table 34 Multiple comparison of active loading (%) in AASLMs (continuous) (data specified in Table 7, page 34)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F10	F1	-.013000*	.0005475	p < 0.05	-.014782	-.011218
	F3	-.034233*	.0005475	p < 0.05	-.036015	-.032451
	F4	-.000241*	.0005475	p < 0.05	.000271	.004375
	F5	-.014911*	.0005475	p < 0.05	-.016693	-.013129
	F6	-.035367*	.0005475	p < 0.05	-.037149	-.033585
	F7	-.003078*	.0005475	p < 0.05	-.004860	-.001296
	F9	-.017533*	.0005475	p < 0.05	-.019315	-.015751
	F11	-.012322*	.0005475	p < 0.05	-.014104	-.010540
	F12	-.019167*	.0005475	p < 0.05	-.020949	-.017385
F11	F1	.010678*	.0005475	p < 0.05	-.005104	.012460
	F3	-.021911*	.0005475	p < 0.05	-.023693	-.020129
	F4	.010811*	.0005475	p < 0.05	.009029	.012593
	F5	-.002589*	.0005475	p < 0.05	-.004371	-.000807
	F6	-.023044*	.0005475	p < 0.05	-.024826	-.021263
	F7	.009244*	.0005475	p < 0.05	.007463	.011026
	F9	-.005211*	.0005475	p < 0.05	-.006993	-.003429
	F10	.012322*	.0005475	p < 0.05	.010540	.014104
	F12	-.006844*	.0005475	p < 0.05	-.008626	-.005063
F12	F1	.006167*	.0005475	p < 0.05	.004385	.007949
	F3	-.015067*	.0005475	p < 0.05	-.016849	-.013285
	F4	.017656*	.0005475	p < 0.05	.015874	.019437
	F5	.004256*	.0005475	p < 0.05	.002474	.006037
	F6	-.016200*	.0005475	p < 0.05	-.017982	-.014418
	F7	.016089*	.0005475	p < 0.05	.014307	.017871
	F9	.001633	.0005475	p < 0.05	-.000149	.003415
	F10	.019167*	.0005475	p < 0.05	.017385	.020949
	F11	.006844*	.0005475	0.101	.005063	.008626

*. The mean difference is significant at the .05 level.

Table 35 Various of stability of AASLMs for particle size, entrapment efficiency (%) and active loading (%) by ANOVA test.

Formulation	Value Label
F1	10% B-Tw80
F3	10% B-P188
F4	10% C-Tw80
F5	10% C-SL
F6	10% C-P188
F7	15% B-Tw80
F9	15% B-P188
F10	15% C-Tw80
F11	15% C-SL
F12	15% C-P188

Table 36 Multiple comparison of stability of particle size in AASLMs (data specified in Table 11-13, page 51-53)

Multiple Comparisons

Dependent Variable: Particle size

Tukey HSD

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F1	F3	38.8021*	.03198	p < 0.05	38.7007	38.9035
	F4	19.0324*	.03198	p < 0.05	18.9309	19.1338
	F5	25.4364*	.03198	p < 0.05	25.3350	25.5379
	F6	43.6375*	.03198	p < 0.05	43.5361	43.7390
	F7	6.7410*	.03503	p < 0.05	6.6299	6.8521
	F9	36.4424*	.03198	p < 0.05	36.3409	36.5438
	F10	15.3145*	.03198	p < 0.05	15.2131	15.4159
	F11	31.2913*	.03198	p < 0.05	31.1898	31.3927
	F12	44.5685*	.03198	p < 0.05	44.4671	44.6699
F3	F1	-38.8021*	.03198	p < 0.05	-38.9035	-38.7007
	F4	-19.7697*	.02860	p < 0.05	-19.8604	-19.6790
	F5	-13.3656*	.02860	p < 0.05	-13.4564	-13.2749
	F6	4.8355*	.02860	p < 0.05	4.7447	4.9262
	F7	-32.0611*	.03198	p < 0.05	-32.1625	-31.9597
	F9	-2.3597*	.02860	p < 0.05	-2.4504	-2.2690
	F10	-23.4876*	.02860	p < 0.05	-23.5783	-23.3969
	F11	-7.5108*	.02860	p < 0.05	-7.6015	-7.4201
	F12	5.7664*	.02860	p < 0.05	5.6757	5.8571
F4	F1	-19.0324*	.03198	p < 0.05	-19.1338	-18.9309
	F3	19.7697*	.02860	p < 0.05	19.6790	19.8604
	F5	6.4041*	.02860	p < 0.05	6.3134	6.4948
	F6	24.6052*	.02860	p < 0.05	24.5145	24.6959
	F7	-12.2914*	.03198	p < 0.05	-12.3928	-12.1900
	F9	17.4100*	.02860	p < 0.05	17.3193	17.5007
	F10	-3.7179*	.02860	p < 0.05	-3.8086	-3.6272
	F11	12.2589*	.02860	p < 0.05	12.1682	12.3496
	F12	25.5361*	.02860	p < 0.05	25.4454	25.6268

Table 36 Multiple comparison of stability of particle size in AASLMs (continuous) (data specified in 11-13, page 51-53)

Multiple Comparisons

Dependent Variable: Particle size

Tukey HSD

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F5	F1	-25.4364*	.03198	p < 0.05	-25.5379	-25.3350
	F3	13.3656*	.02860	p < 0.05	13.2749	13.4564
	F4	-6.4041*	.02860	p < 0.05	-6.4948	-6.3134
	F6	18.2011*	.02860	p < 0.05	18.1104	18.2918
	F7	-18.6955*	.03198	p < 0.05	-18.7969	-18.5940
	F9	11.0059*	.02860	p < 0.05	10.9152	11.0966
	F10	-10.1219*	.02860	p < 0.05	-10.2127	-10.0312
	F11	5.8548*	.02860	p < 0.05	5.7641	5.9455
	F12	19.1320*	.02860	p < 0.05	19.0413	19.2228
F6	F1	-43.6375*	.03198	p < 0.05	-43.7390	-43.5361
	F3	-4.8355*	.02860	p < 0.05	-4.9262	-4.7447
	F4	-24.6052*	.02860	p < 0.05	-24.6959	-24.5145
	F5	-18.2011*	.02860	p < 0.05	-18.2918	-18.1104
	F7	-36.8966*	.03198	p < 0.05	-36.9980	-36.7952
	F9	-7.1952*	.02860	p < 0.05	-7.2859	-7.1045
	F10	-28.3231*	.02860	p < 0.05	-28.4138	-28.2323
	F11	-12.3463*	.02860	p < 0.05	-12.4370	-12.2556
	F12	.9309*	.02860	p < 0.05	.8402	1.0216
F7	F1	-6.7410*	.03503	p < 0.05	-6.8521	-6.6299
	F3	32.0611*	.03198	p < 0.05	31.9597	32.1625
	F4	12.2914*	.03198	p < 0.05	12.1900	12.3928
	F5	18.6955*	.03198	p < 0.05	18.5940	18.7969
	F6	36.8966*	.03198	p < 0.05	36.7952	36.9980
	F9	29.7014*	.03198	p < 0.05	29.6000	29.8028
	F10	8.5735*	.03198	p < 0.05	8.4721	8.6749
	F11	24.5503*	.03198	p < 0.05	24.4489	24.6517
	F12	37.8275*	.03198	p < 0.05	37.7261	37.9289

Table 36 Multiple comparison of stability of particle size in AASLMs (continuous) (data specified in 11-13, page 51-53)

Multiple Comparisons

Dependent Variable: Particle size (Tukey HSD)

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F9	F1	-36.4424*	.03198	p < 0.05	-36.5438	-36.3409
	F3	2.3597*	.02860	p < 0.05	2.2690	2.4504
	F4	-17.4100*	.02860	p < 0.05	-17.5007	-17.3193
	F5	-11.0059*	.02860	p < 0.05	-11.0966	-10.9152
	F6	7.1952*	.02860	p < 0.05	7.1045	7.2859
	F7	-29.7014*	.03198	p < 0.05	-29.8028	-29.6000
	F10	-21.1279*	.02860	p < 0.05	-21.2186	-21.0372
	F11	-5.1511*	.02860	p < 0.05	-5.2418	-5.0604
	F12	8.1261*	.02860	p < 0.05	8.0354	8.2168
F10	F1	-15.3145*	.03198	p < 0.05	-15.4159	-15.2131
	F3	23.4876*	.02860	p < 0.05	23.3969	23.5783
	F4	3.7179*	.02860	p < 0.05	3.6272	3.8086
	F5	10.1219*	.02860	p < 0.05	10.0312	10.2127
	F6	28.3231*	.02860	p < 0.05	28.2323	28.4138
	F7	-8.5735*	.03198	p < 0.05	-8.6749	-8.4721
	F9	21.1279*	.02860	p < 0.05	21.0372	21.2186
	F11	15.9768*	.02860	p < 0.05	15.8860	16.0675
	F12	29.2540*	.02860	p < 0.05	29.1633	29.3447
F11	F1	-31.2913*	.03198	p < 0.05	-31.3927	-31.1898
	F3	7.5108*	.02860	p < 0.05	7.4201	7.6015
	F4	-12.2589*	.02860	p < 0.05	-12.3496	-12.1682
	F5	-5.8548*	.02860	p < 0.05	-5.9455	-5.7641
	F6	12.3463*	.02860	p < 0.05	12.2556	12.4370
	F7	-24.5503*	.03198	p < 0.05	-24.6517	-24.4489
	F9	5.1511*	.02860	p < 0.05	5.0604	5.2418
	F10	-15.9768*	.02860	p < 0.05	-16.0675	-15.8860
	F12	13.2772*	.02860	p < 0.05	13.1865	13.3679
F12	F1	-44.5685*	.03198	p < 0.05	-44.6699	-44.4671
	F3	-5.7664*	.02860	p < 0.05	-5.8571	-5.6757
	F4	-25.5361*	.02860	p < 0.05	-25.6268	-25.4454
	F5	-19.1320*	.02860	p < 0.05	-19.2228	-19.0413
	F6	-.9309*	.02860	p < 0.05	-1.0216	-.8402
	F7	-37.8275*	.03198	p < 0.05	-37.9289	-37.7261
	F9	-8.1261*	.02860	p < 0.05	-8.2168	-8.0354
	F10	-29.2540*	.02860	p < 0.05	-29.3447	-29.1633
	F11	-13.2772*	.02860	p < 0.05	-13.3679	-13.1865

*. The mean difference is significant at the .05 level.

Table 37 Multiple comparison of stability of entrapment efficiency in AASLMs (data specified in Table 14, page 54)

Multiple Comparisons

Dependent Variable: Entrapment efficiency (Tukey HSD)

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F1	F3	-55.4598*	.47113	p < 0.05	-56.9538	-53.9659
	F4	20.1777*	.47113	p < 0.05	18.6837	21.6716
	F5	-15.6714*	.47113	p < 0.05	-17.1654	-14.1774
	F6	-55.4598*	.47113	p < 0.05	-56.9538	-53.9659
	F7	-1.2223	.47113	p < 0.05	-2.7163	.2716
	F9	-55.4598*	.47113	p < 0.05	-56.9538	-53.9659
	F10	11.6424*	.47113	p < 0.05	10.1484	13.1364
	F11	-34.0625*	.47113	p < 0.05	-35.5565	-32.5685
	F12	-55.4598*	.47113	p < 0.05	-56.9538	-53.9659
F3	F1	55.4598*	.47113	p < 0.05	53.9659	56.9538
	F4	75.6375*	.47113	p < 0.05	74.1435	77.1315
	F5	39.7884*	.47113	p < 0.05	38.2945	41.2824
	F6	0.0000	.47113	1.000	-1.4940	1.4940
	F7	54.2375*	.47113	p < 0.05	52.7435	55.7315
	F9	0.0000	.47113	1.000	-1.4940	1.4940
	F10	67.1022*	.47113	p < 0.05	65.6083	68.5962
	F11	21.3973*	.47113	p < 0.05	19.9034	22.8913
	F12	0.0000	.47113	1.000	-1.4940	1.4940
F4	F1	-20.1777*	.47113	p < 0.05	-21.6716	-18.6837
	F3	-75.6375*	.47113	p < 0.05	-77.1315	-74.1435
	F5	-35.8491*	.47113	p < 0.05	-37.3430	-34.3551
	F6	-75.6375*	.47113	p < 0.05	-77.1315	-74.1435
	F7	-21.4000*	.47113	p < 0.05	-22.8940	-19.9060
	F9	-75.6375*	.47113	p < 0.05	-77.1315	-74.1435
	F10	-8.5353*	.47113	p < 0.05	-10.0292	-7.0413
	F11	-54.2402*	.47113	p < 0.05	-55.7341	-52.7462
	F12	-75.6375*	.47113	p < 0.05	-77.1315	-74.1435

Table 37 Multiple comparison of stability of entrapment efficiency in AASLMs (continuous) (data specified in Table 14, page 54)

Multiple Comparisons

Dependent Variable: Entrapment efficiency (Tukey HSD)

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F5	F1	15.6714*	.47113	p < 0.05	14.1774	17.1654
	F3	-39.7884*	.47113	p < 0.05	-41.2824	-38.2945
	F4	35.8491*	.47113	p < 0.05	34.3551	37.3430
	F6	-39.7884*	.47113	p < 0.05	-41.2824	-38.2945
	F7	14.4491*	.47113	p < 0.05	12.9551	15.9430
	F9	-39.7884*	.47113	p < 0.05	-41.2824	-38.2945
	F10	27.3138*	.47113	p < 0.05	25.8198	28.8078
	F11	-18.3911*	.47113	p < 0.05	-19.8851	-16.8971
	F12	-39.7884*	.47113	p < 0.05	-41.2824	-38.2945
F6	F1	55.4598*	.47113	p < 0.05	53.9659	56.9538
	F3	0.0000	.47113	1.000	-1.4940	1.4940
	F4	75.6375*	.47113	p < 0.05	74.1435	77.1315
	F5	39.7884*	.47113	p < 0.05	38.2945	41.2824
	F7	54.2375*	.47113	p < 0.05	52.7435	55.7315
	F9	0.0000	.47113	1.000	-1.4940	1.4940
	F10	67.1022*	.47113	p < 0.05	65.6083	68.5962
	F11	21.3973*	.47113	p < 0.05	19.9034	22.8913
	F12	0.0000	.47113	1.000	-1.4940	1.4940
F7	F1	1.2223	.47113	p < 0.05	-.2716	2.7163
	F3	-54.2375*	.47113	p < 0.05	-55.7315	-52.7435
	F4	21.4000*	.47113	p < 0.05	19.9060	22.8940
	F5	-14.4491*	.47113	p < 0.05	-15.9430	-12.9551
	F6	-54.2375*	.47113	p < 0.05	-55.7315	-52.7435
	F9	-54.2375*	.47113	p < 0.05	-55.7315	-52.7435
	F10	12.8647*	.47113	p < 0.05	11.3708	14.3587
	F11	-32.8402*	.47113	p < 0.05	-34.3341	-31.3462
	F12	-54.2375*	.47113	p < 0.05	-55.7315	-52.7435

Table 37 Multiple comparison of stability of entrapment efficiency in AASLMs (continuous) (data specified in Table 14, page 54)

Multiple Comparisons

Dependent Variable: Entrapment efficiency (Tukey HSD)

(I) Formulation		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
F9	F1	55.4598*	.47113	p < 0.05	53.9659	56.9538
	F3	0.0000	.47113	1.000	-1.4940	1.4940
	F4	75.6375*	.47113	p < 0.05	74.1435	77.1315
	F5	39.7884*	.47113	p < 0.05	38.2945	41.2824
	F6	0.0000	.47113	1.000	-1.4940	1.4940
	F7	54.2375*	.47113	p < 0.05	52.7435	55.7315
	F10	67.1022*	.47113	p < 0.05	65.6083	68.5962
	F11	21.3973*	.47113	p < 0.05	19.9034	22.8913
	F12	0.0000	.47113	1.000	-1.4940	1.4940
F10	F1	-11.6424*	.47113	p < 0.05	-13.1364	-10.1484
	F3	-67.1022*	.47113	p < 0.05	-68.5962	-65.6083
	F4	8.5353*	.47113	p < 0.05	7.0413	10.0292
	F5	-27.3138*	.47113	p < 0.05	-28.8078	-25.8198
	F6	-67.1022*	.47113	p < 0.05	-68.5962	-65.6083
	F7	-12.8647*	.47113	p < 0.05	-14.3587	-11.3708
	F9	-67.1022*	.47113	p < 0.05	-68.5962	-65.6083
	F11	-45.7049*	.47113	p < 0.05	-47.1989	-44.2109
	F12	-67.1022*	.47113	p < 0.05	-68.5962	-65.6083
F11	F1	34.0625*	.47113	p < 0.05	32.5685	35.5565
	F3	-21.3973*	.47113	p < 0.05	-22.8913	-19.9034
	F4	54.2402*	.47113	p < 0.05	52.7462	55.7341
	F5	18.3911*	.47113	p < 0.05	16.8971	19.8851
	F6	-21.3973*	.47113	p < 0.05	-22.8913	-19.9034
	F7	32.8402*	.47113	p < 0.05	31.3462	34.3341
	F9	-21.3973*	.47113	p < 0.05	-22.8913	-19.9034
	F10	45.7049*	.47113	p < 0.05	44.2109	47.1989
	F12	-21.3973*	.47113	p < 0.05	-22.8913	-19.9034
F12	F1	55.4598*	.47113	p < 0.05	53.9659	56.9538
	F3	0.0000	.47113	1.000	-1.4940	1.4940
	F4	75.6375*	.47113	p < 0.05	74.1435	77.1315
	F5	39.7884*	.47113	p < 0.05	38.2945	41.2824
	F6	0.0000	.47113	1.000	-1.4940	1.4940
	F7	54.2375*	.47113	p < 0.05	52.7435	55.7315
	F9	0.0000	.47113	1.000	-1.4940	1.4940
	F10	67.1022*	.47113	p < 0.05	65.6083	68.5962
	F11	21.3973*	.47113	p < 0.05	19.9034	22.8913

*. The mean difference is significant at the .05 level.

Table 38 Test of homogeneity of variances of stability of active loading (%) in F1 (10% beeswax: Tw80) (data specified in Table 15, page 55)

Test of homogeneity of variance

Dependent Variable: Active loading

F	df1	df2	Sig.
2.114	9	80	0.065

Result: Homogeneity of variance ($p = 0.065 > \alpha = 0.05$) implying that variances are equal and homogeneity of variance assumption of ANOVA has been showed. Then ANOVA could test in Turkey's test.

Table 39 Multiple comparison of stability of active loading (%) in F1 (10% beeswax: Tw80) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Temp		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
initial	4C	.007226*	.0008873	p < 0.05	.004898	.009554
	RT	.004174*	.0008873	p < 0.05	.001846	.006502
	45C	.013081*	.0008873	p < 0.05	.010753	.015410
4C	initial	-.007226*	.0008873	p < 0.05	-.009554	-.004898
	RT	-.003052*	.0006274	p < 0.05	-.004698	-.001406
	45C	.005856*	.0006274	p < 0.05	.004209	.007502
RT	initial	-.004174*	.0008873	p < 0.05	-.006502	-.001846
	4C	.003052*	.0006274	p < 0.05	.001406	.004698
	45C	.008907*	.0006274	p < 0.05	.007261	.010554
45C	initial	-.013081*	.0008873	p < 0.05	-.015410	-.010753
	4C	-.005856*	.0006274	p < 0.05	-.007502	-.004209
	RT	-.008907*	.0006274	p < 0.05	-.010554	-.007261

*. The mean difference is significant at the .05 level.

Table 39 Multiple comparison of stability of active loading (%) in F1 (10% beeswax: Tw80) (continuous) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Time		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T0	T30	.003889*	.0008873	p < 0.05	.001561	.006217
	T60	.009456*	.0008873	p < 0.05	.007127	.011784
	T90	.011137*	.0008873	p < 0.05	.008809	.013465
T30	T0	-.003889*	.0008873	p < 0.05	-.006217	-.001561
	T60	.005567*	.0006274	p < 0.05	.003920	.007213
	T90	.007248*	.0006274	p < 0.05	.005602	.008894
T60	T0	-.009456*	.0008873	p < 0.05	-.011784	-.007127
	T30	-.005567*	.0006274	p < 0.05	-.007213	-.003920
	T90	.001681*	.0006274	p < 0.05	.000035	.003328
T90	T0	-.011137*	.0008873	p < 0.05	-.013465	-.008809
	T30	-.007248*	.0006274	p < 0.05	-.008894	-.005602
	T60	-.001681*	.0006274	p < 0.05	-.003328	-.000035

*. The mean difference is significant at the .05 level.

Table 40 Test of homogeneity of variances of stability of active loading (%) in F3 (10% beeswax: P188) (data specified in Table 15, page 55)

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Active loading

F	df1	df2	Sig.
2.099	9	80	0.039

Result: Heterogeneity of variance ($p = 0.039 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 41 One way analysis of stability of active loading in active loading (%) in F3 ((10% beeswax: P188) (data specified in Table 15, page 55)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Temp	Between Groups	25.726	37	.695	.596	0.950 ($p > 0.05$)
	Within Groups	60.674	52	1.167		
	Total	86.400	89			
Time	Between Groups	35.743	37	.966	.992	0.504 ($p > 0.05$)
	Within Groups	50.657	52	.974		
	Total	86.400	89			

Table 42 Test of homogeneity of variances of stability of active loading (%) in F4 (10% cetyl alcohol: Tw80) (data specified in Table 15, page 55)

Test of homogeneity of variance

Dependent Variable: Active loading

F	df1	df2	Sig.
3.009	9	80	0.004

Result: Heterogeneity of variance ($p = 0.004 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 43 One way analysis of stability of active loading in F4 (10% cetyl alcohol: Tw80) (data specified in Table 15, page 55)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Temp	Between Groups	65.019	57	1.141	4.661	$p < 0.05$
	Within Groups	21.381	32	.668		
	Total	86.400	89			
Time	Between Groups	77.167	57	1.330	3.579	$p < 0.05$
	Within Groups	9.233	32	.298		
	Total	86.400	89			

Table 44 Test of homogeneity of variances of stability of active loading (%) in F5 (10% cetyl alcohol: SL) (data specified in Table 15, page 55)

Test of homogeneity of variance

Dependent Variable: Active loading

F	df1	df2	Sig.
4.781	9	80	0.001

Result: Heterogeneity of variance ($p = 0.001 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 45 One way analysis of stability of active loading in F5 (10% cetyl alcohol: SL) (data specified in Table 15, page 55)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Temp	Between Groups	62.519	45	1.275	2.480	$p < 0.05$
	Within Groups	23.881	44	.543		
	Total	86.400	89			
Time	Between Groups	45.483	45	1.341	2.624	$p < 0.05$
	Within Groups	40.917	44	0.75		
	Total	86.400	89			

Table 46 Test of homogeneity of variances of stability of active loading (%) in F6 (10% cetyl alcohol: P188) (data specified in Table 15, page 55)

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Active loading

F	df1	df2	Sig.
1.495	9	80	0.285

Result: Homogeneity of variance ($p = 0.285 > \alpha = 0.05$) implying that variances are equal and homogeneity of variance assumption of ANOVA has been showed. Then ANOVA could test in Turkey's test.

Table 47 Multiple comparison of stability of active loading (%) in F6 (10% cetyl alcohol: P188) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Temp		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
initial	4C	.000252	.0005782	0.972	-.001265	.001769
	RT	.000319	.0005782	0.946	-.001198	.001836
	45C	.000485	.0005782	0.836	-.001032	.002002
4C	initial	-.000252	.0005782	0.972	-.001769	.001265
	RT	.000067	.0004088	0.998	-.001006	.001139
	45C	.000233	.0004088	0.941	-.000839	.001306
RT	initial	-.000319	.0005782	0.946	-.001836	.001198
	4C	-.000067	.0004088	0.998	-.001139	.001006
	45C	.000167	.0004088	0.977	-.000906	.001239
45C	initial	-.000485	.0005782	0.836	-.002002	.001032
	4C	-.000233	.0004088	0.941	-.001306	.000839
	RT	-.000167	.0004088	0.977	-.001239	.000906

Table 47 Multiple comparison of stability of active loading (%) in F6 (10% cetyl alcohol: P188) (continuous) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Time		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T0	T30	.000156	.0005782	0.993	-.001361	.001673
	T60	.000252	.0005782	0.972	-.001265	.001769
	T90	.000648	.0005782	0.678	-.000869	.002165
T30	T0	-.000156	.0005782	0.993	-.001673	.001361
	T60	.000096	.0004088	0.995	-.000976	.001169
	T90	.000493	.0004088	0.626	-.000580	.001565
T60	T0	-.000252	.0005782	0.972	-.001769	.001265
	T30	-.000096	.0004088	0.995	-.001169	.000976
	T90	.000396	.0004088	0.767	-.000676	.001469
T90	T0	-.000648	.0005782	0.678	-.002165	.000869
	T30	-.000493	.0004088	0.626	-.001565	.000580
	T60	-.000396	.0004088	0.767	-.001469	.000676

Table 48 Test of homogeneity of variances of stability of active loading (%) in F7 (15% beeswax: Tw80) (data specified in Table 15, page 55)

Test of homogeneity of variance

Dependent Variable: Active loading

F	df1	df2	Sig.
3.513	9	80	0.001

Result: Heterogeneity of variance ($p = 0.001 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 49 One way analysis of stability of active loading in F7 (15% beeswax: Tw80) (data specified in Table 15, page 55)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Temp	Between Groups	55.317	48	1.252	1.820	$p < 0.05$
	Within Groups	31.083	41	.758		
	Total	86.400	89			
Time	Between Groups	63.983	52	1.230	2.031	$p < 0.05$
	Within Groups	22.417	37	.606		
	Total	86.400	89			

Table 50 Test of homogeneity of variances of stability of active loading (%) in F9 (15% beeswax: P188) (data specified in Table 15, page 55)

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Active loading

F	df1	df2	Sig.
0.973	9	80	0.468

Result: Homogeneity of variance ($p = 0.468 > \alpha = 0.05$) implying that variances are equal and homogeneity of variance assumption of ANOVA has been showed. Then ANOVA could test in Turkey's test.

Table 51 Multiple comparison of stability of active loading (%) in F9 (15% beeswax: P188) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Temp		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
initial	4C	.000211	.0006289	0.987	-.001439	.001861
	RT	.000352	.0006289	0.944	-.001298	.002002
	45C	.000519	.0006289	0.843	-.001132	.002169
4C	initial	-.000211	.0006289	0.987	-.001861	.001439
	RT	.000141	.0004447	0.989	-.001026	.001308
	45C	.000307	.0004447	0.900	-.000859	.001474
RT	initial	-.000352	.0006289	0.944	-.002002	.001298
	4C	-.000141	.0004447	0.989	-.001308	.001026
	45C	.000167	.0004447	0.982	-.001000	.001333
45C	initial	-.000519	.0006289	0.843	-.002169	.001132
	4C	-.000307	.0004447	0.900	-.001474	.000859
	RT	-.000167	.0004447	0.982	-.001333	.001000

Table 51 Multiple comparison of stability of active loading (%) in F9 (15% beeswax: P188) (continuous) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Time		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T0	T30	.000096	.0006289	0.999	-.001554	.001746
	T60	.000233	.0006289	0.982	-.001417	.001883
	T90	.000752	.0006289	0.631	-.000898	.002402
T30	T0	-.000096	.0006289	0.999	-.001746	.001554
	T60	.000137	.0004447	0.990	-.001030	.001304
	T90	.000656	.0004447	0.458	-.000511	.001822
T60	T0	-.000233	.0006289	0.982	-.001883	.001417
	T30	-.000137	.0004447	0.990	-.001304	.001030
	T90	.000519	.0004447	0.650	-.000648	.001685
T90	T0	-.000752	.0006289	0.631	-.002402	.000898
	T30	-.000656	.0004447	0.458	-.001822	.000511
	T60	-.000519	.0004447	0.650	-.001685	.000648

Table 52 Test of homogeneity of variances of stability of active loading (%) in F10 (15% cetyl alcohol: Tw80) (data specified in Table 15, page 55)

Test of homogeneity of variance

Dependent Variable: Active loading

F	df1	df2	Sig.
5.216	9	80	0.001

Result: Heterogeneity of variance ($p = 0.001 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 53 One way analysis of stability of active loading in F10 (15% cetyl alcohol: Tw80) (data specified in Table 15, page 55)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Temp	Between Groups	58.475	36	1.624	3.083	$p < 0.05$
	Within Groups	27.925	53	.527		
	Total	86.400	89			
Time	Between Groups	58.283	36	1.619	3.052	$p < 0.05$
	Within Groups	28.117	53	.531		
	Total	86.400	89			

Table 54 Test of homogeneity of variances of stability of active loading (%) in F11 (15% cetyl alcohol: SL) (data specified in Table 15, page 55)

Test of homogeneity of variance

Dependent Variable: Active loading

F	df1	df2	Sig.
0.967	9	80	0.532

Result: Heterogeneity of variance ($p = 0.043 < \alpha = 0.05$) implying that variances are not equal. ANOVA is still performed and Dunnett's test is used for multiple comparison instead of Turkey's test.

Table 55 One way analysis of stability of active loading in F11 (15% cetyl alcohol: SL) (data specified in Table 15, page 55)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Temp	Between Groups	53.983	49	1.102	1.359	$p < 0.05$
	Within Groups	32.417	40	.810		
	Total	86.400	89			
Time	Between Groups	54.067	49	1.103	1.365	$p < 0.05$
	Within Groups	32.333	40	.808		
	Total	86.400	89			

Table 56 Test of homogeneity of variances of stability of active loading (%) in F12 (15% cetyl alcohol: P188) (data specified in Table 15, page 55)

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Active loading

F	df1	df2	Sig.
1.856	9	80	0.069

Result: Homogeneity of variance ($p = 0.210 > \alpha = 0.05$) implying that variances are equal and homogeneity of variance assumption of ANOVA has been showed. Then ANOVA could test in Turkey's test.

Table 57 Multiple comparison of stability of active loading (%) in F12 (15% cetyl alcohol: P188) (data specified in Table 15, page 55)

(I) Temp		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
initial	4C	.000385	.0005272	0.885	-.000998	.001768
	RT	.000326	.0005272	0.926	-.001057	.001709
	45C	.000459	.0005272	0.820	-.000924	.001843
4C	initial	-.000385	.0005272	0.885	-.001768	.000998
	RT	-.000059	.0003728	0.999	-.001037	.000919
	45C	.000074	.0003728	0.997	-.000904	.001052
RT	initial	-.000326	.0005272	0.926	-.001709	.001057
	4C	.000059	.0003728	0.999	-.000919	.001037
	45C	.000133	.0003728	0.984	-.000845	.001111
45C	initial	-.000459	.0005272	0.820	-.001843	.000924
	4C	-.000074	.0003728	0.997	-.001052	.000904
	RT	-.000133	.0003728	0.984	-.001111	.000845

Table 57 Multiple comparison of stability of active loading (%) in F12 (15% cetyl alcohol: P188) (continuous) (data specified in Table 15, page 55)

Multiple Comparisons

Dependent Variable: Active loading

Tukey HSD

(I) Time		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T0	T30	.000115	.0005272	0.996	-.001268	.001498
	T60	.000426	.0005272	0.851	-.000957	.001809
	T90	.000630	.0005272	0.632	-.000754	.002013
T30	T0	-.000115	.0005272	0.996	-.001498	.001268
	T60	.000311	.0003728	0.838	-.000667	.001289
	T90	.000515	.0003728	0.515	-.000463	.001493
T60	T0	-.000426	.0005272	0.851	-.001809	.000957
	T30	-.000311	.0003728	0.838	-.001289	.000667
	T90	.000204	.0003728	0.947	-.000774	.001182
T90	T0	-.000630	.0005272	0.632	-.002013	.000754
	T30	-.000515	.0003728	0.515	-.001493	.000463
	T60	-.000204	.0003728	0.947	-.001182	.000774

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