การพัฒนาน้ำยาบ้วนปากฟลูโอซิโนโลนอะเซโทไนด์ไมโครอิมัลชัน

นางสาวภัทราวดี ชูแก้วรุ่งโรจน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

DEVELOPMENT OF FLUOCINOLONE ACETONIDE MICROEMULSION MOUTHWASH

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutics
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2016

Thesis Title	DEVELOPMENT OF FLUOCINOLONE ACETONIDE MICROEMULSION MOUTHWASH
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ภัทราวดี ชูแก้วรุ่งโรจน์ : การพัฒนาน้ำยาบ้วนปากฟลูโอซิโนโลนอะเซโทไนด์ไมโครอิมัลชัน (DEVELOPMENT OF FLUOCINOLONE ACETONIDE MICROEMULSION MOUTHWASH) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร. พรเพ็ญ วีระวัฒกานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ภญ. ร.ต.ท. ดร. วลัยศิริ ม่วงศิริ, 66 หน้า.

โรคไลเคนพลานัสในช่องปาก (Oral lichen planus) เป็นโรคที่มีอาการอักเสบเรื้อรังที่เยื่อบุในช่องปาก เชื่อว่าเกิดจากความผิดปกติของระบบภูมิคุ้มกัน แผลในช่องปากชนิดที่มีการฝ่อและการกร่อนของเนื้อเยื่อมักเป็นสาเหตุของความเจ็บปวด มีการนำยาคอร์ติโคสเตียรอยด์เฉพาะที่เพื่อลดอาการอักเสบและความเจ็บปวด และมีรายงานว่าตัวยาฟลูโอซิโนโลนอะเซโทไนด์มีประสิทธิภาพในการลดความรุนแรงของแผลชนิดที่มีการฝ่อและการกร่อน โดยไม่มีอาการข้างเคียงที่รุนแรง ดังนั้นยาฟลูโอซิโนโลนอะเซโทไนด์ในรูปแบบน้ำยาบ้วนปากจึงเป็นตำรับที่น่าสนใจในการรักษาโรคไลเคนพลานัสในช่องปาก ไมโครอิมัลชันจึงถูกเลือกมาเพื่อเตรียมเป็นน้ำยาบ้วนปากเพื่อเพิ่มค่าการละลายและความคงตัวของตัวยาฟลูโอซิโนโลนอะเซโทไนด์ วัตถุประสงค์ของการศึกษานี้คือเพื่อตั้งตำรับน้ำยาบ้วนปากในรูปของไมโครอิมัลชันและพรีไมโครอิมัลชันเข้มข้นที่มีตัวยาฟลูโอซิโนโลนอะเซโทไนด์ ซึ่งได้ประเมินความคงตัวทางกายภาพและเคมีของตำรับน้ำยาบ้วนปาก และกำหนดวันที่ควรใช้ก่อนของตำรับ จากการสร้างเฟสไดอะแกรมซึ่งประกอบด้วยน้ำมันกานพลูหรือน้ำมันเปปเปอร์มินท์เพื่อตั้งตำรับไมโครอิมัลชัน พบว่าการใช้สารลดแรงตึงผิวผสมที่อัตราส่วน 2:1 ของทวีน 80 กับโพลีเอทิลีนไกลคอล 400 ทำให้ได้เฟสไดอะแกรมที่มีพื้นที่ไมโครอิมัลชันกว้างกว่าการใช้สารลดแรงตึงผิวผสมที่อัตราส่วน 1:1 ส่วนประกอบที่เหมาะสมได้แก่ น้ำมันกานพลู 3.5% สารลดแรงตึงผิวผสม 31.5% และน้ำ 65% ซึ่งมีค่าการละลายของยาฟลูโอซิโนโลนอะเซโทไนด์ในช่วงที่ยอมรับได้และสามารถเตรียมไมโครอิมัลชันที่มีตัวยาฟลูโอซิโนโลนอะเซโทไนด์ 0.1% นอกจากนี้ยังเตรียมพรีไมโครอิมัลชันเข้มข้นด้วยอัตราส่วนเดียวกัน จากนั้นได้ทำการศึกษาความคงตัวทางกายภาพและเคมีของตำรับไมโครอิมัลชันและพรีไมโครอิมัลชันเข้มข้น และศึกษาผลต่อความคงตัวทางเคมีเมื่อมีการเติมสารต้านออกซิเดชัน หลังจากเก็บตำรับน้ำยาบ้วนปากเป็นเวลา 6 เดือน พรีไมโครอิมัลชันเข้มข้นและไมโครอิมัลชันทุกตำรับมีความคงตัวทางกายภาพที่ดี อย่างไรก็ตามพบว่าในพรีไมโครอิมัลชันเข้มข้นมีปริมาณยาฟลูโอซิโนโลนอะเซโทไนด์ลดลงมากกว่าในไมโครอิมัลชัน อาจอธิบายได้จากในไมโครอิมัลชันมีการกักเก็บยาฟลูโอซิโนโลนอะเซโทไนด์อยู่ในเฟสน้ำมันภายใน จึงทำให้แยกตัวยาออกจากสารไม่บริสุทธิ์ที่เจือปนหรือสารกลุ่มที่เกิดปฏิกิริยาได้ง่ายที่สามารถเร่งการสลายตัวของยา ในขณะที่สารเหล่านี้กระจายตัวทั่วในตำรับพรีไมโครอิมัลชันเข้มข้น นอกจากนี้การกำหนดวันที่ควรใช้ก่อนของไมโครอิมัลชันยาฟลูโอซิโนโลนอะเซโทไนด์ได้ที่อายุ 12 เดือน ซึ่งยาวกว่าพรีไมโครอิมัลชันเข้มข้นยาฟลูโอซิโนโลนอะเซโทไนด์

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต .....
		ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
สาขาวิชา	เภสัชกรรม	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
ปีการศึกษา	2559	

5776125433 : MAJOR PHARMACEUTICS

KEYWORDS: MICROEMULSION, PREMICROEMULISON CONCENTRATE, FLUOCINOLONE ACETONIDE

PATARAVADEE CHUKAEWRUNGROJ: DEVELOPMENT OF FLUOCINOLONE ACETONIDE MICROEMULSION MOUTHWASH. ADVISOR: ASSOC. PROF. PORNPEN WERAWATGANONE, Ph.D., CO-ADVISOR: ASST. PROF. WALAISIRI MUANGSIRI, Ph.D., 66 pp.

Oral lichen planus (OLP) is a common chronic mucocutaneous inflammatory disease. Atrophic lesions and erosions are most likely to cause pain. Topical corticosteroids have been widely used for reducing inflammation and pain. Fluocinolone acetonide (FA) was reported to successfully reduce severity of atropic and erosive lichen planus without serious complication. Therefore FA mouthwash became an interesting preparation for OLP treatment. Microemulsions was selected for preparing FA mouthwash to improve solubility and stability of the drug. Aims of this study were to formulate mouthwashes in form of microemulsion and premicroemulsion concentrate containing FA. Then physical and chemical stability of the mouthwashes were evaluated and beyond-use date was determined. Pseudo-ternary phase diagrams containing clove oil or peppermint oil were constructed in order to formulate buccal microemulsions of FA. The use of surfactant mixture (S_{mix}) (2:1) ratio of Tween80 to PEG400 provided a larger microemulsion region in the phase diagram compared with S_{mix} (1:1) ratio. The suitable combinations were 3.5% clove oil: 31.5% S_{mix}: 65% water which provided acceptable FA solubility and could be prepared 0.1% FA microemulsion. Moreover, FA premicroemulsion concentrate was prepared according to this ratio. Physical and chemical stability of microemulsion and premicroemulsion concentrates were investigated. Then effect of antioxidants on the chemical stability of FA was also determined. After the FA mouthwashes were kept for 6 months, all FA premicroemulsion concentrates and FA microemulsions exhibited good physical stability. However the content of FA in premicroemulsion concentrates were found to decrease more than that in microemulsions. It could be explained that entrapment of FA in the internal oil phase could exclude FA from impurities or reactive species catalyzing the degradation while these reactants freely dispersed in premicroemulsion concentrates. Beyond-use date of FA microemulsions without and with antioxidants were indicated to be 12 months which was longer than that of FA premicroemulsion concentrates.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Pharmaceutics	Co-Advisor's Signature .....
Academic Year:	2016	

ACKNOWLEDGEMENTS

First of all, I would like to express appreciation to my advisor, Associate Professor Pornpen Werawatganone and my co-advisor, Assistant Professor Walaisiri Muangsiri, for their valuable advises, kindness, and encouragement throughout my study.

I would like to express grateful thanks to all members of my thesis committee for their valuable discussion.

I would like to sincerely thanks to all staffs and lab members Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University for facilities and instrument support.

Special thanks are giving to Graduate school Chulalongkorn University for providing CU graduate school thesis grant. I gratefully acknowledge a part of financial support from the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

I would like to express my profound gratitude to Pharmacy Department, Siriraj Hospital for permission of my education as well as Siriraj development scholarship for providing my tuition fee.

Finally, I am most grateful to my family and all friends for their love, caring, encouragement, kindness supporting throughout my life.

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CHAPTER I

INTRODUCTION

Oral lichen planus (OLP) is a common chronic mucocutaneous inflammatory disease. Various forms of oral lichen planus appear clinically: reticular papular, plaque-like, atrophic, erosive, and bullous lesion. OLP represents a cell-mediated immune response with an inflammatory infiltrating cell population composed of T lymphocytes (Epstein et al., 2003). Topical corticosteroids have been widely used for reducing inflammation and pain. Due to the use of systemic corticosteroids is limited by their toxicity; therefore, potent topical corticosteroids are becoming increasingly useful in the treatment of OLP (Thongprasom and Dhanuthai, 2008).

Fluocinolone acetonide (FA) is a corticosteroid used topically in the treatment of various skin disorders. FA was used at 0.1% concentration for OLP therapy without serious complication. It can improve from severity of atrophic and erosive lichen planus (Buajeeb, Pobrurksa, and Kraivaphan, 2000).

Degradation pathways of FA are oxidation, hydrolysis with pH dependence and photolysis. Degradation of FA is catalyze by hydrogen ion, hydroxide ion and trace metal ions (Kenley et al., 1987; Miolo et al., 2005). FA has been prepared in cream, gel, solution, and ointment dosage forms for topical use. For extensive lesion of OLP, FA mouthwash should be suitable. However, FA is classified as a water insoluble compound. Proper pharmaceutical techniques and safety for buccal use are required for FA mouthwash preparation.

Microemulsions are clear, stable, and isotropic mixtures of oil, water, surfactant, and co-surfactants at appropriate ratio. Microemulsions can incorporate both lipophilic and hydrophilic drug. Drugs incorporated in microemulsions can partition between the

aqueous and hydrophobic phases, depending on their lipophilicity (Azeem et al., 2009). Microemulsions are considered as good liquid vehicles for drug delivery system. Their advantages include the thermodynamic stability, ease of preparation, increase of drug solubilization, improving drug stability, high drug loading capacity, and penetration-enhancing ability with small droplet size range of 10-140 nm (Lopes, 2014). Moreover microemulsions have a very low interfacial tension, which results in excellent contact with the skin surface as well as buccal mucosa. Premicroemulsion concentrates are mixtures of drugs, oils, and surfactants without aqueous phase. Premicroemulsion concentrates can provide spontaneous microemulsions when diluted with a certain amount of water and properly mixed (Li et al., 2005). Therefore, microemulsion preparation seems to be a promising technique to improve solubility of stability of the FA.

In this study, FA was incorporated into microemulsions and premicroemulsion concentrates to prepare mouthwashes and then evaluated its physical and chemical stability to determine beyond-use date of the preparation.

The objectives of present study were as following:

1. To formulate microemulsion and premicroemulsion concentrate containing FA mouthwashes.
2. To evaluate physical and chemical stability of FA microemulsion and premicroemulsion concentrate.
3. To determine beyond-use date of of FA microemulsion and premicroemulsion concentrate.

CHAPTER II

LITERATURE REVIEW

1. Oral lichen planus

Oral lichen planus (OLP) is a common chronic mucocutaneous inflammatory disease and condisdered as a T-cell mediated autoimmune disease. The Increase of TH1 cytokines production is an early step in OLP. Activated T cells are attracted and migrate towards the oral epithelium. Cytokines secreted by keratinocytes such as tumor necrosis factor-alpha (TNF-α) and interleukins-1 (IL-1), IL-8, IL-10 and IL-12 are chemotactic for lymphocytes. The T cells bind to keratinocytes and then lead to apoptosis which destroys the epithelial basal cells. Long term of OLP may result from the activation of the inflammatory mediator nuclear factor kappa B (NF-κB) which may cause keratinocyte hyperproliferation and leads to form white lesions. (Thongprasom and Dhanuthai, 2008).

OLP is often characterized by a bandlike lymphocytic infiltrate in the lamina propia and liquefactive degeneration of basal keratinocytes TNF-α, one of the proinflammatory cytokines, has been reported to play a role in the pathogenesis and inflammatory process of OLP. The oral lesions are often asymptomatic but the atrophic-erosive form of OLP can cause symptoms ranging from burning sensation to severe pain, such as difficulty in speaking, eating and swallowing.

Lichen planus commonly affects the oral mucosa. Mucosal lesion is usually multiple bilateral. Symptomatic OLP is mainly encountered with erosive form and approximately 90.9% of the patients have multiple oral sites of involvement. In old patients the erosive presentations showed significantly longer duration, more affected

sites (oral, genital, oesophageal) and greater prevalence than the reticular or atrophic ones.

OLP can presents as small, raised, white, papules or plaques. Atrophic lesions and erosions are most likely forms to cause pain (Scully and Carrozzo, 2008). The most common affected areas are buccal mucosae, tongue, gingiva, labial mucosa and vermilion of the lower lip. Lesions on palate, floor of the mouth and upper lip are uncommon. Mucosal lesion commonly forms white papules, then gradually enlarges and coalesces to form either a reticular, annular or plaque-like pattern (Al-Hashimi et al., 2007). The gingivae is the common site of erythematous and erosive OLP. Two main types of OLP, reticular and erosive, are mentioned (Paul C. Edwards, 2002). The reticular form is the most common type of OLP. It presents as interlacing white keratotic line (Whickham's striae) with an erythematous border. Reticular form and plaque-like are usually asymptomatic. Erosive OLP is the second most common type. It presents combination of erythematous and ulcerated areas surrounded by radiating keratotic striae. Patients with this form present with symptomps ranging from episodic pain to severe discomfort.

Management of OLP

Good hygiene is concerned to reduce severity of OLP symptoms. The main objective of treatment is to reduce the length and severity of symptomatic outbreak. Treatment of OLP depends on symptoms, the extent of oral involvement, medical history and other factors. Patients with reticular or asymptomatic OLP lesions usually require no active treatment, while patients with symptomatic lesions may require treatment, usually with drug.

Topical corticosteroids

The most widely accepted treatment for lesion of OLP is using topical or systemic corticosteroids to modulate the patient's immune response. Topical drug

treatment is preferred due to fewer side effect. The topical corticosteroids are widely used in the treatment of OLP to reduce pain and inflammation. Potent topical corticosteroid are the first line of treatment for symptomatic OLP at any site. Moderate to high potency topical corticosteroids are effective in OLP such as triamcinolone, fluocinolone acetonide, fluocinonide, mometasone and clobetasol. Various preparations of topical drugs such as oral pastes, ointments, solutions, sprays or mouthwashes have been employed for the treatment of OLP. Different formulations, dosage forms and potency of topical steroids provided different efficacy for the treatment of OLP. Fungal overgrowth of normal oral flora by Candida is the common side-effect arise from topical corticosteroids. In addition, minor other side-effects are bad taste, nausea, dry mouth and sore throat (Thongprasom and Dhanuthai, 2008).

Example of topical corticosteroid preparations for symptomatic the treatment of OLP are triamcinolone acetonide mouthwash, triamcinolone oral paste, fluocinolone acetonide oral paste, fluocinolone acetonide gel and fluocinonide gel. One of the problems of topical corticosteroids in oral treatments is difficulty for application and ability to reach posterior oral places and to cover extensive and multiple areas (Aguirre et al., 2004). Solution dosage form can diminish this problem since it can be used as oral rinse to access various laboriously applied sites (Scully and Carrozzo, 2008). Systemic corticosteroids usually reserved for cases are failed using topical drugs should be taper to reduce adverse effect.

2. Fluocinolone acetonide

Fluocinolone acetonide (FA) is a synthetic hydrocortisone derivative which is used topically for treatment of inflammatory disease. FA is classified as moderate potency topical corticosteroids. FA is insoluble in water, but soluble in acetone,

methanol and ethanol. FA preparations are available in cream, gel, lotion, ointment, and solution dosage forms (PubChem, 2016).

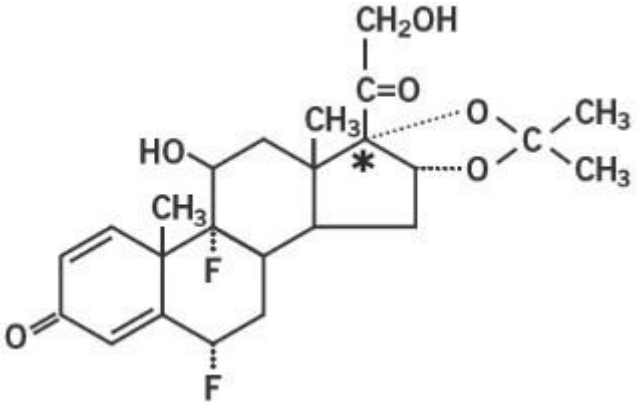


Figure 1 Chemical structure of fluocinolone acetonide

The primary chemical reactivity of steroids occurs at C17 (* marked in Figure 1) side chain leading to degradation. Moreover transition metal, hydroxide and hydrogen ions can catalyze degradation of the steroids, including oxidation and hydrolysis can cause degradation. The degradation of FA increased with temperature at all pH values. The degradation rate constant was observed minimum at pH 4. In addition, FA degradation occurred in aqueous phase of the oil-in-water cream samples (Kenley et al., 1987). Therefore possibility of FA degradation might increase when it was surrounded by aqueous medium.

Another degradation pathway of FA is photodegradation with UV-A or UV-B light. There is reported that FA is very unstable to UV light. FA is quite photolabile, particularly when exposed to UV-B. Therefore exposure to sunlight or fluorescent lamps can cause degradation and loss of therapeutic activity due to chemical modification of the FA molecule (Miolo et al., 2005).

FA is used topically in the treatment of various skin disorders. FA was used at 0.1% concentration in oral base for OLP therapy without serious complication except candidiasis. The lesion is improved from severity of atrophic and erosive lichen planus after treated with FA gel and oral base formulations (Buajeeb, Kraivaphan, and

Pobrurksa, 1997). In addition, FA gel 0.1% was reported that be a safe and effective in the treatment OLP (Buajeeb et al., 2000). The efficacy of 0.1% FA is result from the local anti-inflammatory effect. Moreover some advantages may result from anti-immunologic properties of suppressing T-cell function (Buajeeb et al., 2000).

3. Microemulsions

Microemulsions are defined as a colloidal dispersion system composed of oil, water and amphiphile (surfactant without or with co-surfactant) which is an isotropic and thermodynamically stable liquid solution (Lawrence and Rees, 2012). Microemulsions are considered as a pseudo-ternary system and distinguished from macroemulsions as following three main points. Firstly, macroemulsions are thermodynamically unstable that can lead to phase separation but microemulsions have good thermodynamic stability. Secondly, macroemulsions have droplet size in range of 1-10 μm which are cloudy appearance while microemulsions are clear and transparent resulting from very small droplet size around 10-140 nm or 20-200 nm. Thirdly, microemulsions can form spontaneously, whereas macroemulsions require a lot of energy input due to higher amount of surfactant (Azeem et al., 2009; Lawrence and Rees, 2012).

Suitable ratios of the three components (oil, water and surfactant) can produce microemulsions. The advantages of microemulsions include thermodynamic stability, the ease of preparation, the possibility of incorporating both hydrophilic and lipophilic drugs, the increase of drug loading and the penetration-enhancing ability (Lopes, 2014). There are three types of microemulsions depening on the compositions. Volume fraction of each composition affects types of microemulsions. A low volume fraction of oil leads oil-in-water microemulsions. On the contrary, a low volume fraction of

water leads to water-in-oil microemulsions. An equal amount of water and oil results in bicontinuous microemulsions.

The formation of microemulsions depends on the reduction of oil-water interfacial tension owing to the large amount of surfactants. Microemulsions cause the change in surface area because of the large number of very small droplets formed. A negative free energy of the formation is achieved by large reduction in the surface tension. Therefore most of microemulsification is spontaneous and obtained dispersion is thermodynamically stable.

The phase behavior of conventional microemulsions containing oil, water and surfactant can be studied and each corner of the diagram represents 100% of each composition. However, most of the microemulsion formulation contained surfactants and cosurfactants which pseudoternary phase diagrams are obtained.

The pseudo-ternary phase diagrams are constructed in order to identify the boundaries of the different phases as a function of the three compositions. The pseudoternary phase diagrams show the borderline of microemulsion region that represents the ratio of the compositions resulting in microemulsions as shown in Figure 2. The difference of phases present for a particular mixture can be visually assessed. The extent of microemulsion formation is limited because every combination of the components cannot produce microemulsions. The phase diagrams are usually prepared from the titration of the third composition into series of binary compositions, and then the mixture is evaluated after each addition. Microemulsion region represents sufficient surfactants to promote the formation of microemulsions.

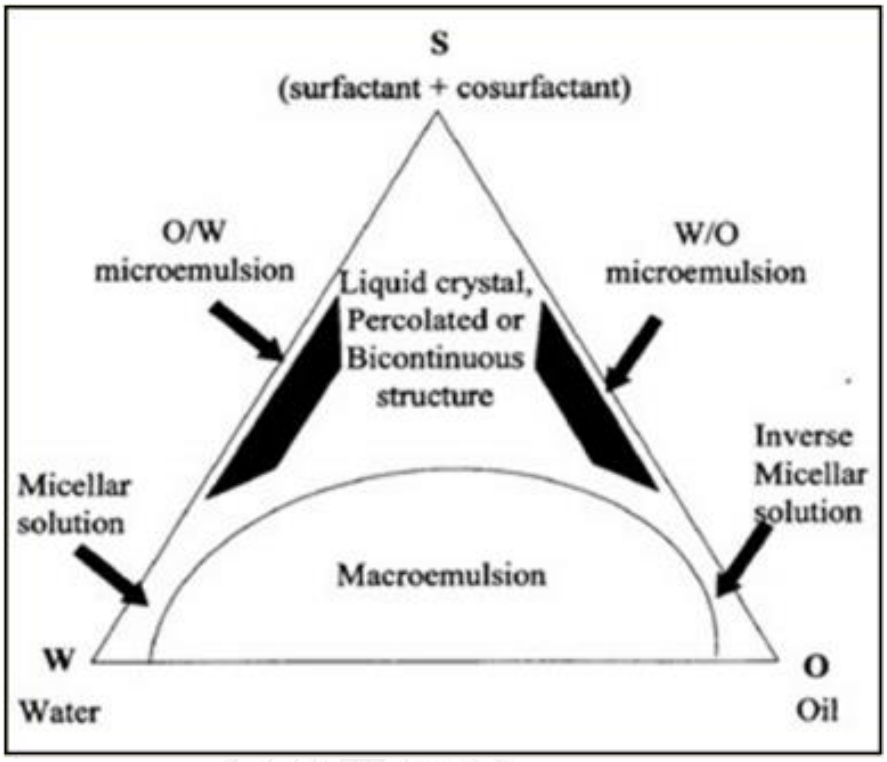


Figure 2 Pseudoternary phase diagram microemulsion region

Both non-ionic and ionic surfactants are used to stabilize microemulsions. However non-ionic surfactants are more intensively employed due to good stability, acid-base insensitive and low toxicity. Examples of non-ionic surfactants are polysorbates, sorbitan fatty acid, polyoxyethlene derivatives of triglycerides, pegylated fatty alcohols and fatty acid and poloxamer. Desirable properties of surfactants are great reduction of surface tension in order to disperse the oil phase to nano-sized droplets, and can make flexible film coated internal phase droplets. Besides surfactants having proper hydrophile-lipophile balance (HLB) value can provide stable microemulsions. Combination of surfactants providing high and low HLB value can give desired HLB value that proper to form microemulsions. Surfactant and cosurfactant ratio is a key factor influencing the phase properties. (Azeem et al., 2009)

The role of co-surfactants is to increase interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film due to the void space

among surfactant molecules (Constantinides and Scalart, 1997). Moreover, the benefit of adding co-surfactants is to reduce the amount of surfactants in formulation. The co-surfactants are also amphiphilic with an affinity for both oil and aqueous phases and can partition into the surfactant interfacial monolayer present at the oil-water interface.

Premicroemulsion concentrate

Self-microemulsifying drug delivery system (SMEDDS) or premicroemulsion concentrate or microemulsion preconcentrate can be described as a mixture consisting of drugs, oils, and surfactants. When diluted with water and with gently agitated, the premicroemulsion concentrate spontaneously forms clear isotropic solution, which is oil-in-water microemulsions (Li et al., 2005). This premicroemulsion concentrate may improve stability due to absence of aqueous phase. Therefore, some degradations such as hydrolysis may be retarded. The probable advantages of this formulation is longer shelf-life, ease of transportation and higher solubility.

CHAPTER III

MATERIALS AND METHODS

Materials

- Fluocinolone acetonide reference standard (Fluka, Lot.LRAA2173)
- Fluocinolone acetonide (Siam Pharmaceutical, Thailand, Lot.00673)
- Clove oil (Symrise, Germany, Lot. 11910004)
- Peppermint oil (Anhui, China, Lot. 20150422)
- Tween 80 (Croda, Singapore, Lot. 27376)
- Polyethylene glycol 400 (PEG 400) (Fluka, Lot. BCBM2199V)
- Caprylic/capric triglyceride (Lexol GT865, INOLEX)
- Medium chain triglyceride (waglinol 3/9280, Malaysia, Lot.1408155)
- Acetonitrile HPLC grade (Labscan, Thailand, Lot.15100264)
- Water HPLC grade (Labscan, Thailand, Lot. 16090268)
- Butylated hydroxyl toluene (S.Tong, Thailand, Lot.150226)
- disodium ethylenediaminetetraacetate (EDTA) (Ajax, Australia, Lot. 0903328)
- sodium metabisulfite (S.Tong, Thailand, Lot.45160356P0)

Apparatus

- Analytical balance (MettlerToledo AG285, Germany)
- High performance liquid chromatography (HPLC) instrument equipped with the following
    - Liquid chromatograph pump (LC-20AB, binary pump, Shimadzu, Japan)
    - UV-VIS detector (SPD-20A, Shimadzu, Japan)

- Auto sampler (SIL-20ATH, Shimadzu ,Japan)
- C18 column (Grace, 5µm, 250x4.6mm, reverse phase column)

- pH meter (MettlerToledo, sevenCompact, Germany)
- Nanosizer (Zetasizer, Nano-ZS with software version 7.11, Malvern, UK)
- Ultrasolnic Bath (GT sonic , China)
- Vortex mixer (Genie-2, Scientific industries, USA)
- Magnetic stirrer (Fisher Scientific, USA)
- Rotator (Chatcharee Holding, Thailand)

Methods

1. Analytical method of fluocinolone acetonide

High performance liquid chromatographic (HPLC) method was employed as an analytical method for FA. The quantitative determination of FA was performed on a reversed-phase HPLC component system from Shimadzu™ consisting of a LC-20AB binary pump, a SPD-20D multiple wavelength detector, a SIL-20ATH auto sampler and LCsolution software. The conditions for HPLC analysis was modified from British Pharmacopoeia 2013.

1.1 Chromatographic conditions

To remove the interference of oil phase in microemulsion on FA peak in the HPLC chromatogram, the analysis method was modified by adjusting mobile phase ratio. Chromatographic condition for analysis of FA was as following:

Column : Apollo® C_{18} reverse phase HPLC column (4.6x250mm, 5 µm)

Mobile phase : isocratic system composed of acetonitrile : water (50:50 v/v)

Flow rate : 1.0 ml/min

UV detector : wavelength 238 nm

Injection volume : 20 µL

Retention time : 6.9-7.0 min

The samples were diluted to get appropriate concentration and filtered through 0.45 µm membrane filter before injection.

1.2 Standard solution

Reference standard of FA (98.7%) was accurately weighed and dissolved in acetonitrile. This stock solution was further diluted with acetonitrile:water (50:50) to obtain standard solutions with the final concentrations of FA at 0.5, 1, 2.5, 5, 7.5 and 10 mg/100ml.

1.3 Sample solution

FA microemulsion was accurately weighed and dissolved in the mobile phase, acetonitrile : water (50:50). This solution was further diluted using the mobile phase until the final concentration of FA was in the range of 0.5-10 mg/100ml.

1.4 Verification of the HPLC method

The HPLC analysis condition was validated with typical parameters, which are linearity, accuracy, precision and selectivity. (ICH topic Q2(R1), 2005)

Linearity

Six concentrations of FA solutions were prepared from 98.7% fluocinolone acetonide reference compound and analyzed. The relation between peak areas and concentrations were plotted and least square linear regression (R^2) was calculated. The test was done for three replications. Acceptable criteria R^2 value is 0.99.

Accuracy

Spiked samples were prepared in triplicate at three concentration levels, 2.5, 5, 10 mg/100ml. The percent recovery was calculated. Acceptable criteria percent recovery is 98-102.

Precision

Within run precision

Six concentrations of FA solutions (three replicates/concentration), were prepared and analyzed. The precision of each concentration was determined from the percent coefficient of variation (%CV) which should be not greater than 3.

Between run precision

Six concentrations of standard solutions were prepared and analyzed in three different days. The precision of each concentration was determined from the percent coefficient of variation (%CV) which should be not greater than 3.

Specificity

FA microemulsions and blank microemulsions were diluted using the mobile phase and injected to the HPLC system. Peak purity index was determined from their chromatogram. The peak purity index should be not less than 0.99.

2. Formulation of fluocinolone acetonide microemulsion

2.1 Solubility studies for screening of microemulsion components

Solubility of FA in different oils (clove oil, peppermint oil, medium chain triglyceride, and capric/caprylic triglyceride) were determined. An excess amount of FA was added to 3 mL of various oils filled in amber glass vials and the vials were rotated 360° for 24 hours at room temperature (25±2°C). The samples were filtered using 0.45 µm membrane filter, properly diluted using the mobile phase and analyzed using HPLC method to determine FA concentration. The oils providing high solubility of FA were selected in order to be used in the preparation of microemulsion.

2.2 Construction of pseudo-ternary phase diagram

In order to determine the concentration ranges of components in microemulsions, pseudo-ternary phase diagrams were constructed using water titration method. Surfactant mixtures, Tween 80 and Polyethylene glycol 400 (PEG 400), were

prepared at the ratio of 1:1 and 2:1 by weight. Then each ratio of the surfactant mixtures was mixed with the selected oils at the ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 by weight. Water was gradually added drop by drop to the mixtures under moderately stirring until visual changes were observed in the samples from turbid to transparent and from transparent to turbid. Concentrations of the three components were used for preparing pseudo-ternary phase diagrams. Area in the phase diagram was defined to be clear region and turbid or phase-separate region. Only the transparent region was termed as the microemulsion existence region.

2.3 Selection of the suitable microemulsions

Based on the pseudo-ternary phase diagrams, suitable ratios of oil, surfactant mixture and water for preparation of oil-in-water microemulsions were selected. The suitable ratio was selected from the transparent region of the phase diagram. The microemulsion at this ratio must be able to dissolve FA not less than 0.12%w/v which is 20% greater than our expected concentration in the final preparation. FA solubility in these MEs was determined using the same manner as previous study (2.1).

2.4 Preparation of microemulsions containing FA

Based on the selected ratios from 2.3, FA microemulsions were prepared from premicroemulsion concentrate containing FA dissolved in the oil and the surfactant mixture. A certain amount of water was added into the premicroemulsion concentrate to obtain 0.1% FA microemulsion preparation. Visual appearance and precipitation of microemulsion were monitored. Preliminary stability study of the preparation was also performed at 40°C storage temperature. The preparations of premicroemulsion concentrate and microemulsions providing clear solution were selected for further study.

3. Stability study of premicroemulsion concentrate and microemulsion containing FA

3.1 Preparation of FA premicroemulsion concentrate and FA microemulsion without antioxidant

Based on the selected preparations from 2.4, surfactant, cosurfactant and oil were accurately weighed and mixed together to form clear single phase. Accurate weight of FA was added into the mixtures and stirred to obtain clear solution of premicroemulsion concentrate. Microemulsion containing 0.1% FA was prepared by adding suitable amount of water into the premicroemulsion concentrate. Physical and chemical stability of the premicroemulsion concentrate and the microemulsion were monitored.

3.2 Preparation of FA premicroemulsion concentrate and FA microemulsion containing antioxidants

3.2.1 FA premicroemulsion concentrate and FA microemulsion containing 0.1% butylated hydroxytoluene (BHT)

The FA premicroemulsion concentrate was prepared as described in 3.1. BHT was dissolved in the premicroemulsion concentrate. Then the microemulsion was obtained once water was added. The stabilities of both premicroemulsion concentrate and microemulsion were monitored.

3.2.2 FA microemulsion containing 0.1% disodium ethylenediaminetetraacetate (EDTA)

The FA premicroemulsion concentrate was prepared as described in 3.1. disodium EDTA was dissolved in water phase before added to the premicroemulsion concentrate to obtain microemulsion. The stabilities of the microemulsion were monitored.

3.2.3 FA microemulsion containing 0.1% sodium metabisulfite

The FA premicroemulsion concentrate was prepared as described in 3.1. Sodium metabisulfite was dissolved in water phase before added to the

premicroemulsion concentrate to obtain microemulsion. The stabilities of the microemulsion were monitored.

3.3 Physical and chemical stabilities

These premicroemulsion concentrates and microemulsions were filled in 10 mL amber glass vials, sealed and stored in ovens at 30ºC and 40ºC (75%RH) for 6 months.

3.3.1 Physical stability

Visual appearance, color, clearness and precipitation of the samples kept at 30ºC and 40ºC were observed. Zeta potential, particle size, size distribution and pH of the samples kept at 30 ºC were determined. Zeta potential, particle size and size distribution were measured by dynamic light scattering method using Zetasizer™ (Zetasizer™ Nano-ZS with software version 7.11, Malvern, UK). The pH values of the samples were recorded using a pH meter (METTLER TOLEDO™, SevenCompact, Germany) at room temperature.

3.3.2 Chemical stability

Three batches of each formulation were prepared, filled in amber glass vials and kept at 30±2ºC and 75±5 %RH. Quantitative assay of FA was determined using HPLC method at 0, 1, 2, 3 and 6 month(s).

3.4 Shelf-life and beyond-use date determination

Because premicroemulsion concentrates and microemulsions were considered to use the same criteria as FA topical solution, 90-110% label amount was indicated in USP29. Shelf-life and beyond-use date were determined by regression analysis. Ninety-five percent lower confidence limit was calculated from the equation 1. The shelf-life of each batch was estimated from the time at intersection between the regression curve of 95% lower confidence limit and 90% drug remaining.

$$X = Y \pm t_{df,0.95}(S_{yx})\sqrt{\frac{1}{N} + \frac{(x-\overline{X})^2}{\sum(x-\overline{X})^2}}$$ Equation 1

Where X=two-sided confidence limit interval ,Y= predicted potency of drug (from linear regression) , $t_{df,0.95}$ *= T score,* S_{yx} *= sum of square of time range N = sample size , x = time (month) and* $\overline{X}$ *= time (average).*

Beyond-use date was defined from the shortest shelf-life of each formulation and should be not greater than twice of the stability study period.

CHAPTER IV

RESULTS AND DISCUSSIONS

1. Analysis of FA

In this study, the HPLC method (BP, 2013) had been modified and verified.

1.1 Linearity

The representative calibration curve data of FA are shown in Table A-1 in Appendix A. The plot of FA concentrations versus the peak area of the drug exhibited the linear correlation in the concentration range of 0.5-10 mg/100mL. The coefficient of determination (R^2) was 1.000 as shown in Figure 3 . This result indicated that the HPLC method was acceptable for quantitative analysis of FA in the studied range.

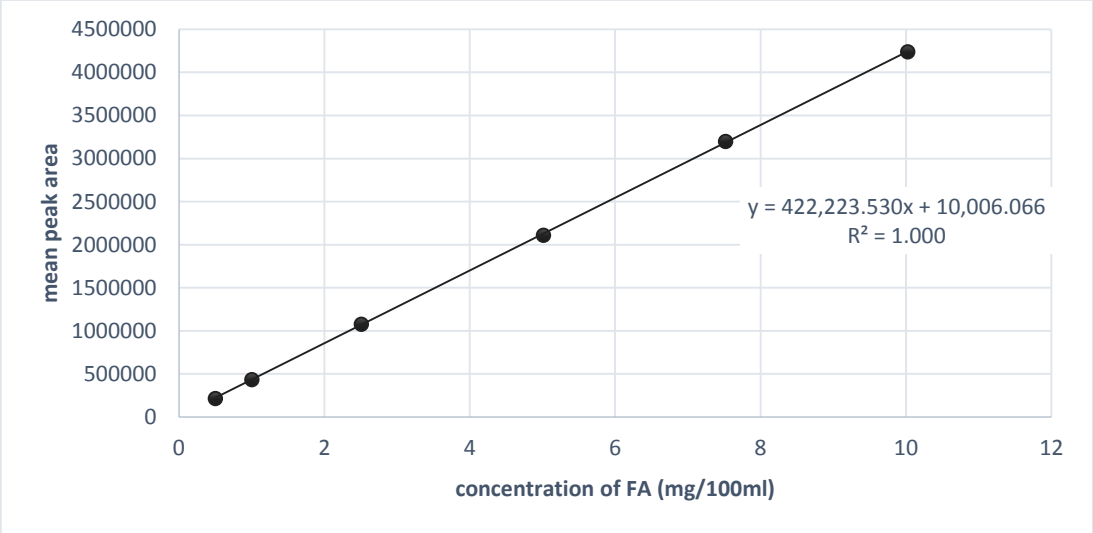


Figure 3 Calibration curve of FA standard solution using modified HPLC method

1.2 Accuracy

The accuracy of an analytical procedure expresses the closeness between a conventional true value or an accepted reference value and the value found. The accuracy assessed using 3 concentration levels covering the specified range.

This modified HPLC method could be used for FA analysis in the studied range. Since the percentage of analytical recovery was in the range of 98.88 – 99.27 as shown in Table 1.

Table 1 Data of accuracy of FA analyzed by modified HPLC method

Concentration (mg/100mL)	**% Analytical recovery**			**Mean ± SD**
	Set 1	Set 2	Set 3	
2.5292	99.750	97.947	98.921	98.873 ± 0.902
5.0540	99.389	99.085	99.331	99.268 ± 0.162
7.5712	99.128	99.110	99.390	99.209 ± 0.157

1.3 Precision

The precision of an analytical procedure expresses the closeness between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision assesses using 3 concentration levels covering the specified range.

The precision was determined both within run and between run, which were expressed as the present coefficient of variation (%CV) in

Table 2 and Table 3. The coefficient of variation values of within run and between run were in the range of 0.158-0.904 % and 0.263-0.634%, respectively, as presented in

Table 2 and Table 3. This indicated that the HPLC method was precise for FA analysis in the studied range.

Table 2 Data of within run precision of FA analyzed by modified HPLC method

FA Concentration (mg/100mL)	Calculated concentration of FA			Mean	SD	%CV
	Set1	Set2	Set3			
2.5292	2.523	2.477	2.502	2.501	0.023	0.913
5.054	5.023	5.008	5.020	5.017	0.008	0.163
7.5712	7.505	7.504	7.525	7.511	0.012	0.158

Table 3 Data of between run precision of FA analyzed by modified HPLC method.

FA Concentration (mg/100mL)	Calculated concentration of FA			Mean	SD	%CV
	Set1	Set2	Set3			
2.5196	2.568	2.581	2.575	2.575	0.007	0.265
5.0392	5.014	4.977	4.965	4.986	0.025	0.508
7.5588	7.620	7.528	7.550	7.566	0.048	0.636

1.4 Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of the other components in the sample. There was no interference from other components with the peak area of FA at the purity index value of FA peak of 0.9999 as shown in Figure 4. This implied that the modified HPLC method was specific for analysis of FA in the samples.

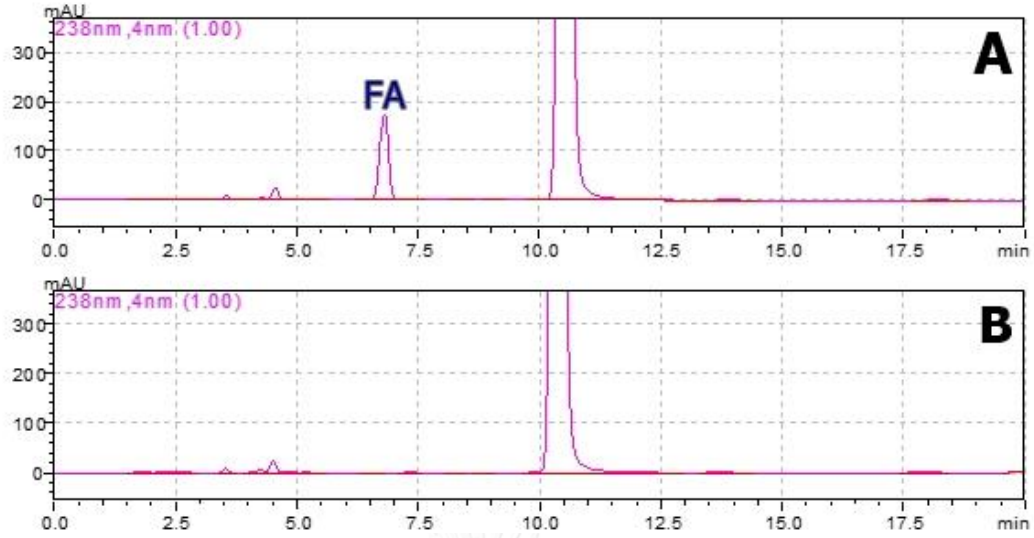


Figure 4 Chromatogram of microemulsion (A) FA-loaded microemulsion shows FA peak at 6.8-7.0 min and (B) blank microemulsion shows no interference at the retention time of FA.

The method has linear response in stated range and is accurate and precise. The described method can be used as stability indicating method for FA assay in microemulsion and premicroemulsion concentrate.

2. Formulation of fluocinolone acetonide microemulsion

2.1 Solubility studies for screening of microemulsion components

Owning to developing mouthwash preparation, various components were chosen from general regard as safe category. The high solubility of FA in oil phase is important to develop microemulsions providing high drug loading. In order to screen appropriate oil phase for preparation of microemulsions, the solubility of FA in different oils was determined and the results are shown in Table 4. The selection of oils in microemulsion formulation, medium chain triglycerides and capric/caprylic triglyceride, were preferred due to reproducibility property of semisynthetic oils. In oil-in-water microemulsion, FA, a lipophilic drug, should reside in oil phase. (Azeem et al., 2009) Even though, medium chain triglyceride and capric/caprylic triglyceride have been

widely used in microemulsion preparation but these oils did not dissolve FA well. Due to low solubility of FA, these oils would provide low drug loading capacity. In contrast, high solubility of FA in clove oil (25.22 mg/ml) and peppermint oil (9.5 mg/ml) were observed. Consequently, clove oil and peppermint oil were selected as the oil phase for the development of microemulsion systems in next study. Both clove oil and peppermint oil have good odor and taste. Besides clove oil and peppermint oil have been widely used in dental preparations, meaning that they are proper to be used in this mouthwash preparation.

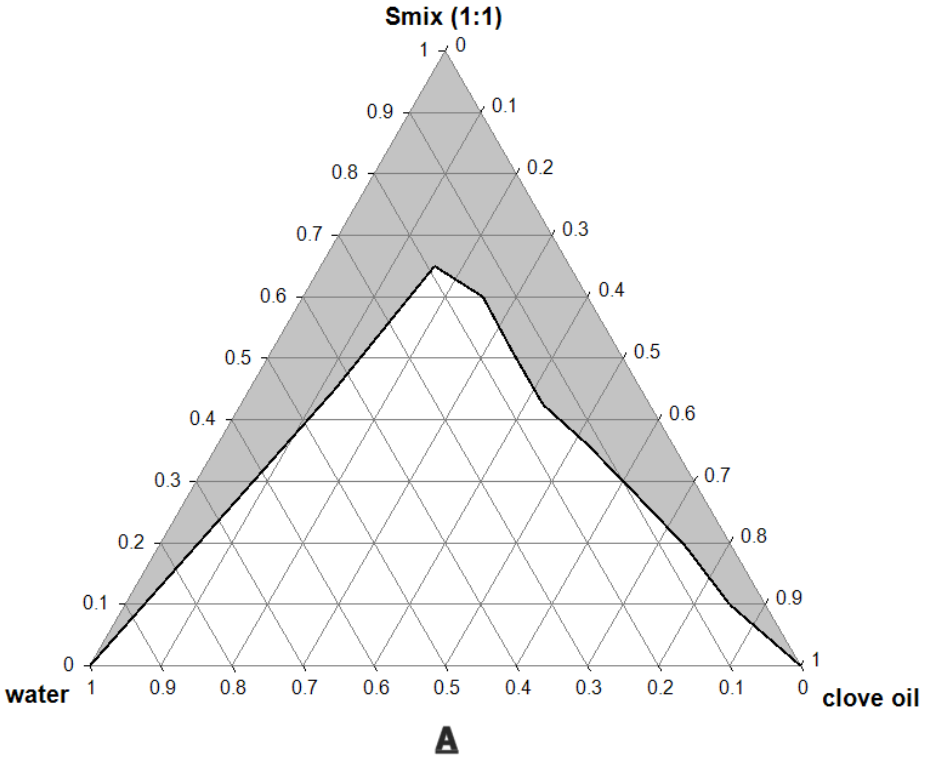
Table 4 Solubility of FA in different oils (n=3, mean ± SD)

Oil	**Solubility (mg/ml)**
Clove oil	25.22 ± 1.32
Peppermint oil	9.50 ± 0.65
Medium chain triglyceride	1.18 ± 0.11
Capric/caprylic triglyceride	**1.32 0.11**

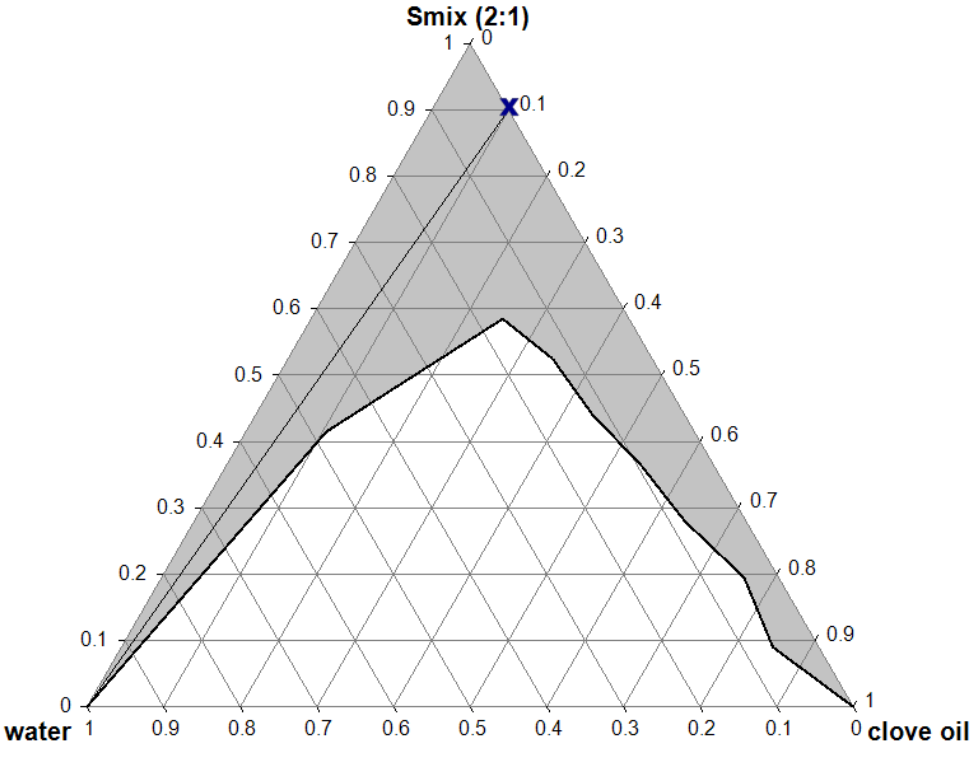
2.2 Construction of pseudo-ternary phase diagrams

The pseudo-ternary phase diagrams were constructed to determine the existing region of microemulsion. Clove oil or peppermint oil were used as the oil phase in the phase diagram. S_{mix} of 1:1 or 2:1 (Tween 80: PEG 400) was used as surfactant mixtures.

When clove oil was the oil phase, the phase diagrams containing S_{mix} 1:1 and 2:1 are presented in figure 5A and 5B, respectively. With the used of peppermint oil as the oil phase, the phase diagrams containing S_{mix} 1:1 and 2:1 are shown in figure 5C and 5D, respectively.


Smix (1:1)
water
clove oil
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

A


Smix (2:1)
water
clove oil
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

B

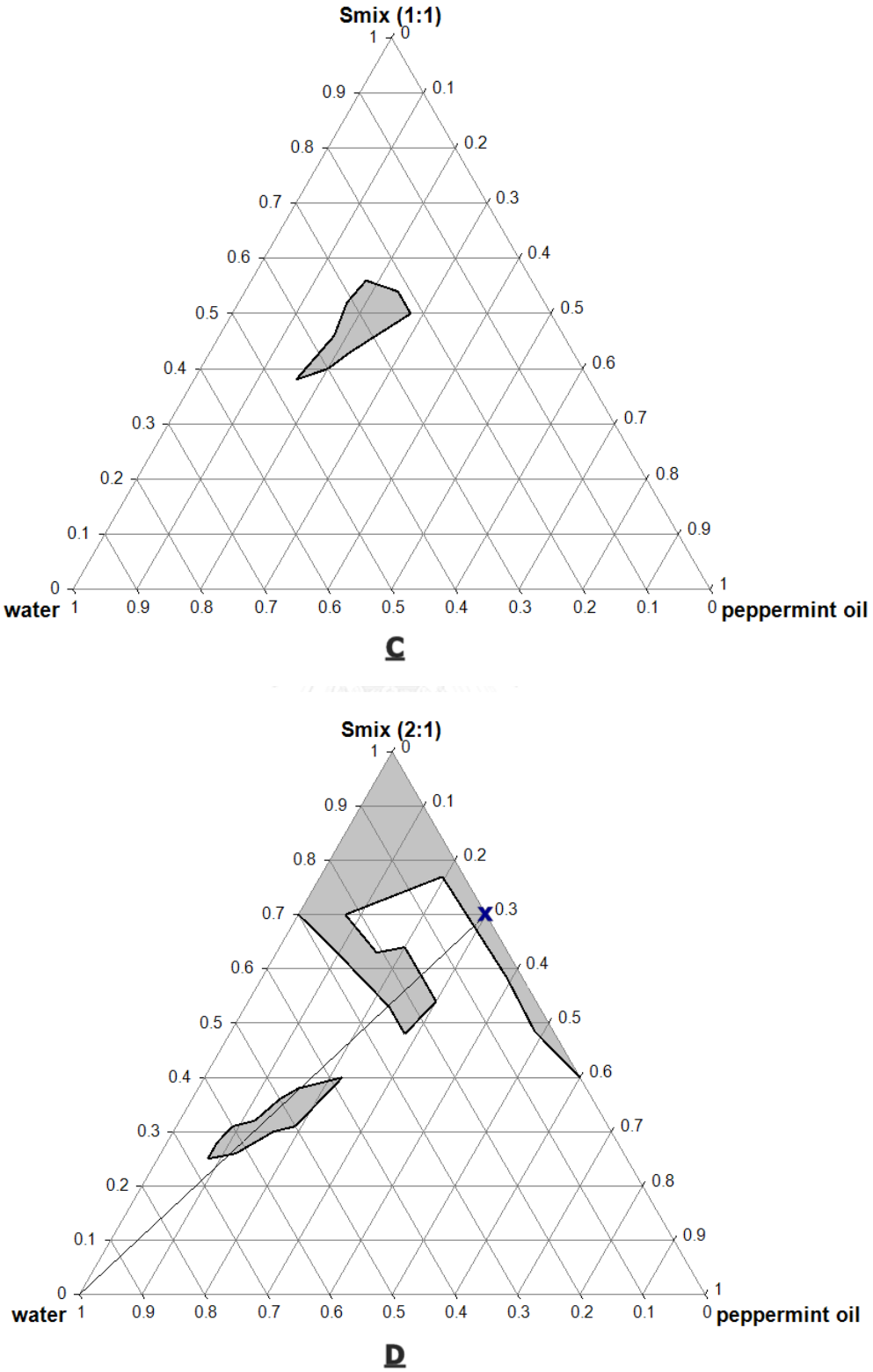


Figure 5 Pseudo-ternary phase diagrams composed of clove oil (A,B) or peppermint oil (C,D), surfactant mixture (S_{mix}) and water and shaded area representing ME region

The weight ratio of surfactant to co-surfactant affected the microemulsion existing region. The diagrams containing S_{mix} (2:1) provided a larger microemulsion region than the ones containing S_{mix} (1:1) meaning that the increase of surfactant in the surfactant mixture enhanced micelle formation which would consequently increase the solubilizing capacity of oil the microemulsions (Butani, Yewale, and Misra, 2014). The S_{mix} ratio of 2:1 was selected to continue in next study.

However, decreasing in interfacial tension plays an important roles to form microemulsions, increasing ratio of surfactant to cosurfactant may improve formulating microemulsions. (Kawakami et al., 2002)

2.3 Selection of the suitable microemulsions

In this study, the criteria for selection of suitable combination microemulsion region were based on their physicochemical properties (clear or transparent, no precipitation, low viscosity and no phase separation) when they were diluted with a certain amount of water. Regarding the phase diagrams (figure 5B and 5D), a premicroemulsion concentrate of 30% peppermint oil: 70% S_{mix} (3:7) and a premicroemulsion concentrate of 10% clove oil : 90% S_{mix} (1:9) were selected (X marked on Figure 5B and 5D). Clear preparations were obtained from these ratios, when they were in forms of premicroemulsion concentrate (mixture of oil and S_{mix}) and diluted microemulsion.

The selected premicroemulsion concentrates were diluted with water at different amount as presented in Table 5 and mixed well until clear oil-in–water microemulsions were obtained. Then solubility of FA in these microemulsions were determined and the result is shown in Table 5.

Table 5 Solubilities of FA in different microemulsions (n=3, mean ± SD)

Oil phase	Oil (%w/w)	S_{mix} (2:1) (%w/w)	Water (%w/w)	Solubility of FA (mg/ml)
Peppermint oil	15	35	50	3.20 ± 0.29
Clove oil	1.67	15	83.33	0.70 ± 0.00
	2.67	24	73.33	1.01 ± 0.00
	3.33	30	66.67	1.14 ± 0.02
	3.5	31.5	65	1.31 ± 0.05
	4	36	60	1.73 ± 0.03

Since the final concentration of FA in microemulsion is 0.1%w/v, then the expected solubility of FA in the microemulsion was about 20% greater than this final concentration. The criteria of this study for a suitable microemulsion were minimum amount of S_{mix} providing FA solubility not less than 1.2 mg/ml and clear preparation. The amount of S_{mix} was minimized in order to limit unfavorable taste and risk of irritation. The selected microemulsions were 3.5% clove oil : 31.5% S_{mix} : 65% water and 15% peppermint oil : 35% S_{mix} : 50% water.

2.4 Preparation of premicroemulsion concentrate and microemulsion containing fluocinolone acetonide.

FA premicroemulsion concentrates and FA microemulsion were prepared in the selected preparations from 2.3 clear mixture without phase separation or precipitation was obtained at initial as shown in Figure 6.

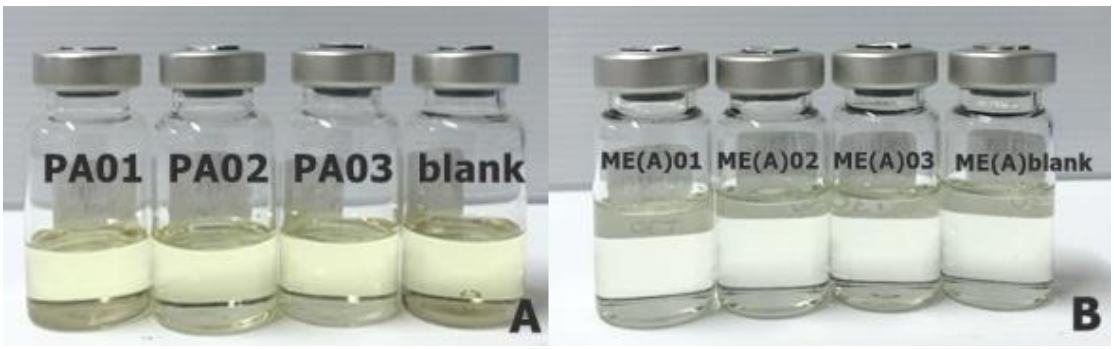


Figure 6 Physical appearance of FA preparations (A) premicroemulsion concentrates and (B) microemulsions

However, premicroemulsion concentrate and microemulsion containing peppermint oil were excluded because preliminary stability experiment was shown that the preparation containing peppermint oil (stored at 40°C), became turbid within 4 weeks. Due to high temperature, microemulsion existence region change, the selected ratio could not provide clear and stable microemulsion. Therefore only the premicroemulsion concentrate and the microemulsion containing clove oil were selected for further studies.

3. Stability study of premicroemulsion concentrate and microemulsion containing FA

The selected FA premicroemulsion concentrate and microemulsion containing clove oil were prepared in the system without and with antioxidants as presented in *Table 6* and Table 7.

Table 6 Formulation of FA premicroemulsion concentrates

Premicroemulsion concentrates	FA (%w/w)	Clove oil (%w/w)	S_{mix} (%w/w)		Antioxidant (%w/w)
			Tween 80	PEG 400	
PA	0.2857	10	60	30	-
PB	0.2857	10	60	30	0.1 (BHT)

Table 7 FA microemulsion prepared from premicroemulsion concentrates

Preparation	PA (%w/w)	PB (%w/w)	Antioxidant (%w/w)	Water (%w/w)
ME(A)	35	-	-	65
ME(B)	-	35	0.035* (BHT)	65
ME(C)	35	-	0.1 (sodium EDTA)	65
ME(D)	35	-	0.1 (sodium metabisulfite)	65

*0.035% BHT was already in PB.

3.1 Physical stability

After six months at both 30 ± 2ºC and 40 ± 2ºC, physical appearances of FA microemulsions and premicroemulsion concentrates were unchanged in term of transparency and phase separation. Also, precipitation of the drug was not detected.

All FA premicroemulsion concentrates and FA microemulsions exhibited good physical stability during six-month study period at 30 ± 2 ºC/75%RH ± 5%RH. There was no significant change of pH value as shown in Table 8. The particle size of drug

free microemulsions and drug loaded microemulsions were determined and there was no significant difference observed in average particle size after loading the drug. Moreover, size distribution and zeta potential of the preparations were not significant change after six month storage. In addition, pH values of microemulsions were stable around six months.

Table 8 Physical stability of FA-loaded microemulsions preparation

Preparation		**pH**	**Size Z-average (d.nm)**	**Size distribution PdI**	**Zeta potential (mV)**
Blank ME(A)	Initial	3.71	15.43	0.214	-1.56
	6 month	3.82	14.27	0.245	-2.18
ME(A)	Initial	4.21	14.54	0.22	-4.2
	6 month	3.42	10.94	0.192	-3.14
ME(B)	Initial	4.18	14.78	0.2	-2.34
	6 month	3.53	11.98	0.260	-2.68
ME(C)	Initial	4.3	15.39	0.281	-1.96
	6 month	3.72	14.32	0.208	-1.49
ME(D)	Initial	3.95	14.28	0.221	-2.38
	6 month	3.83	11.17	0.195	-2.33

3.2 Chemical stability

Three batches of each formulation were prepared and evaluated. The percent remaining of FA in the preparations versus time was plotted and shown in Figure 7 and Figure 8. At 30±2°C and 75±5%RH, percent remaining of FA in all preparations

was greater than 90 over 6 month storage time. However, FA degradation in premicroemulsion concentrates (PA and PB) was found to be greater than that in microemulsions (ME(A) and ME(B)).

The FA premicroemulsion concentrates could be considered as aqueous free solutions and FA was dissolved in clove oil. Impurities or reactive species in clove oil such as aldehyde, heavy metals and phenol may have potential to directly catalyze degradation of FA molecules in the solutions.

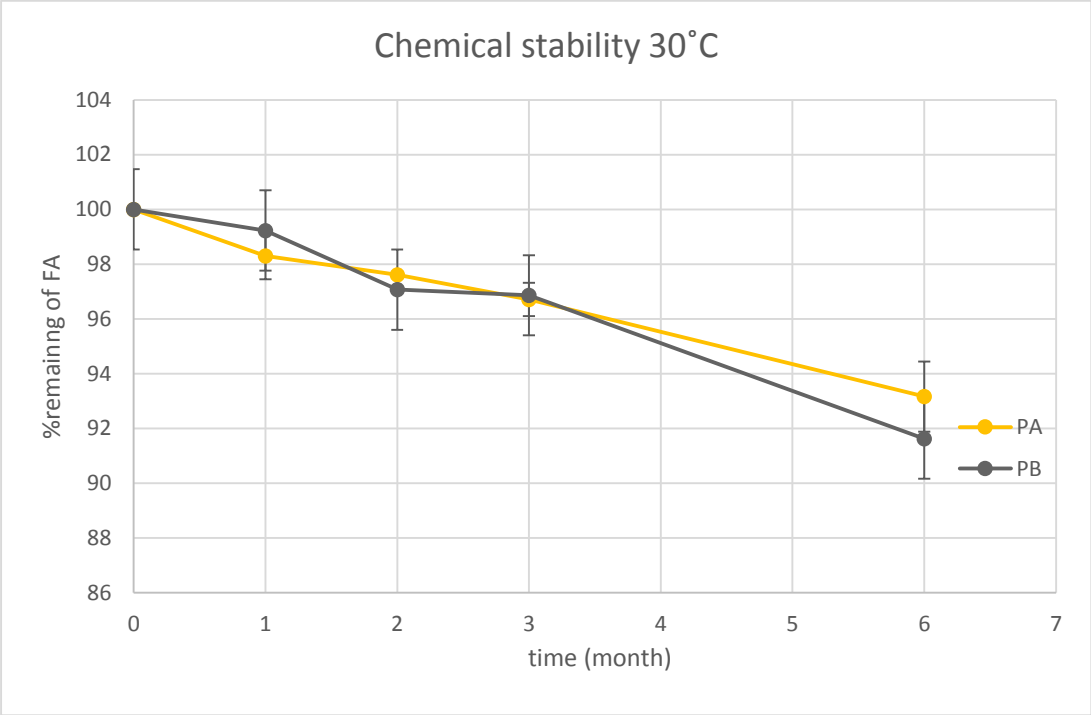


Figure 7 Chemical stability of FA premicroemulsion concentrates stored at 30˚C

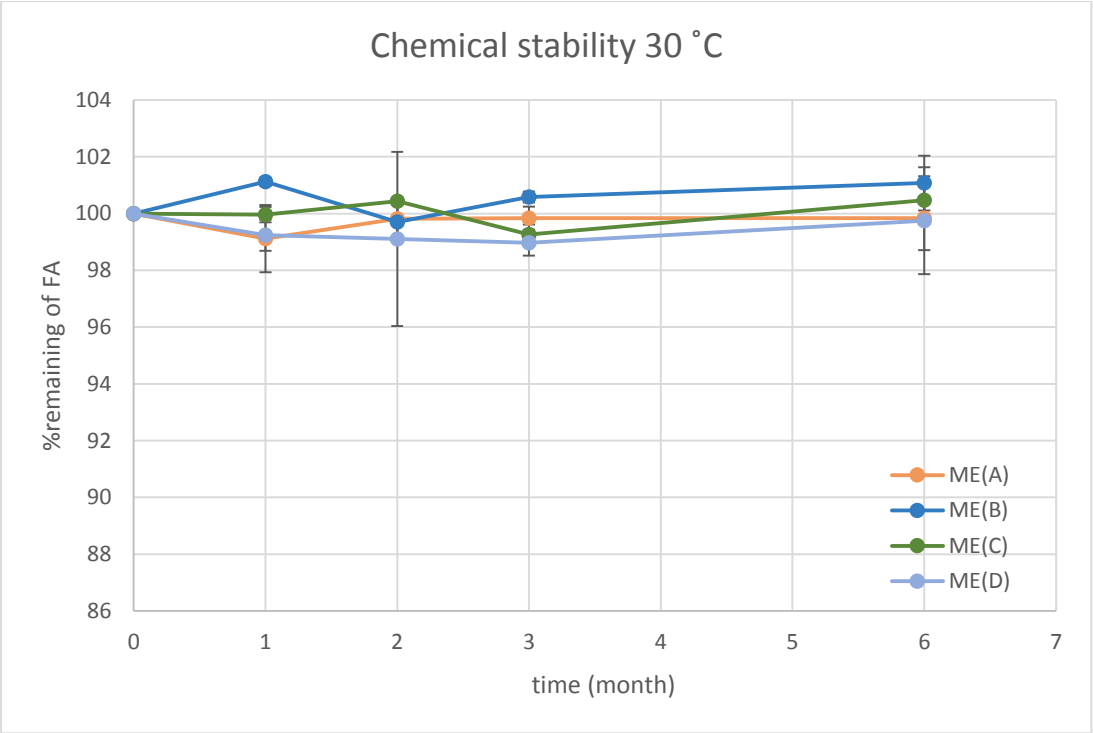


Figure 8 Chemical stability of FA-loaded microemulsions stored at 30˚C

When clove oil was prepared in microemulsion preparations, these impurities or reactive species may prefer to diffuse into water phase due to their polarity while FA was still retained in the microdroplet of oil resulting in less degradation observed. Moreover, steric hindrance of surfactant molecules surrounding the microdroplet of oil in microemulsions could impede reactive species to attack FA located in the oil phase. Similar to chloramphenicol-loaded microemulsions, chloramphenicol molecules were screened from the bulk water and its stability increased (Lv, Zheng, and Tung, 2005). However, further study is needed to prove this speculation.

Photodegradation of fluocinolone acetonide is another degradation pathway. Although all formulations were filled in amber glass vials, wavelengths affecting to FA are both UV-A (320-400 nm) and UV-B (290-320 nm) some wavelengths may pass through the vials. (Miolo et al., 2005). Microdroplets of oil in microemulsion has light scattering property; hence, can reduce light exposure to drug molecules incorporated in internal oil phase. Photo-sensitive chemicals such as resveratrol were protected in

nanoemulsions; therefore, encapsulation of the drug can improve chemical stability. (Davidov-Pardo and McClements, 2015) In addition, photodegradation process was reduced in isotretitoin-loaded microemulsion as compared to the methanol solution meaning that photostability of the drug-loaded microemulsion was improved. (Patel et al., 2011) The effect of photooxidation might take place during the sample preparation for HPLC analysis. Therefore, chemical stability of FA-loaded microemulsion was improved when compared with FA premicroemulsion concentrate.

BHT seemed to be ineffective to retard the degradation of FA in both premicroemulsion concentrate and microemulsion. Moreover, the addition of sodium EDTA and sodium metabisulfite in microemulsions rarely improved the stability of FA in microemulsion as shown in Figure 8. It could be due to antioxidant activity of clove oil. Clove oil is more effective antioxidant with higher H_2O_2 scarvenging activity when compared with these antioxidants (BHT, sodium EDTA, sodium metabisulfite), therefore, antioxidation effect of the antioxidants might be covered by the effect of clove oil. (Gülçin, Elmastaş, and Aboul-Enein, 2012)

Nevertheless, all of FA microemulsions were chemically stable since FA concentrations remained constant through 6 months. Therefore, no significant difference of percent FA remaining between formulations with and without antioxidant was observed.

After FA premicroemulsion concentrates and microemulsions were stored at 40ºC, more degradation of FA in premicroemulsion concentrates and micromemulsions was observed when compared to the samples stored at 30ºC. FA in microemulsions were also found to be more stable than that in the premicroemulsion concentates as shown in Figure 9 and Figure 10.

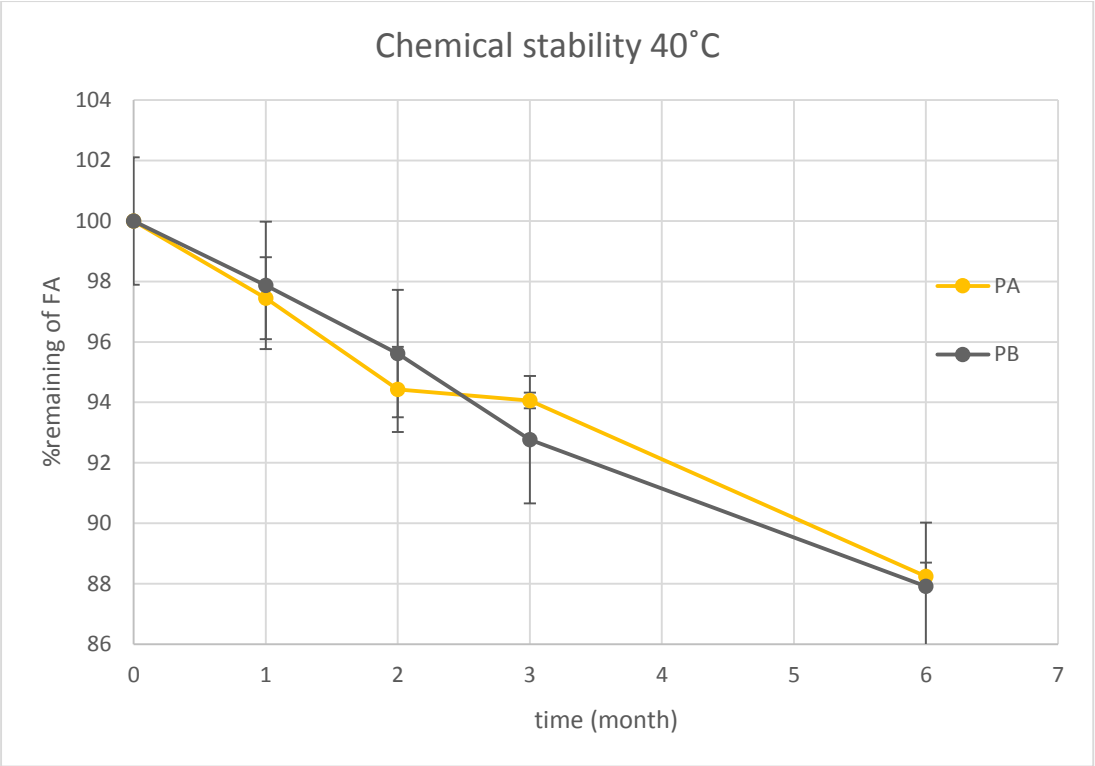


Figure 9 Chemical stability of FA premicroemulsion concentrates stored at 40˚C

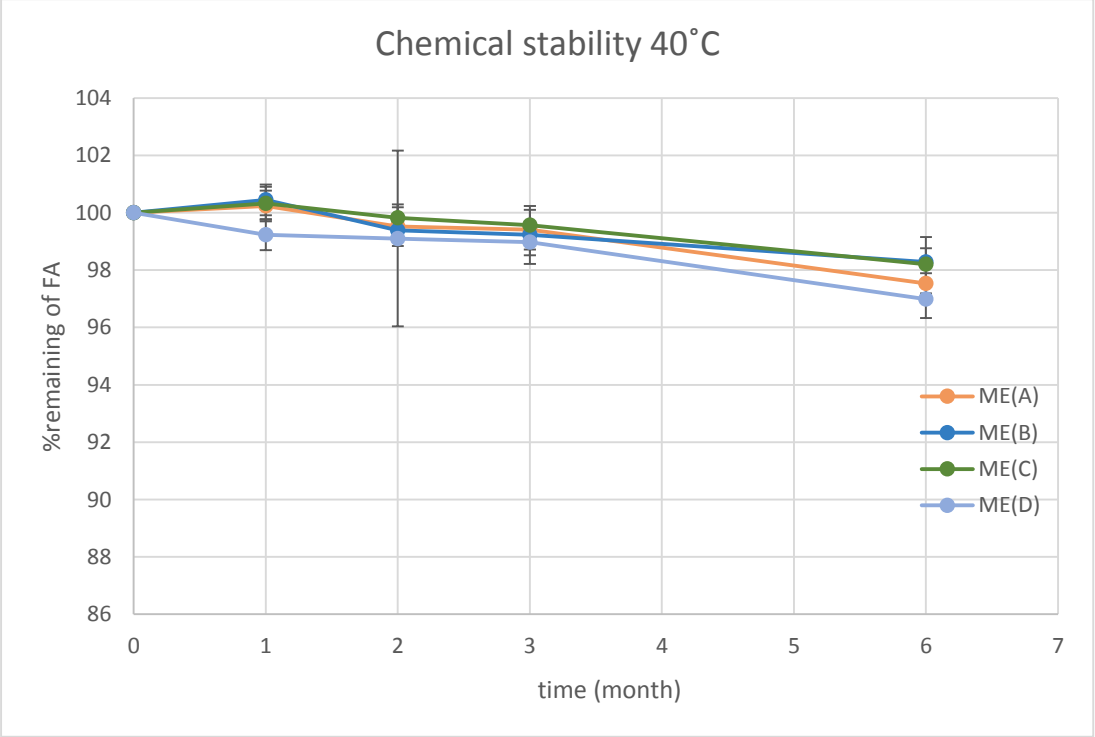


Figure 10 Chemical stability of FA microemulsions stored at 40˚C

3.3 Shelf-life and beyond-use date determination

From chemical stability data at 30 ºC, shelf-life and beyond-use date were determined using regression analysis. A typical plot of shelf life determination is presented in Figure 11. The shelf-life of each batch was estimated from the time at intersection of 95% lower confidence limit regression curve and 90% remaining. Shelf-life of all FA microemulsions was longer than that of FA premicroemulsion concentrates (Table 9). The shortest shelf-life of FA microemulsions was longer than 12 months which was twice of 6 months. Then, the beyond-use date of FA microemulsions was indicated to be 12 months.

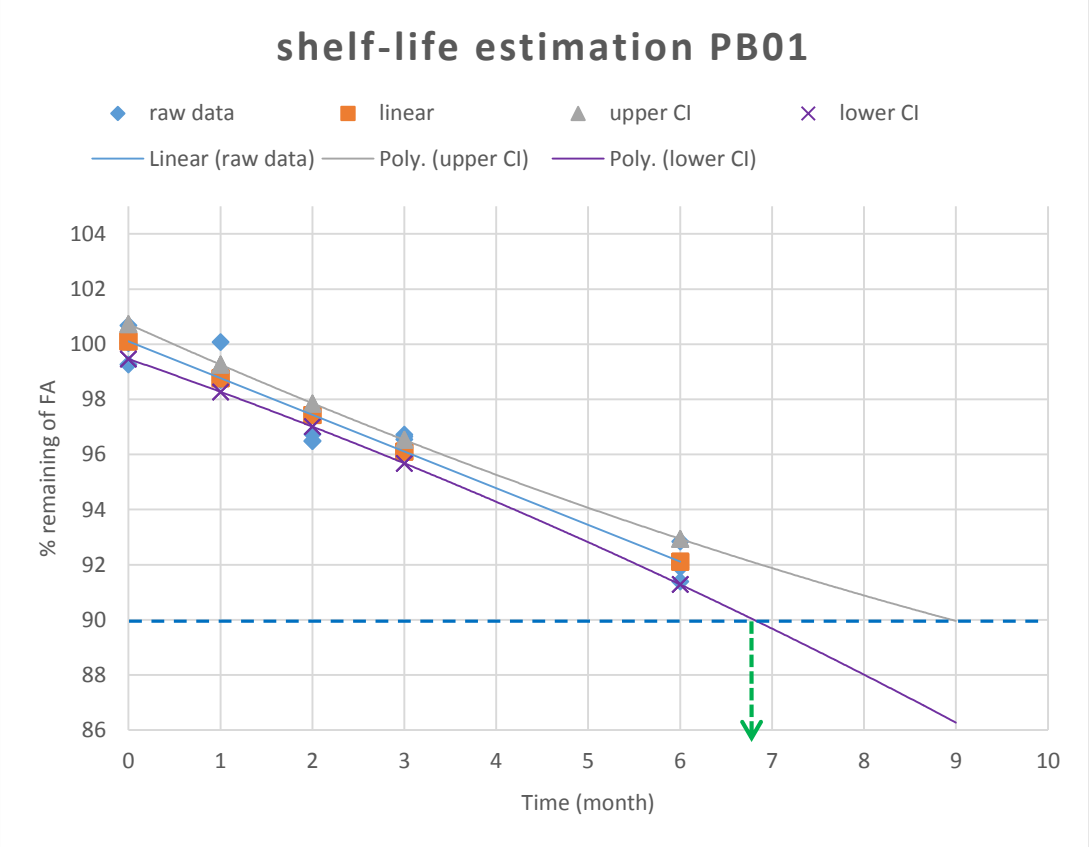


Figure 11 Typical plot shelf-life estimation from lin ear regression using lower confident interval

Table 9 shelf-life estimation of each FA formulation

Formulations		Batch	Shelf-life (months)	Beyond-use date (months)
Premicroemulsion concentrate	PA	PA01	7.1	7.1
		PA02	7.7	
		PA03	7.3	
	PB	PB01	6.8	6.3
		PB02	6.7	
		PB03	6.3	
Microemulsion	ME(A)	ME(A)01	14.5	12
		ME(A)02	23	
		ME(A)03	16.4	
	ME(B)	ME(B)01	15	12
		ME(B)02	16.8	
		ME(B)03	19.2	
	ME(C)	ME(C)01	17.8	12
		ME(C)02	21.5	
		ME(C)03	19.8	
	ME(D)	ME(D)01	16.8	12
		ME(D)02	17.2	
		ME(D)03	15	

CHAPTER V

CONCLUSION

FA, a poorly water-soluble drug, was incorporated in microemulsions in order to improve water solubility and stability. Pseudo-ternary phase diagrams of oil, surfactant and water were constructed using water titration method. Peppermint oil or clove oil was added as oil phase. Tween 80 and PEG 400 were surfactant and co-surfactant, respectively. S_{mix} ratio 2:1 of surfactant: co-surfactant provided large microemulsion region which was suitable for this preparation. Then, combinations of oil-in-water microemulsions were selected from the pseudo-ternary phase diagrams. These combinations contained 3.5% clove oil : 31.5% S_{mix} : 65% water by weight and 15% peppermint oil : 35% S_{mix} : 50% water by weight. FA was loaded in the selected oil-in-water microemulsions at 0.1% w/w. The FA microemulsion containing peppermint oil was dropped off because it turned turbid in 4 weeks. Premicroemulsion concentrate containing clove oil and S_{mix} loaded with FA was prepared at this selected ratio. FA microemulsion was obtained after a certain amount of water was added into the premicroemulsion concentrate. After FA microemulsions and premicroemulsion concentrates were kept for 6 months at 30°C, all formulations presented physically stable, but FA content in microemulsions remained unchanged (~100% remaining) while FA content in premicroemulsion concentrate was 90-94% remaining. Increase of temperature accelerated the loss of FA in the preparations. Stability of FA was not improved by the addition of antioxidants. Beyond-use date of FA microemulsions was 12 months while that of premicromulsion concentrates was 6-7 months. According to the stability study, microemulsion mouthwash of FA is more likely to be developed

for clinical use. For further study, efficacy and safety of FA microemulsions should be evaluated.

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APPENDIX

Appendix A

Analysis of FA

Linearity

Table A- 1 The representative calibration curve data of FA

Concentration (mg/100mL)	Peak area of FA			Mean	SD	%CV
	Set1	Set2	Set3			
0.501192	216401	217665	216021	216695.6667	860.7005	0.3972
1.002384	434479	437498	436217	436064.6667	1515.2539	0.3475
2.50596	1078488	1077321	1076677	1077495.333	918.0002	0.0852
5.01192	2110820	2107744	2109165	2109243	1539.4827	0.0730
7.51788	3197206	3198129	3199719	3198351.333	1271.1673	0.0397
10.02384	4241556	4231907	4239890	4237784.333	5157.6326	0.1217

Appendix B

Chemical stability

Table B- 1 % remaining of FA in premicroemulsion concentrates kept at 30°C

Formulation	months				
	0	1	2	3	6
PA	100.00	98.30	97.61	96.71	93.17
PA01	100.00	98.00	97.57	96.04	92.38
PA02	100.00	99.25	97.51	97.23	94.64
PA03	100.00	97.64	97.73	96.85	92.47
PB	100.00	99.23	97.07	96.86	91.63
PB01	100.00	99.25	96.57	96.64	92.05
PB02	100.00	98.68	96.67	97.08	91.63
PB03	100.00	99.76	97.97	N/A	91.20

Table B- 2 % remaining of FA in premicroemulsion concentrates kept at 40°C

Formulation	months				
	0	1	2	3	6
PA	100.00	97.44	94.43	94.06	88.24
PA01	100.00	97.62	95.04	93.77	88.00
PA02	100.00	96.01	92.82	94.17	88.76
PA03	100.00	98.70	95.43	94.24	87.97
PB	100.00	97.87	95.61	92.77	87.91
PB01	100.00	97.73	94.80	91.38	88.96
PB02	100.00	97.48	94.46	92.98	86.93
PB03	100.00	98.39	97.58	93.94	87.84

Table B- 3 % remaining of FA in microemulsions kept at 30°C

Formulation	months				
	0	1	2	3	6
ME-A	100.00	99.12	99.81	99.84	99.84
ME(A)01	100.00	99.21	99.30	99.91	98.61
ME(A)02	100.00	100.26	100.47	100.20	100.83
ME(A)03	100.00	97.89	99.66	99.40	100.09
ME-B	100.00	101.12	99.70	100.58	101.08
ME(B)01	100.00	101.23	99.78	100.64	100.38
ME(B)02	100.00	101.18	99.02	100.74	100.66
ME(B)03	100.00	100.96	100.30	100.37	102.18
ME-C	100.00	99.96	100.44	99.26	100.47
ME(C)01	100.00	100.21	100.45	98.87	100.60
ME(C)02	100.00	100.00	100.53	99.54	99.58
ME(C)03	100.00	99.67	100.34	99.38	101.24
ME-D	100.00	99.24	99.10	98.97	99.75
ME(D)01	100.00	98.81	101.80	99.06	101.83
ME(D)02	100.00	99.05	99.73	99.38	99.25
ME(D)03	100.00	99.85	95.77	98.48	98.16

Table B- 4 % remaining of FA in microemulsions kept at 40°C

Formulation	months				
	0	1	2	3	6
ME-A	100.00	100.24	99.52	99.41	97.54
ME(A)01	100.00	100.11	99.05	99.03	97.40
ME(A)02	100.00	100.82	100.28	100.21	97.27
ME(A)03	100.00	99.78	99.21	98.99	97.93
ME-B	100.00	100.45	99.38	99.23	98.28
ME(B)01	100.00	100.92	99.16	98.07	97.79
ME(B)02	100.00	100.54	99.75	99.76	97.78
ME(B)03	100.00	99.88	99.25	99.86	99.28
ME-C	100.00	100.33	99.82	99.56	98.21
ME(C)01	100.00	100.99	99.42	99.44	97.79
ME(C)02	100.00	100.07	99.69	99.57	97.98
ME(C)03	100.00	99.93	100.34	99.68	98.84
ME-D	100.00	99.24	99.10	98.97	96.99
ME(D)01	100.00	98.81	101.80	99.06	96.67
ME(D)02	100.00	99.05	99.73	99.38	97.74
ME(D)03	100.00	99.85	95.77	98.48	96.55

Appendix c

Physical stability

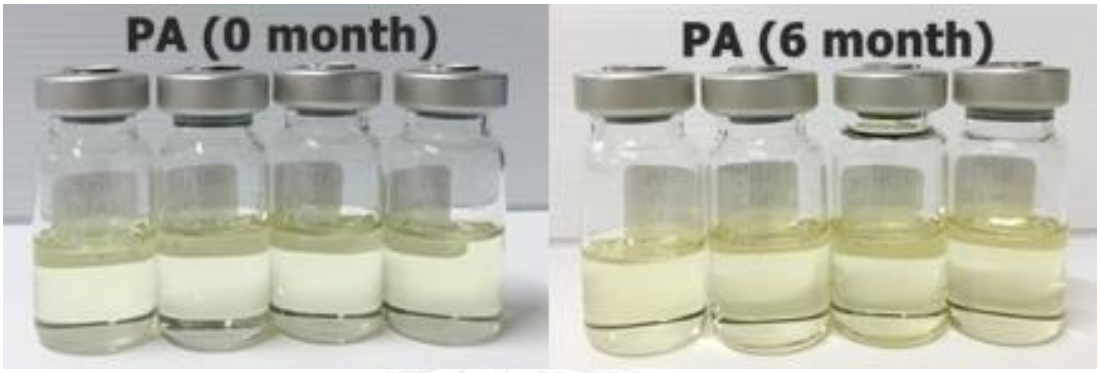

Figure C- 1 Physical Appearance of premicroemulsion concentrates "PA" kept at 30 °C

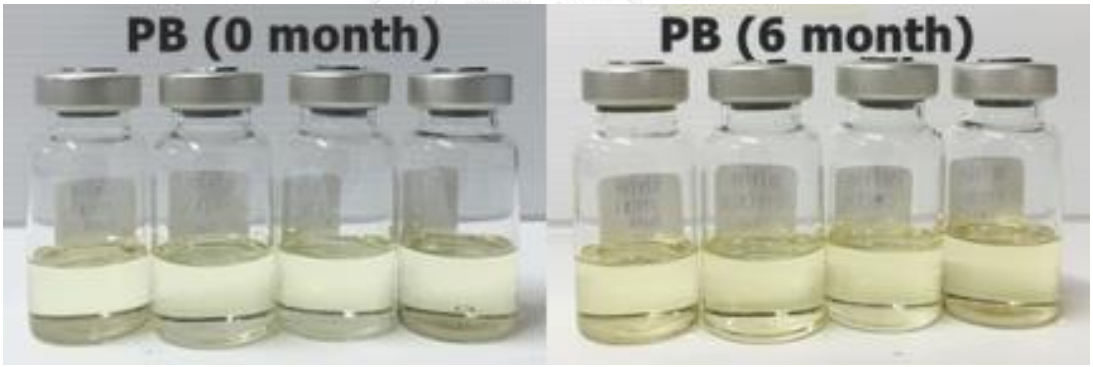

Figure C- 2 Physical Appearance of premicroemulsion concentrates "PB" kept at 30 °C

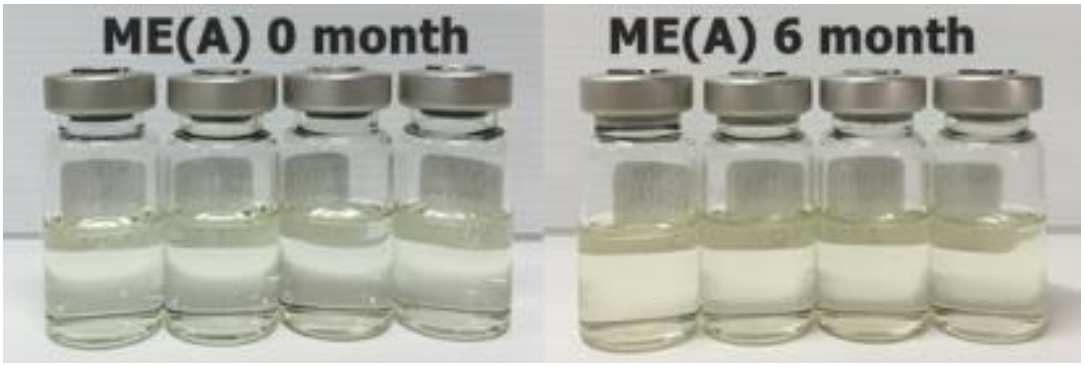

Figure C- 3 Physical Appearance of microemulsions "ME(A)" kept at 30 °C

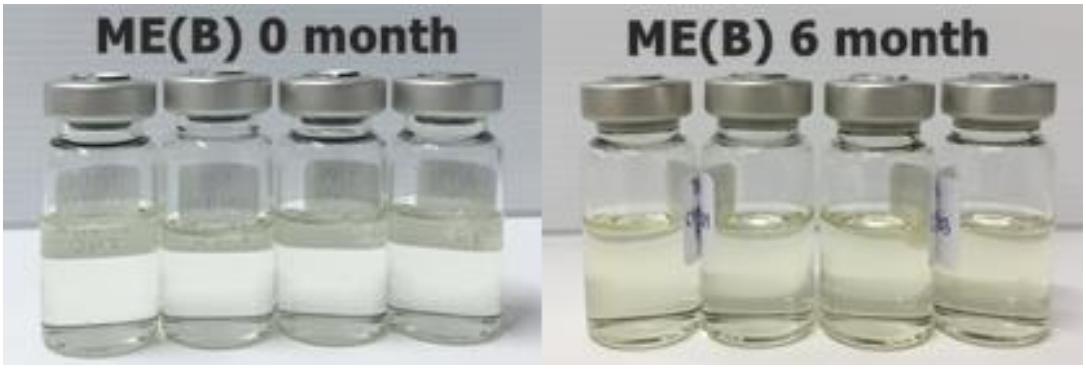

Figure C- 4 Physical Appearance of microemulsions "ME(B)" kept at 30°C

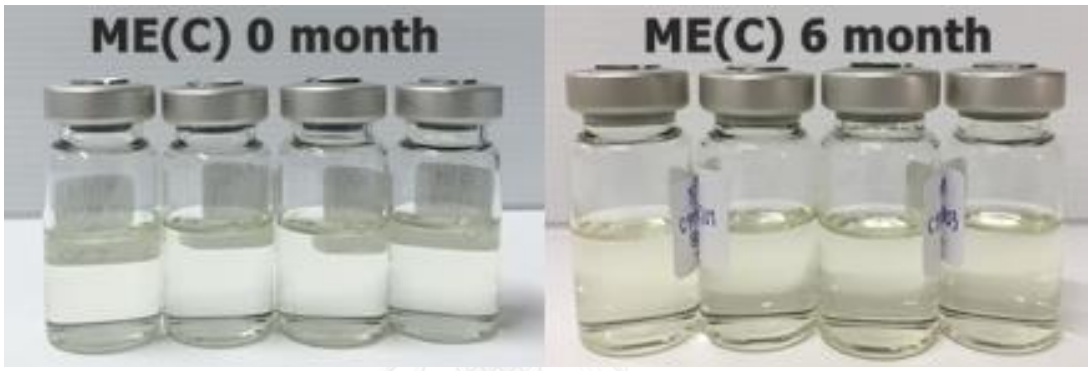

Figure C- 5 Physical Appearance of microemulsions "ME(C)" kept at 30°C

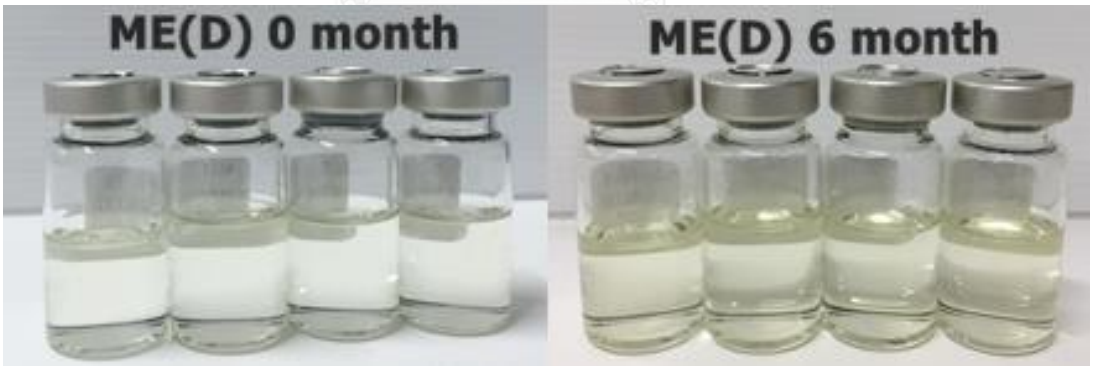

Figure C- 6 Physical Appearance of microemulsions "ME(D)" kept at 30°C

Appendix D

Shelf-life determination

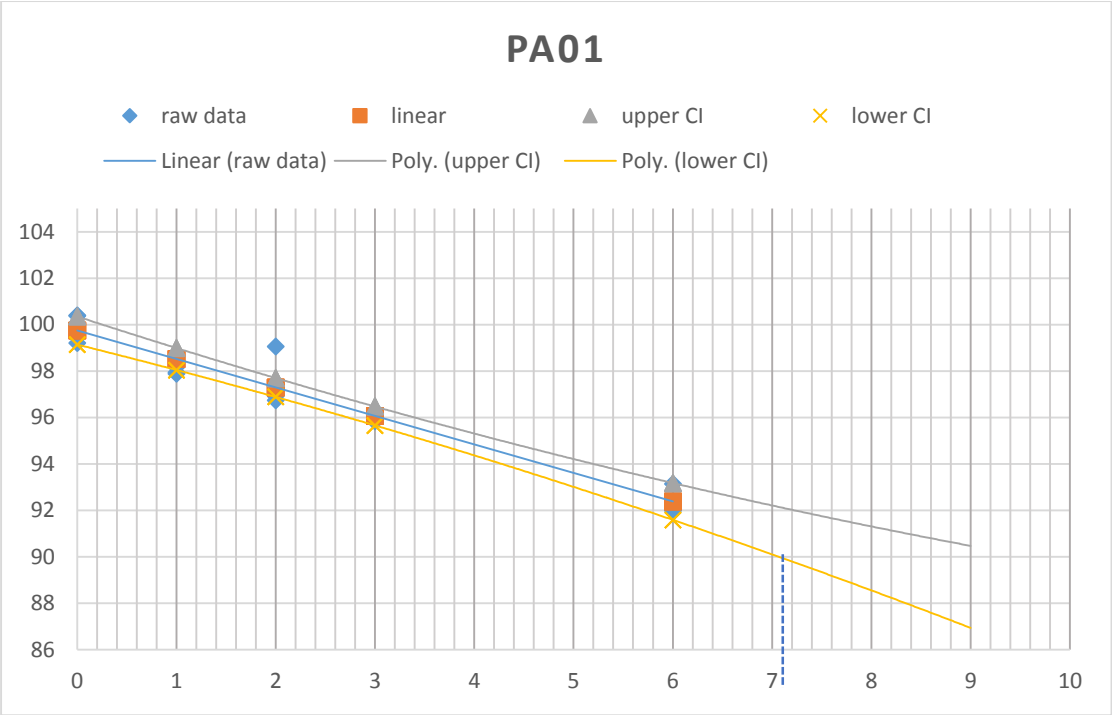

Figure D- 1 shelf-life estimation using 95% confident interval of Premicroemulsion concentrate "PA01"

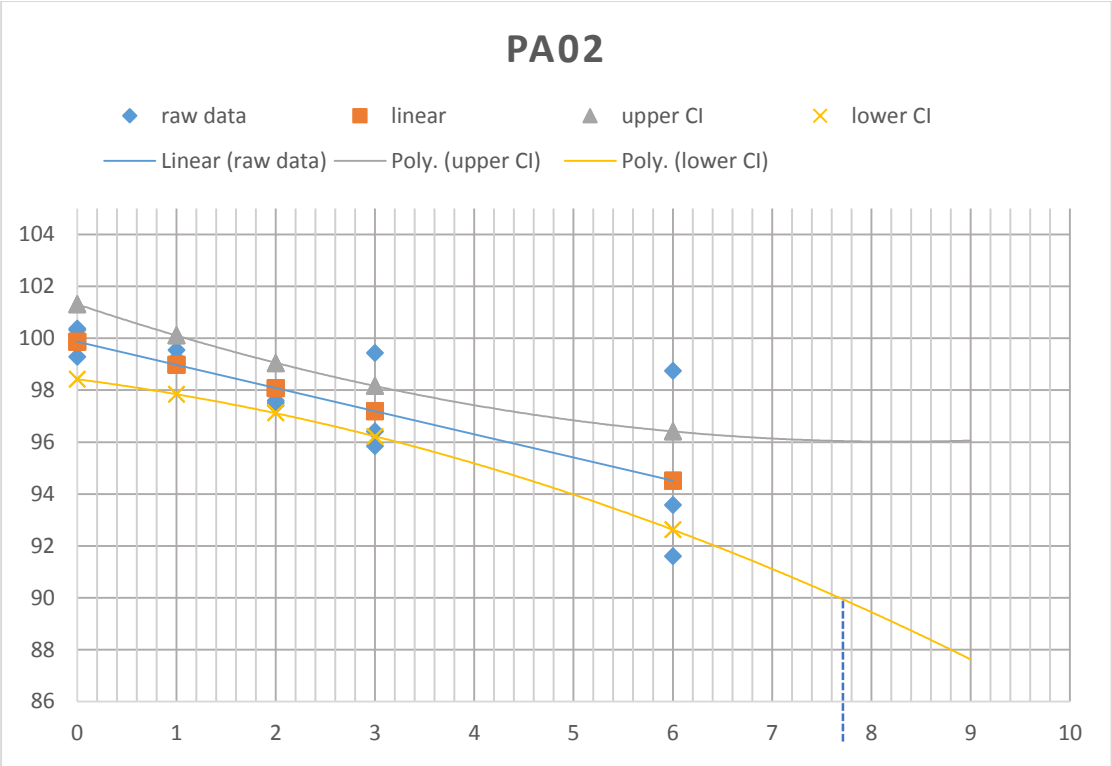

Figure D- 2 shelf-life estimation using 95% confident interval of Premicroemulsion concentrate "PA02"

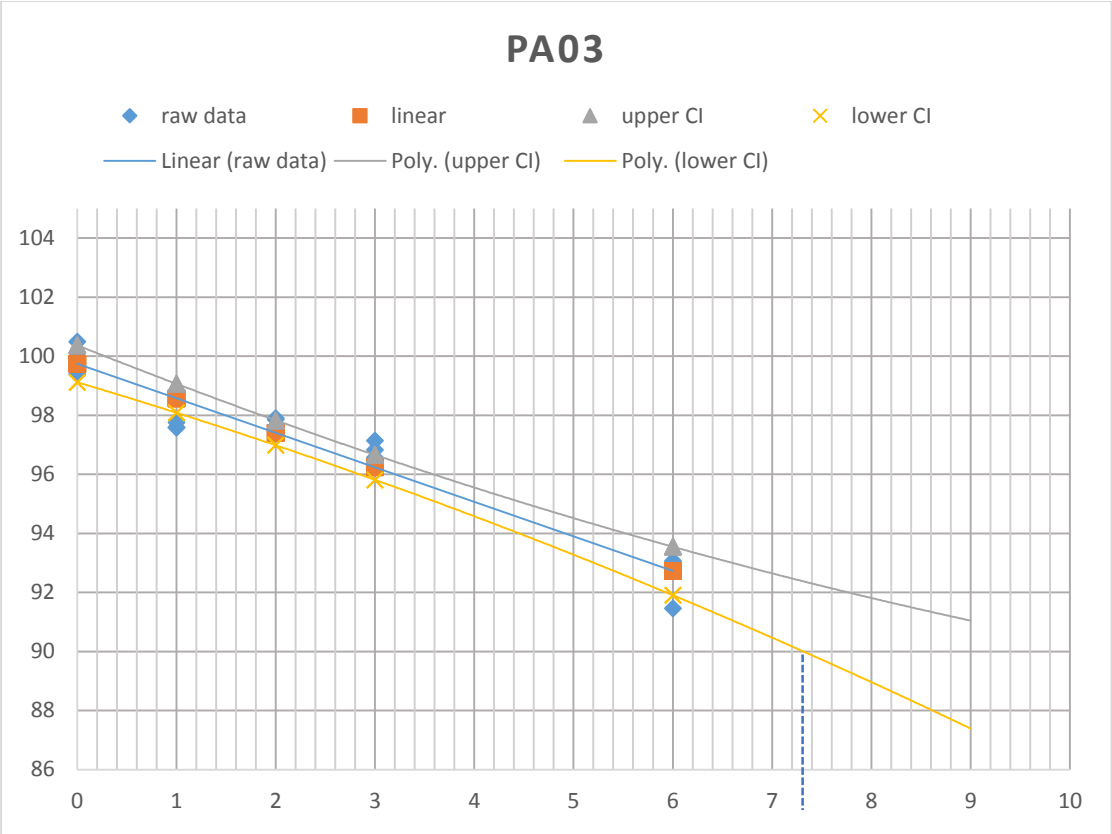

Figure D- 3 shelf-life estimation using 95% confident interval of Premicroemulsion concentrate "PA03"

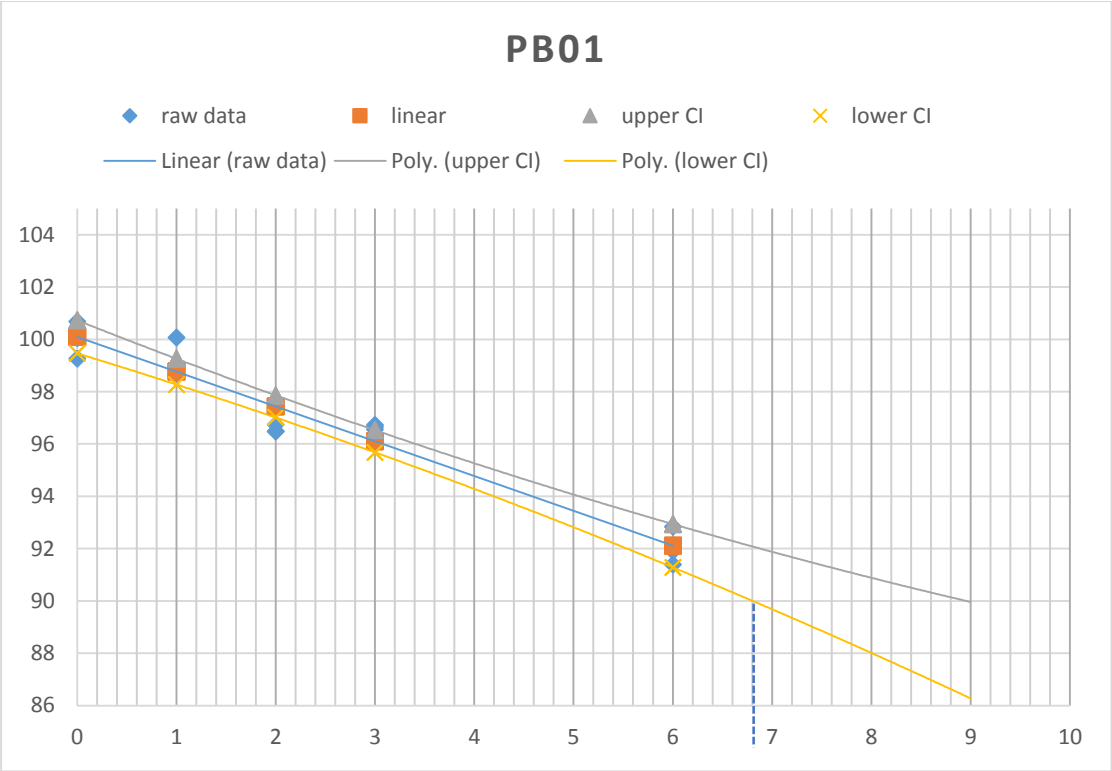

Figure D- 4 shelf-life estimation using 95% confident interval of Premicroemulsion concentrate "PB01"

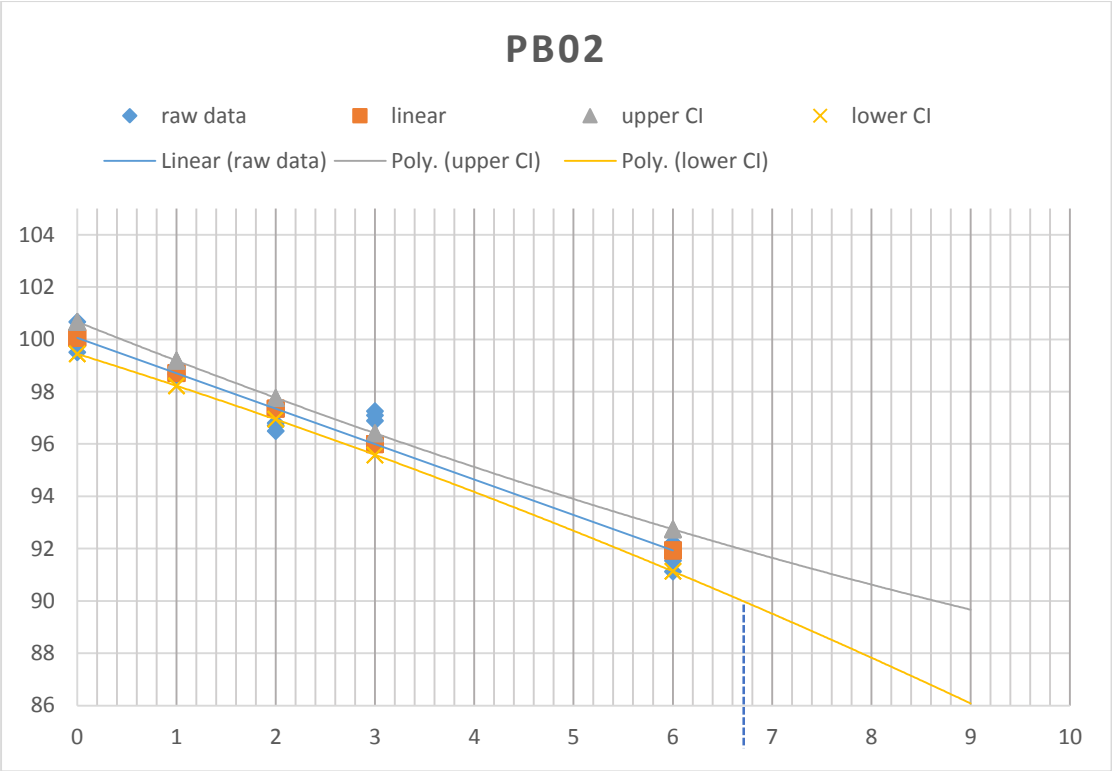

Figure D- 5 shelf-life estimation using 95% confident interval of Premicroemulsion concentrate "PB02"

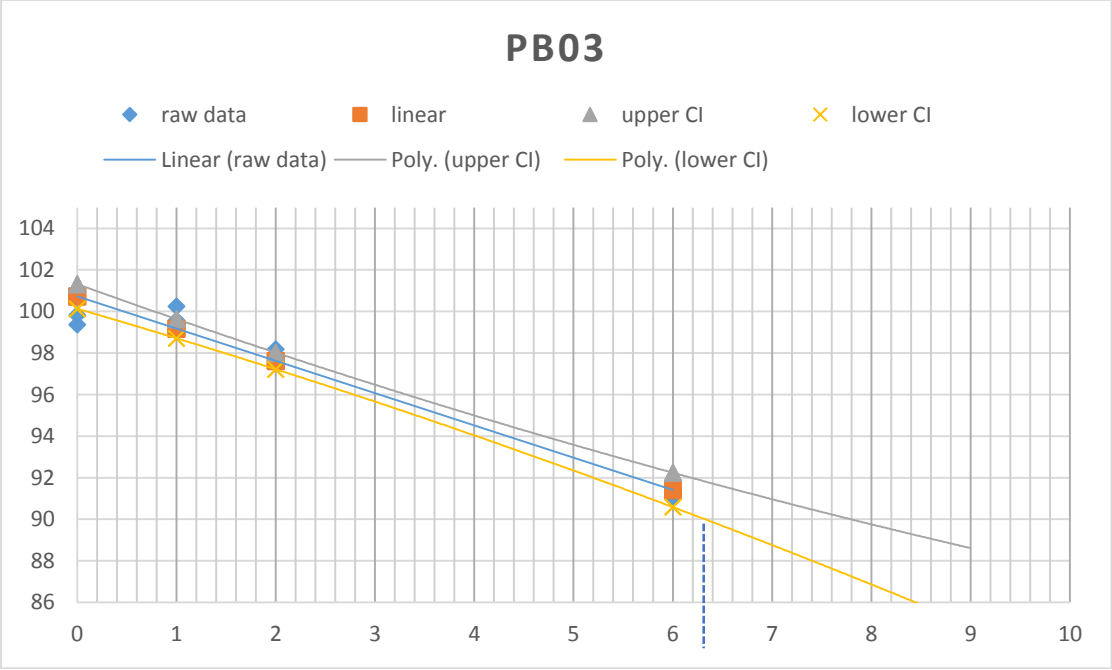

Figure D- 6 shelf-life estimation using 95% confident interval of Premicroemulsion concentrate "PB03"

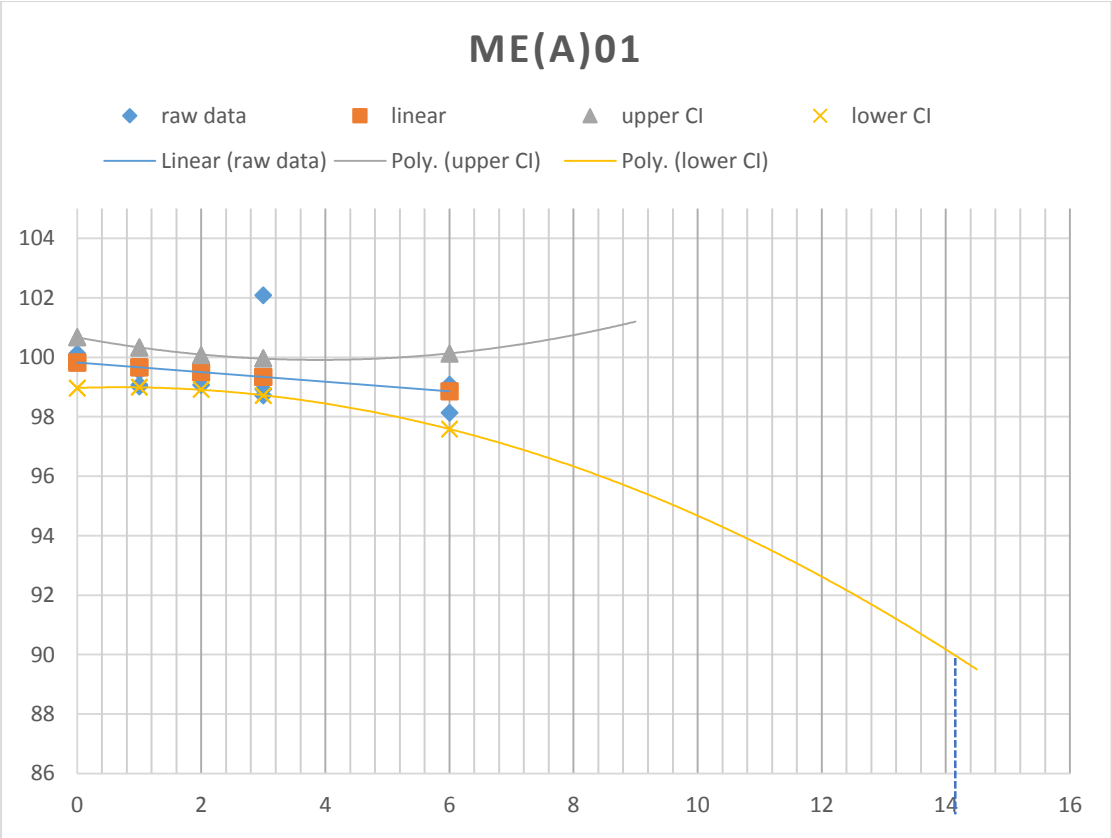

Figure D- 7 shelf-life estimation using 95% confident interval of microemulsion "ME(A)01"

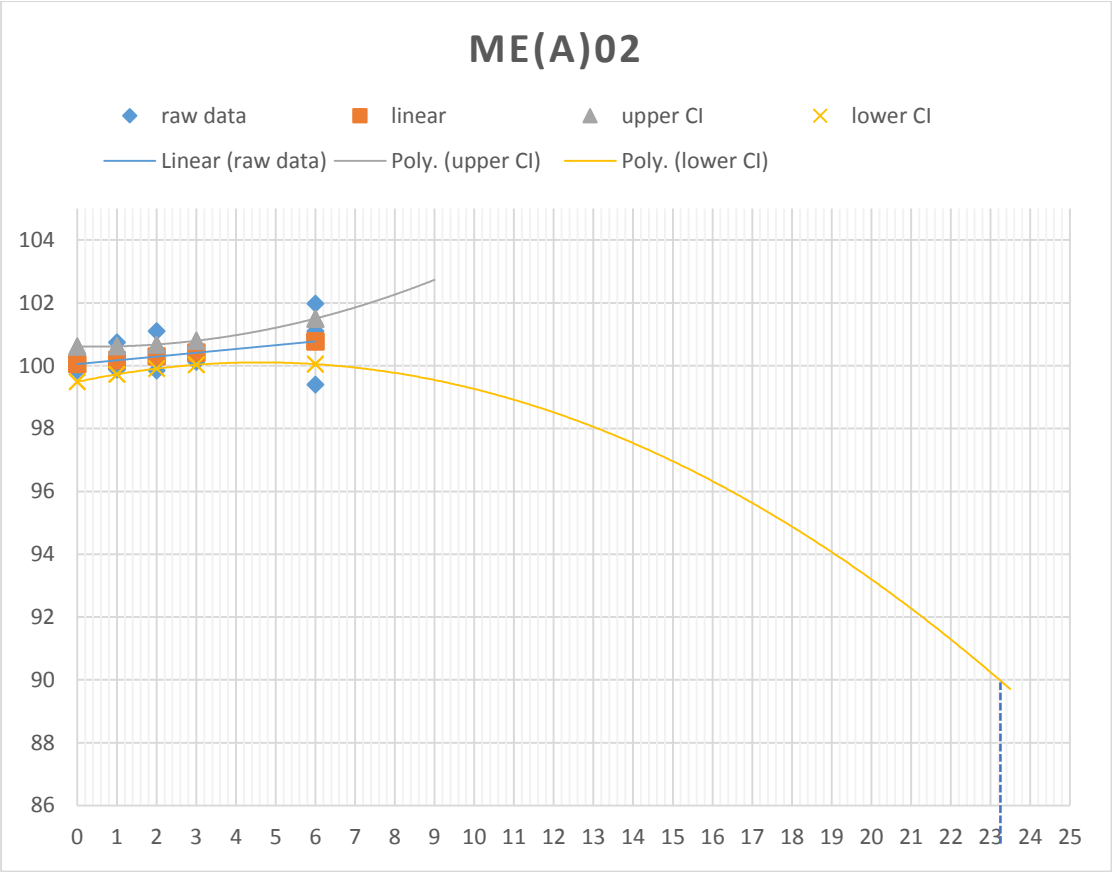

Figure D- 8 shelf-life estimation using 95% confident interval of microemulsion "ME(A)02"

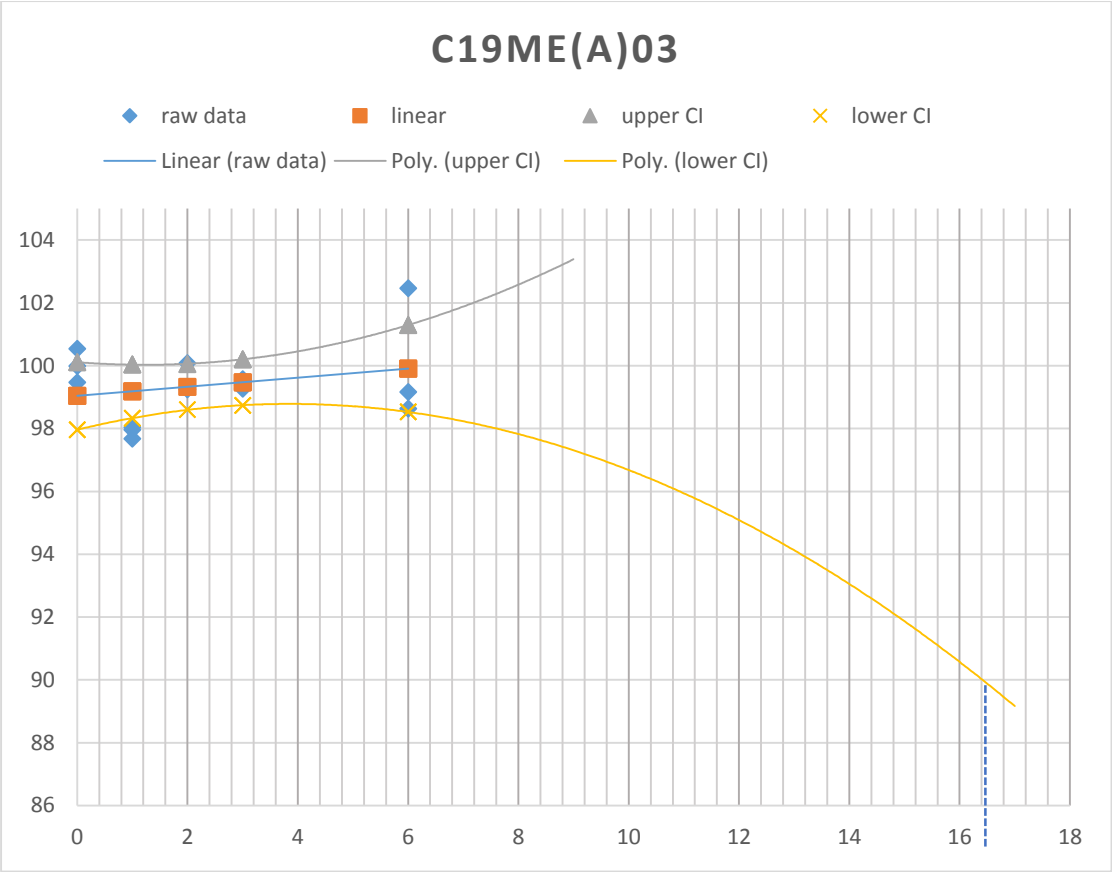

Figure D- 9 shelf-life estimation using 95% confident interval of microemulsion "ME(A)03"

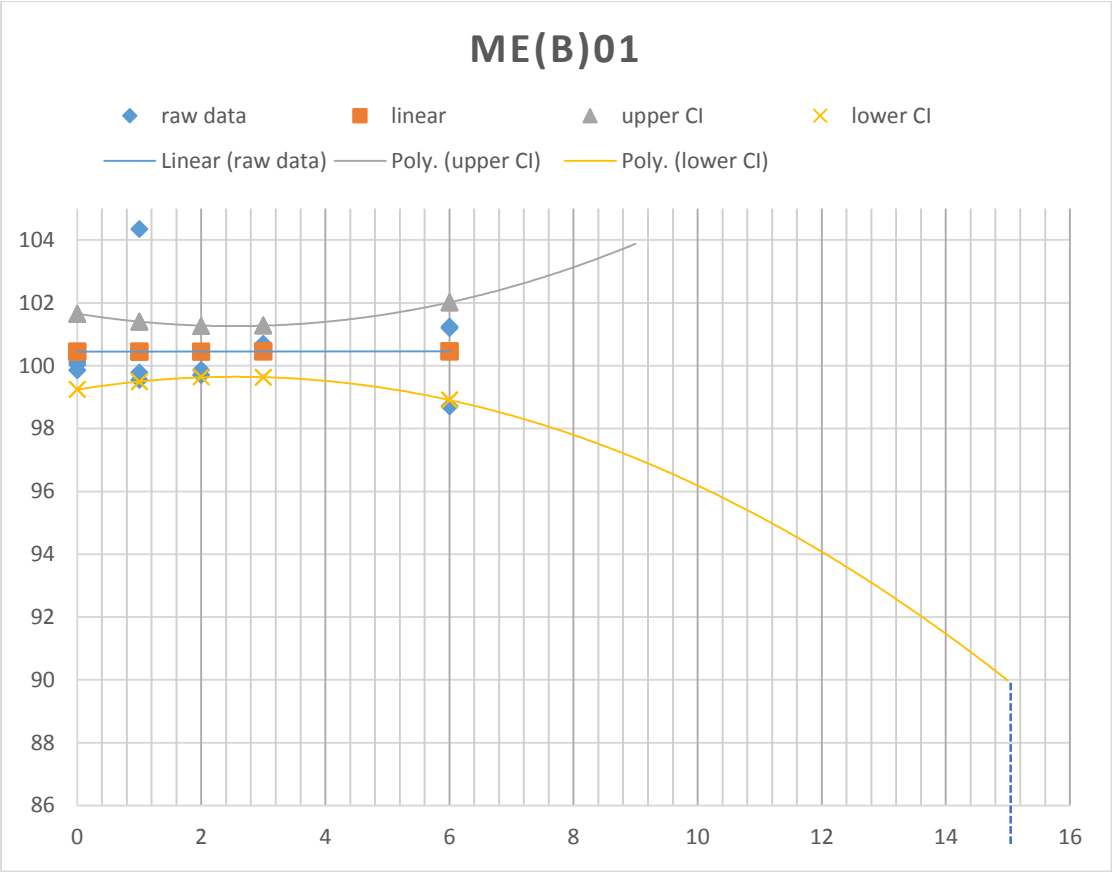

Figure D- 10 shelf-life estimation using 95% confident interval of microemulsion "ME(B)01"

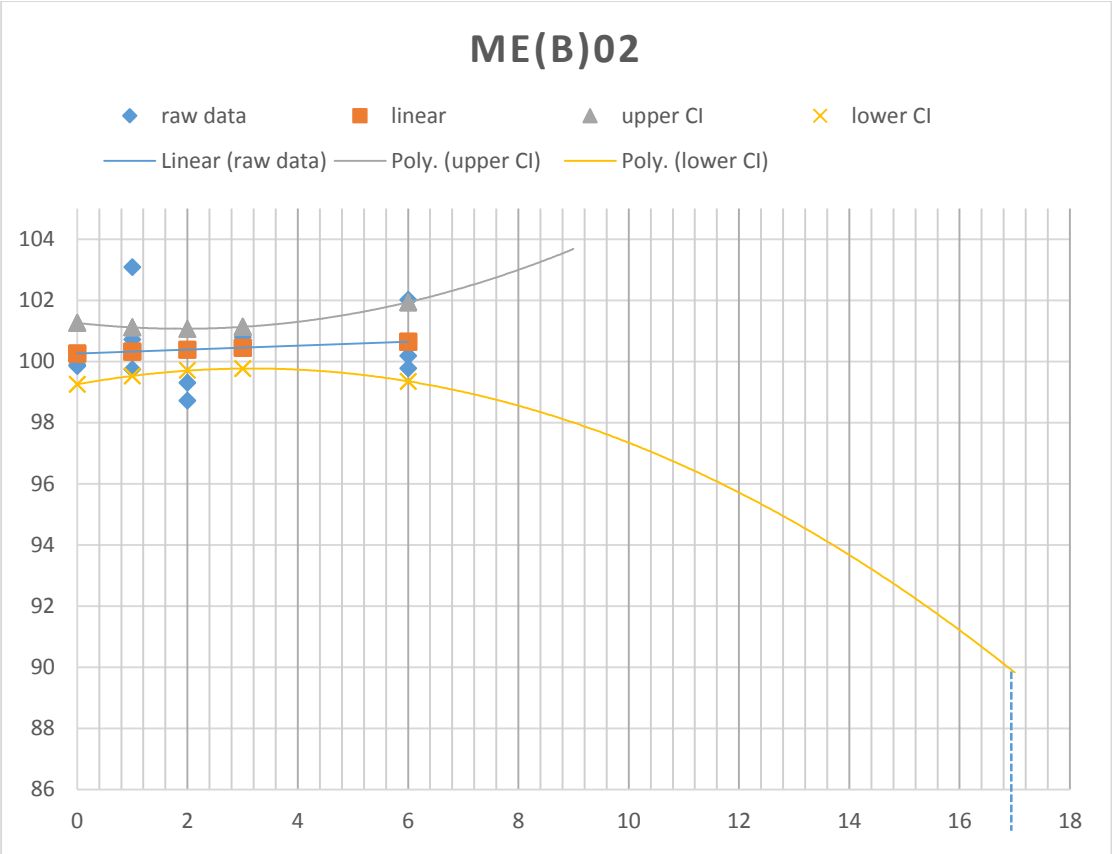

Figure D- 11 shelf-life estimation using 95% confident interval of microemulsion "ME(B)02"

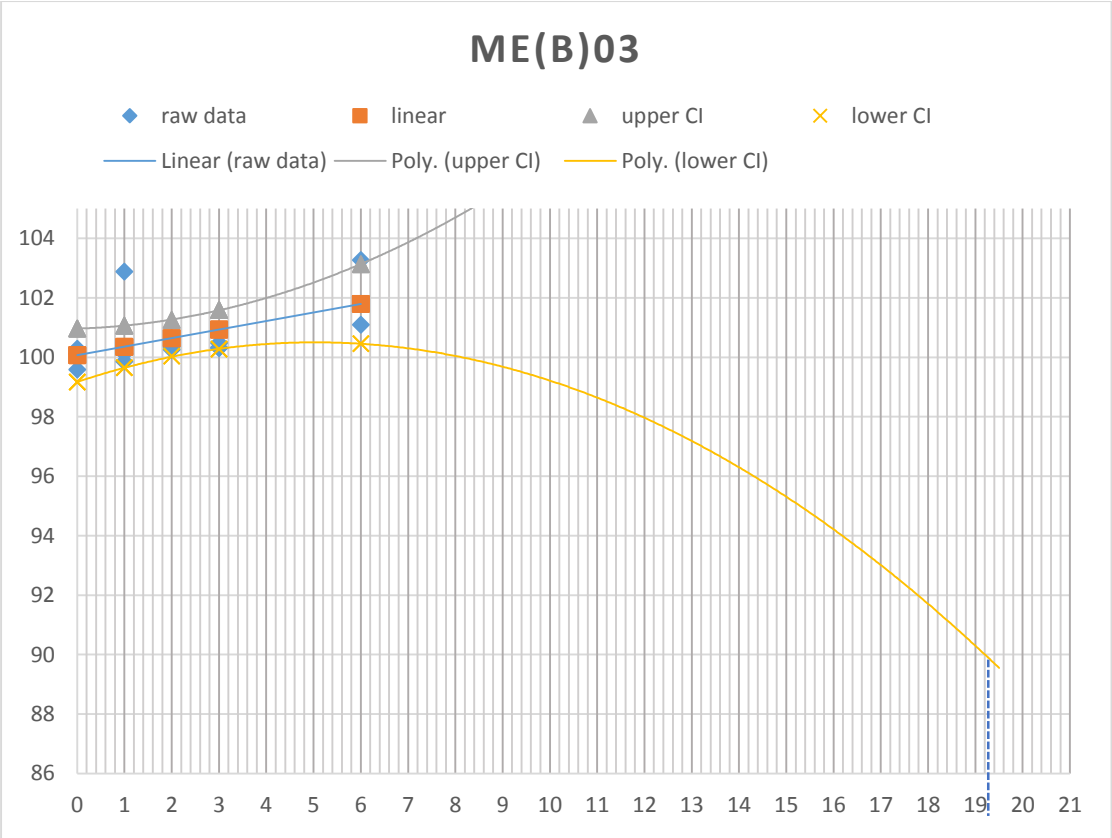

Figure D- 12 shelf-life estimation using 95% confident interval of microemulsion "ME(B)03"

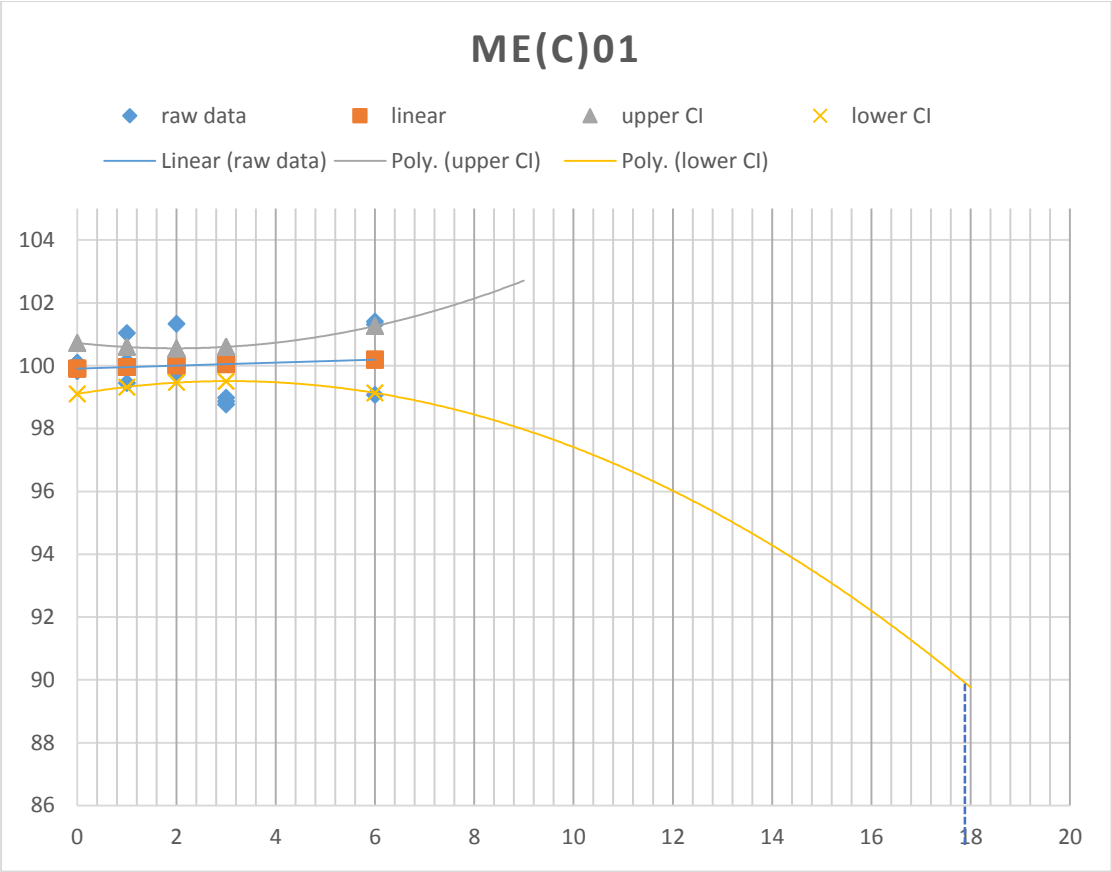

Figure D- 13 shelf-life estimation using 95% confident interval of microemulsion "ME(C)01"

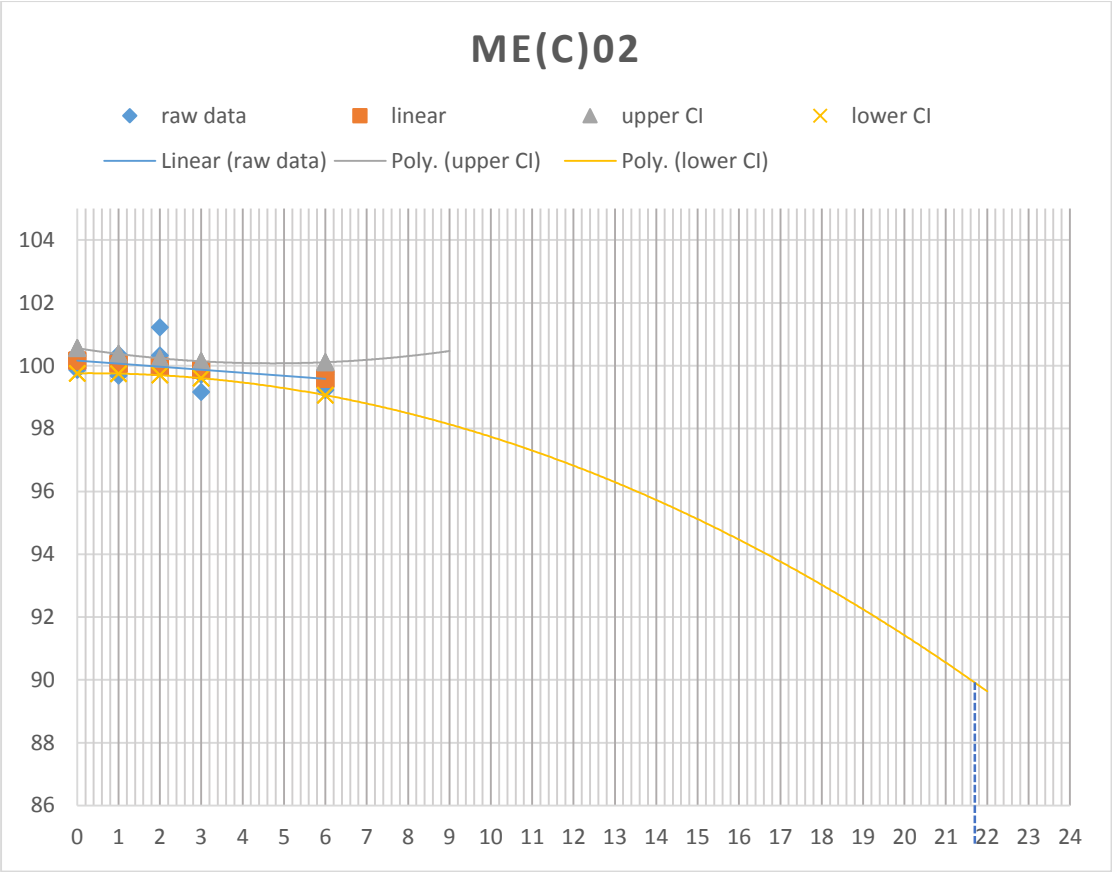

Figure D- 14 shelf-life estimation using 95% confident interval of microemulsion "ME(C)02"

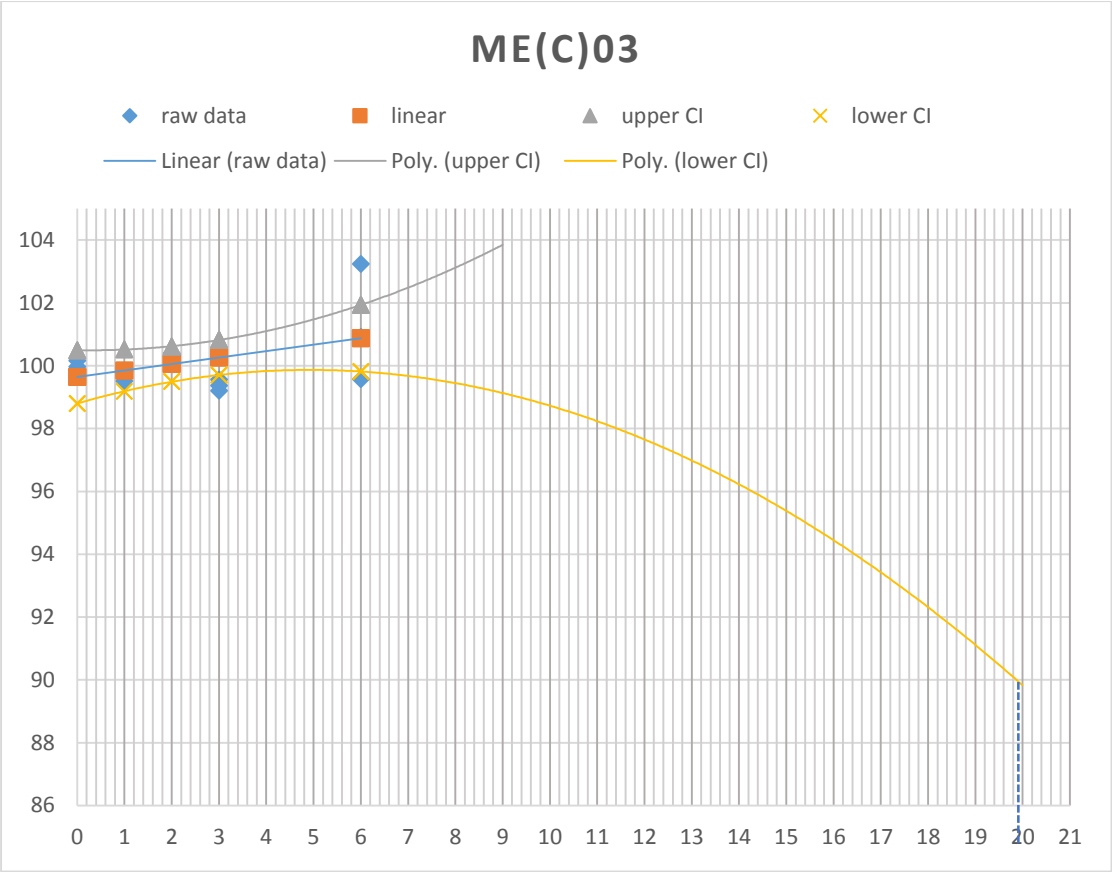

Figure D- 15 shelf-life estimation using 95% confident interval of microemulsion "ME(C)03"

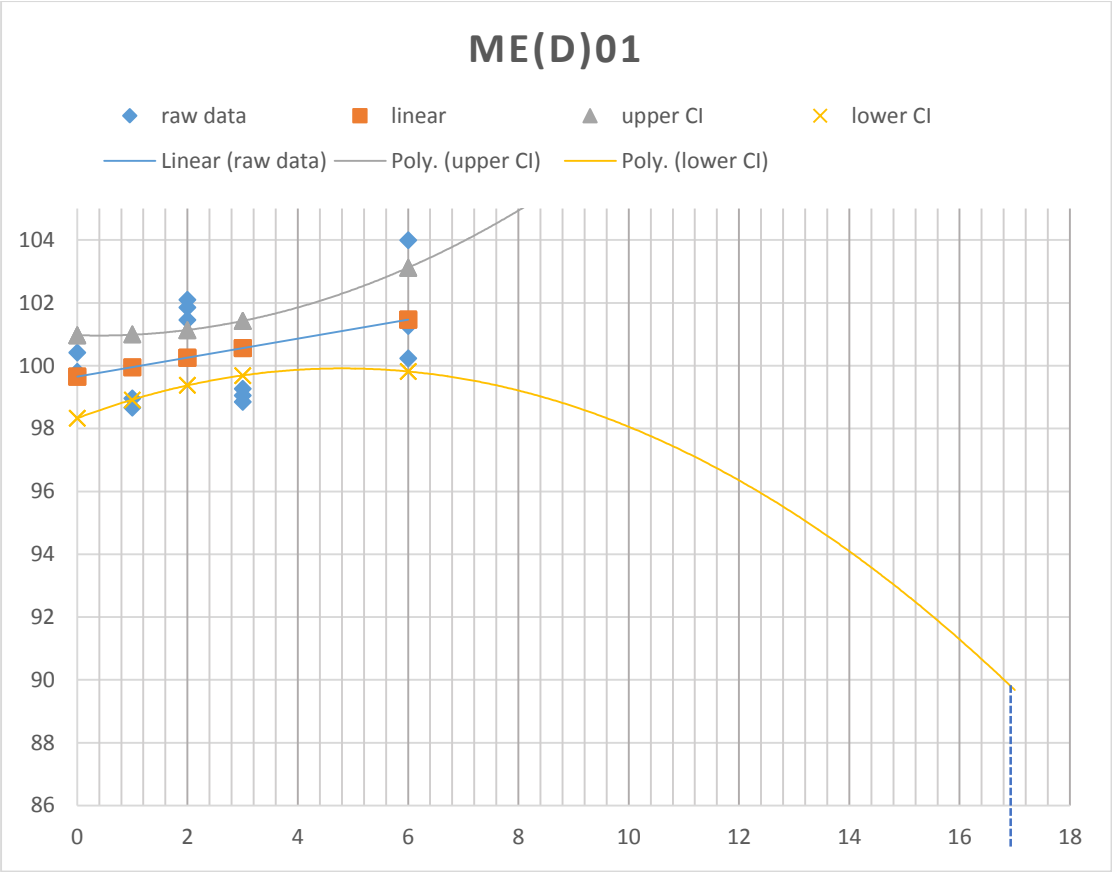

Figure D- 16 shelf-life estimation using 95% confident interval of microemulsion "ME(D)01"

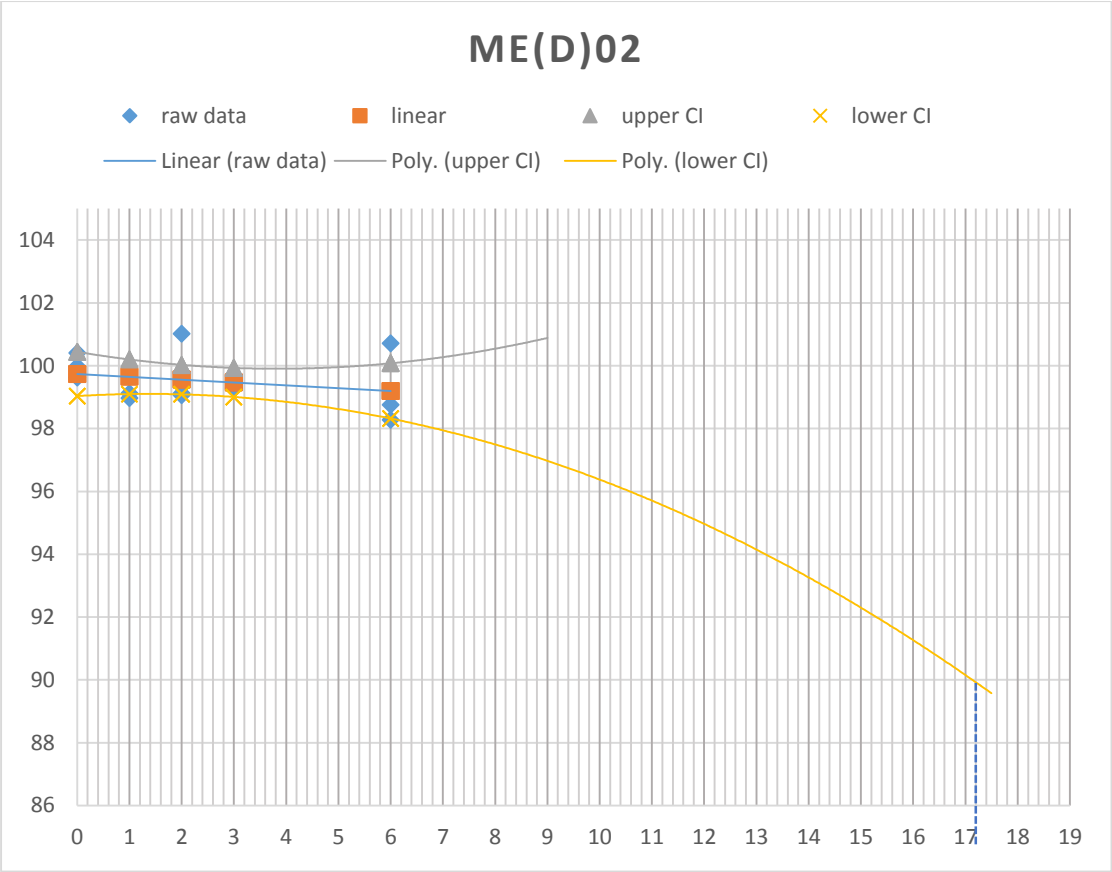

Figure D- 17 shelf-life estimation using 95% confident interval of microemulsion "ME(D)02"

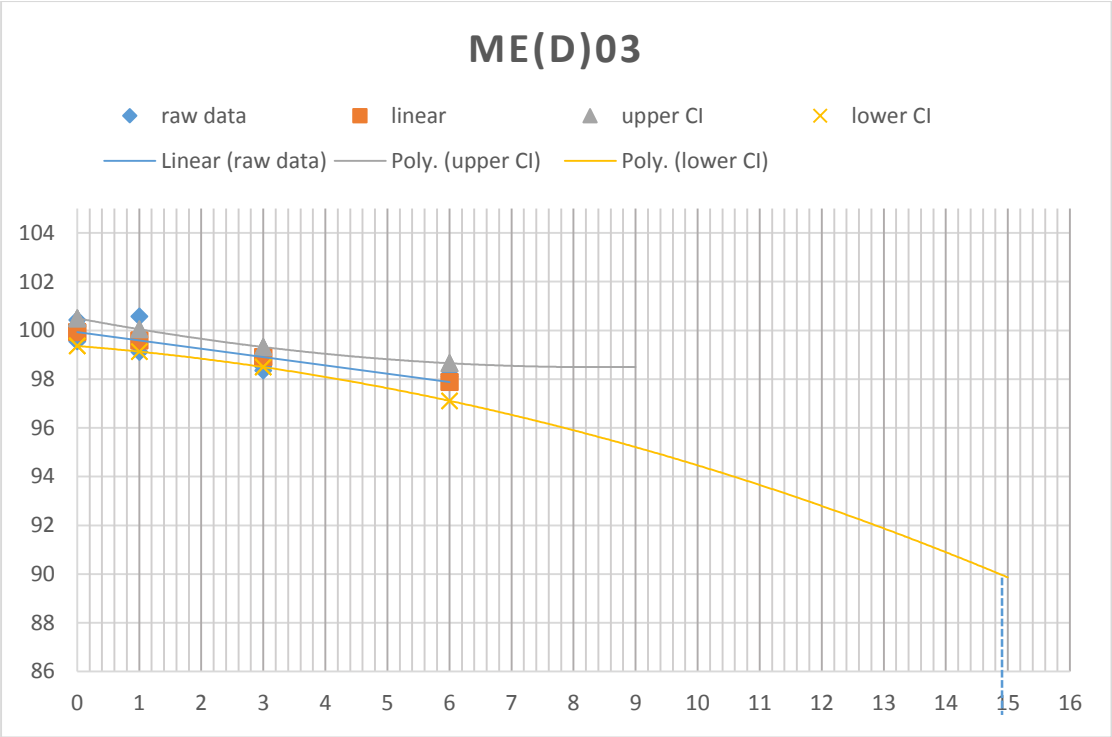

Figure D- 18 shelf-life estimation using 95% confident interval of microemulsion "ME(D)03"

VITA

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