Formulation Development of Nicotinamide Microemulsions Using Natural Oils as Oil Phase

Nang Hnin Ei Hlaing

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science Program in Cosmetic Sciences (International Program)

Prince of Songkla University

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Academic Year 2018

ABSTRACT

Concerning the conservation of ecosystem, biodegradable and environmental friendly chemicals have been chosen to formulate as green microemulsions (MEs). The objective of this research was to investigate phase behavior of nonionic surfactants with natural oils on formation of green MEs. In this study, MEs were formulated from olive oil or virgin coconut oil with various ratios of surfactant mixture (S_{mix}) between polyoxyethylene (20) sorbitan monooleate (Tween 80) and sorbitan monooleate (Span 80). The formulation scanning was carried out based on hydrophilic lipophilic deviation (HLD) concept followed by titration method. From the obtained pseudotenary phase diagrams, surfactant:cosurfactant (S:CoS) ratios of 0.6:0.4 and 0.7:0.3 were chosen as S_{mix}. The compositions of blank MEs were 45% w/w of oil, 50% w/w of S_{mix} and 5% w/w of water. The visual and technical characterization of formulations indicated that all MEs were water-in-oil (w/o) type. After accelerating stability test, 3% w/w of nicotinamide was added to blank MEs to obtain MEO1-N, MEO2-N, MEC1-N and MEC2-N. They were characterized as w/o MEs. Afterward, samples were kept at 4°C, room temperature (28 ± 2°C) and 45°C for three months to study physical and chemical stabilities. MEO2-N got phase separation since the first month of storage at all conditions due to enlarged entropy. The drug remaining content was assayed by HPLC method and more than 90% of active was detected in MEO1-N, MEC1-N and MEC2-N kept at 4°C and room temperature. High temperature, 45°C, caused discoloration in all formulations. The *in vitro* release profiles of MEO1-N and MEC1-N were compared with 3% w/w nicotinamide solution (NCT sol). After 12 h, NCT sol reached a plateau while MEO1-N and MEC1-N provided the sustained release profiles which were best fitted to the Higuchi model. The release rate of MEO1-N was higher than MEC1-N

because of the board intermolecular area of fatty acid chain allowing nicotinamide to diffuse better. The *in vitro* release kinetics of after three months stored MEs also followed the Higuchi model and the release rate was significantly different to freshly prepared MEs ($p < 0.05$, *t*-test). The current observation revealed that not only HLB but also the degree of saturation of fatty acids, water-to-lipid ratio and S:CoS ratio influenced the formation of MEs type and stability. The *in vitro* release rate was affected by the oil type when the same S_{mix} was applied. These natural oil microemulsions were promising as nanocarriers of nicotinamide. Moreover, they could be further observed for incorporation of other active ingredients and effectiveness of active delivery to the skin.

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Nang Hnin Ei Hlaing

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
ºC	degree Celsius
µm	micrometer(s)
µS	micro siemen(s)
cm^2	square centimeter(s)
et al.	et alia, and others
°F	Fahrenheit
g	gram(s)
h	hour(s)
HLD	Hydrophilic-lipophilic deviation
HPLC	high performance liquid chromatography
ICH	International Conference on Harmonisation
i.e.	id est, that is
LOD	limit of detection
LOQ	limit of quantitation
m^2	square meter(s)
ME	microemulsion(s)
mg	milligram(s)
mL	milliliter(s)
mm	millimeter(s)
OECD	Organization for Economic Co-operation and Development
PBS	phosphate buffer solution
pH	the negative logarithm of the hydrogen ion concentration
PI	polydispersity index
o/w	oil-in-water
R^2	correlation of determination
rpm	revolution(s) per minute
RSD	relative standard deviation

LIST OF ABBREVIATIONS AND SYMBOLS (cont.)

RT	room temperature
s	second(s)
SC	stratum corneum
SD	standard deviation
SEM	standard error of mean
TEM	transmission electron microscopy
UV	Ultra Violet
w/o	water-in-oil
w/w	weight by weight

CHAPTER 1

INTRODUCTION

1. Background and rationale

The major determinant of human's skin color is a pigment called melanin, which is produced by special cells known as melanocytes. Red haemoglobins and yellow carotenoids also distribute some color to skin. Melanins are quinoid polymers of two types, i.e. yellow or red phaeomelanins and brown or black eumelanins. Both are produced by oxidation of tyrosine by tyrosinase enzyme to 3,4-dihydroxyphenylanine (DOPA) and then dehydrogenated to L-DOPA quinone. Afterward, the synthesis pathway separates to form either phaeomelanins or eumelanins. In the presence of cysteine or glutathione, L- DOPA quinone interacts with the amino acid and then phaeomelanin synthesis will be dominant (Harry 1982; Cazorla 2014).

Skin lightening products are highly demanded especially in Asia and now globally since most consumers perceive that brighter skin tone is charming and attractive. This perception leads both genders especially in urban to higher consume the brightening products in comparison to the last centuries. Modern cosmetic trends are invested in the skin lightening products to expand the inclination for radiant and flawless skin. Many advertisements are pushing consumers towards the use of skin lightening products (Peltzer et al. 2016). Skin lightening agents have been widely used in the cosmetic field and clinic therapy. They are supposed to either lighten skin color for aesthetic purpose or to treat medical conditions such as melasma, freckles, rosacea and senile lentigines (Ong and Maibach 2014).

Several lightening agents show different mechanisms to lessen skin pigment. Nicotinamide, the biologically active amide of niacin (vitamin B3), exhibits as a depigmenting agent (Baumann 2014). Nicotinamide has been demonstrated to suppress melanosome transfer to epidermal keratinocytes, by up to 68% *in vitro* model (Hakozaki et al. 2002). However, the water loving vitamin nicotinamide is trouble to reach the basal layer of epidermis because the stratum corneum (SC) is hydrophobic in

nature (Barry 2001). The lipid bilayer structure of SC is formidable to water and many chemical substances. Many extensive researches to enhance the delivery of actives through stratum corneum have been investigated.

Previous study by Boonme and coworkers indicated that microemulsion formulation could provide nicotinamide to accumulate in pig skin higher than permeate into the receptor solution (Boonme et al. 2016). Obviously, microemulsion is one of the interesting formulations to delivery active substances for achieving efficiency because of the high solubilization capacity for both hydrophilic and lipophilic compounds (Lawrence and Rees 2012).

Microemulsion is thermodynamically stable colloidal system of two unmixable phases which can form a single phase with the help of a surfactant or a mixture of surfactant and cosurfactant. Microemulsions can originate simultaneously without high energy requirement and time consuming. The tiny droplet size usually below 140 nm makes optically clear and low viscosity which behaves Newtonian flow. The ideal characteristics of microemulsion appeal many utilities in different technological fields (Solans et al. 1997; Lawrence and Rees 2012).

Surfactant and cosurfactant are used for producing interfacial film and reducing interfacial tension between different phases. The selection of the surfactants will be intensely dependent on the type and nature of the products and intended purpose. In cosmetic products, the amount of surfactant should be minimized to evade irritation potential. Surfactants which interact strongly with stratum corneum proteins have a higher potential to cause erythema and itching (Som et al. 2012; Jackson et al. 2014). Surfactants must meet environmental compatibility and toxicological safety. Nonionic surfactants based on esters have low risks for skin toxicity and the most frequently used in cosmetic formulations (Rieger 1997; Lawrence and Rees 2012; Kaur and Mehta 2017).

Oils are the basis component of topical products and commonly used in cosmetology and dermatology. Various plant-derived oils possess numerous fatty acids and bioactive compounds which are beneficial for skin. Plant-derived oils are evolving as an alternative to mineral oils in current cosmetic fields. Comparison study of mineral-derived (paraffin and petrolatum) and plant-derived oils (almond oil and jojoba oil) found that plant-derived oils showed better penetration in the stratum

corneum. The study reported that the presence of fatty acids enhanced the penetration of plant-derived oils while mineral oils lack fatty acids (Choe et al. 2017). Olive oil and virgin coconut oil have been long used for promoting hair and skin health due to moisturizing, antiaging, anti-inflammatory and antimicrobial effects (Burnett et al. 2017). Both oils are abundant in fatty acids and bioactive compounds.

Nowadays, a "green" lifestyle is interesting for consumers due to high concern about global environment. Several products including skin-care products have to be designed for both efficient benefits and eco-friendly consciousness in order to provide high response to consumer's expectation. MEs prepared from biodegradable components have been of interest due to eco-friendly awareness (Hloucha et al. 2009). Therefore, developed nicotinamide-loaded microemulsions using biodegradable and natural components were of interest.

The present work aimed to develop nicotinamide-loaded microemulsions formulated from vegetable oils (i.e. olive oil and virgin coconut oil) with the blends of Tween 80 (polyoxyethylene (20) sorbitan monooleate) as a surfactant and Span 80 (sorbitan monooleate) as a cosurfactant. Blank microemulsions were prepared, physically characterized and tested for the accelerating stability. Afterward, the stable formulations were selected to load nicotinamide. Following three months stability study stored at 4°C, room temperature (28 ± 2°C) and 45°C, the amount of active remaining in physically stable formulations were analyzed. Subsequently, the release kinetics of nicotinamide was investigated in comparison with simple nicotinamide solution.

2. Objectives of the research

- To formulate biodegradable green microemulsions.
- To utilize and evaluate environmentally friendly vegetable oils as oil phase of microemulsions.
- To investigate the optimal ratios of Tween 80 and Span 80 required for microemulsion formation.
- To characterize physicochemical properties of blank and nicotinamide-loaded microemulsions.
- To study chemical stability of nicotinamide-loaded microemulsions.
- To assess *in vitro* release of nicotinamide-loaded microemulsions compared with a simple solution.

CHAPTER 2

LITERATURE REVIEW

1. Skin and skin color

Skin, the outer covering of the body, is the largest sensory and contact organ of human body and serves as primary protection from external factors. Its other functions are thermoregulation, synthesis of vitamin D and prevention of excessive water loss. As shown in Figure 1, the skin is composed of two main layers namely- epidermis and dermis (Harry 1982; McGrath et al. 2004). However, hypodermis or subcutaneous tissue, underneath the dermis layer, may be considered as the third layer of the skin in some other literatures.

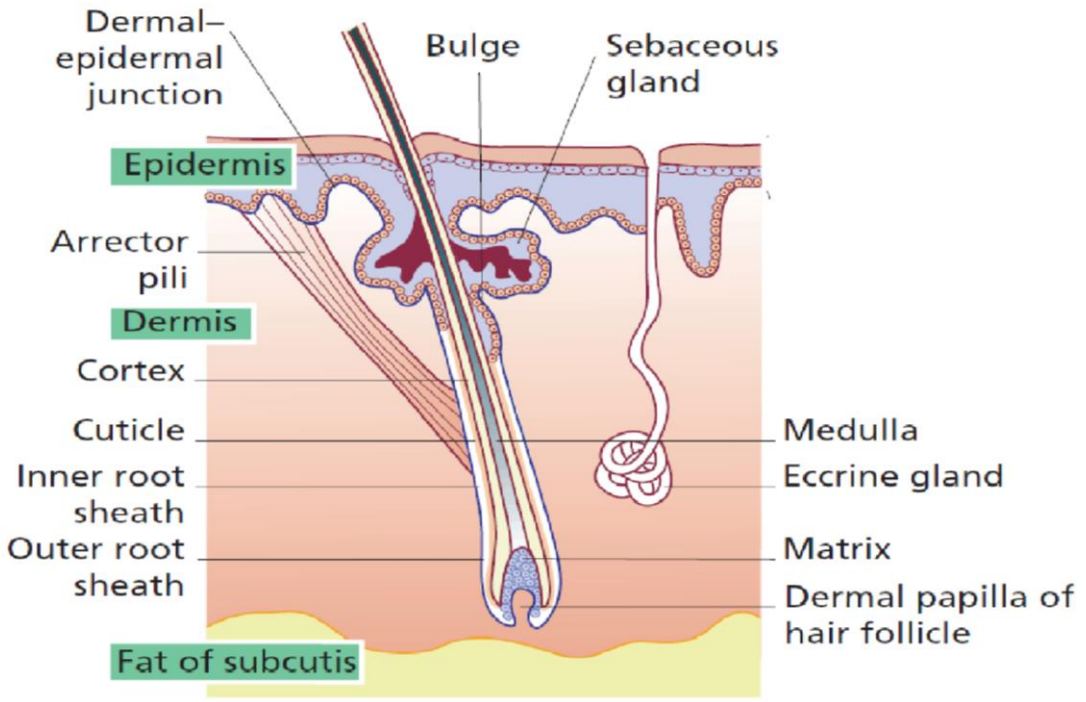


Figure 1. Anatomy of skin (McGrath et al. 2004).

Epidermis is the outermost layer of the skin and made up of layers of keratinocyte cells. The keratinocytes develop at the basal layer and rise to top, where they become dead, hard, and flattened cells. Therefore, epidermis contains both living and dead cells. Additionally, it is constantly renewing itself. Dermis accounts for almost 90 % of the skin thickness. Two types of proteins, i.e., collagen and elastin fibers are found in dermis layer. It has sensual organs for touch, pressure, pain and temperature, blood vessels, nerve fibers, hair follicles, sebaceous and sweat glands. Its vascular network supplies the avascular epidermis with nutrients (McGrath et al. 2004)

Stratum corneum (SC) is a protective outer layer of epidermis made up of multilayered wall-liked structure of flattened keratinized dead cells. The main barrier function of the SC is due to epidermal differentiation, protein matrix and lipid layer. When the basal layer divides to form keratinocytes and migrate to the upper, the membrane coating granules (MCG) and the keratohyalin granules begin to appear in the intercellular region. After these latter degrade, keratinocytes are fully cornified with fibrous keratin at the final stages of epidermal differentiation (Abraham 1997; McGrath et al. 2004).

When cornified keratinocytes move upward to SC layer, they transform to flat enucleated proteinaceous cells (corneocytes). The epidermal keratin filaments form a complex cytoskeleton network in the corneocytes to develop a highly resistant and insoluble outer envelope. Corneocytes are joined together by multiple protein links called corneodesmosomes. The horny cell lipid envelope (HCE) acts as a template for the lamellar lipid structure of the intercellular lipids. This becomes “brick (corneocytes) and mortar (intercellular lipid)” structure and physical barrier to prevent water loss and entry of exogenous materials. Other cells of epidermis such as melanocytes, Langerhans cells and Merkel cells are also found in the basal layer (Abraham 1997; McGrath et al. 2004). The anatomy structure of epidermis is illustrated in Figure 2.

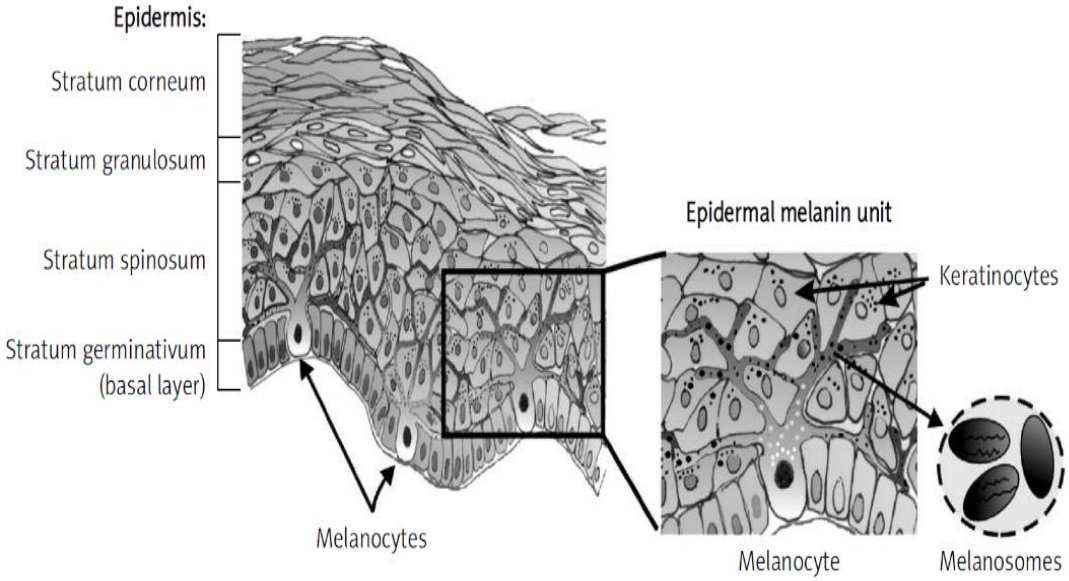


Figure 2. Anatomy of epidermis structure (Cichorek et al. 2013).

Melanocytes are dendritic cells which extend between the adjacent keratinocytes. Organelles located in the cytoplasm of melanocytes are called melanosomes. They produce melanin, a major pigment which determines the skin color. Following melanogenesis, the melanosomes containing pigment migrate to the tips of the dendritic and are phagocytized by the surrounding keratinocytes as illustrated in Figure 2. The pigmented keratinocytes move from the lower layer up to the surface and give the skin color according to the type, size and distribution of melanin saturated in the keratinocytes (Harry 1982; Cichorek et al. 2013).

Melanin can serve as natural sunscreen to prevent skin harmful from UV rays. Melanin production is primarily controlled by the tyrosinase enzyme. It hydroxylates tyrosine to L-DOPA (3,4-dihydroxyphenylanine) and then oxidizes L-DOPA to dopaquinone (Harry 1982; Cichorek et al. 2013). At that point, the pathway diverges to produce either red/yellow type of melanin (pheomelanin), or brown/black melanin (eumelanin) as exhibited in Figure 3.

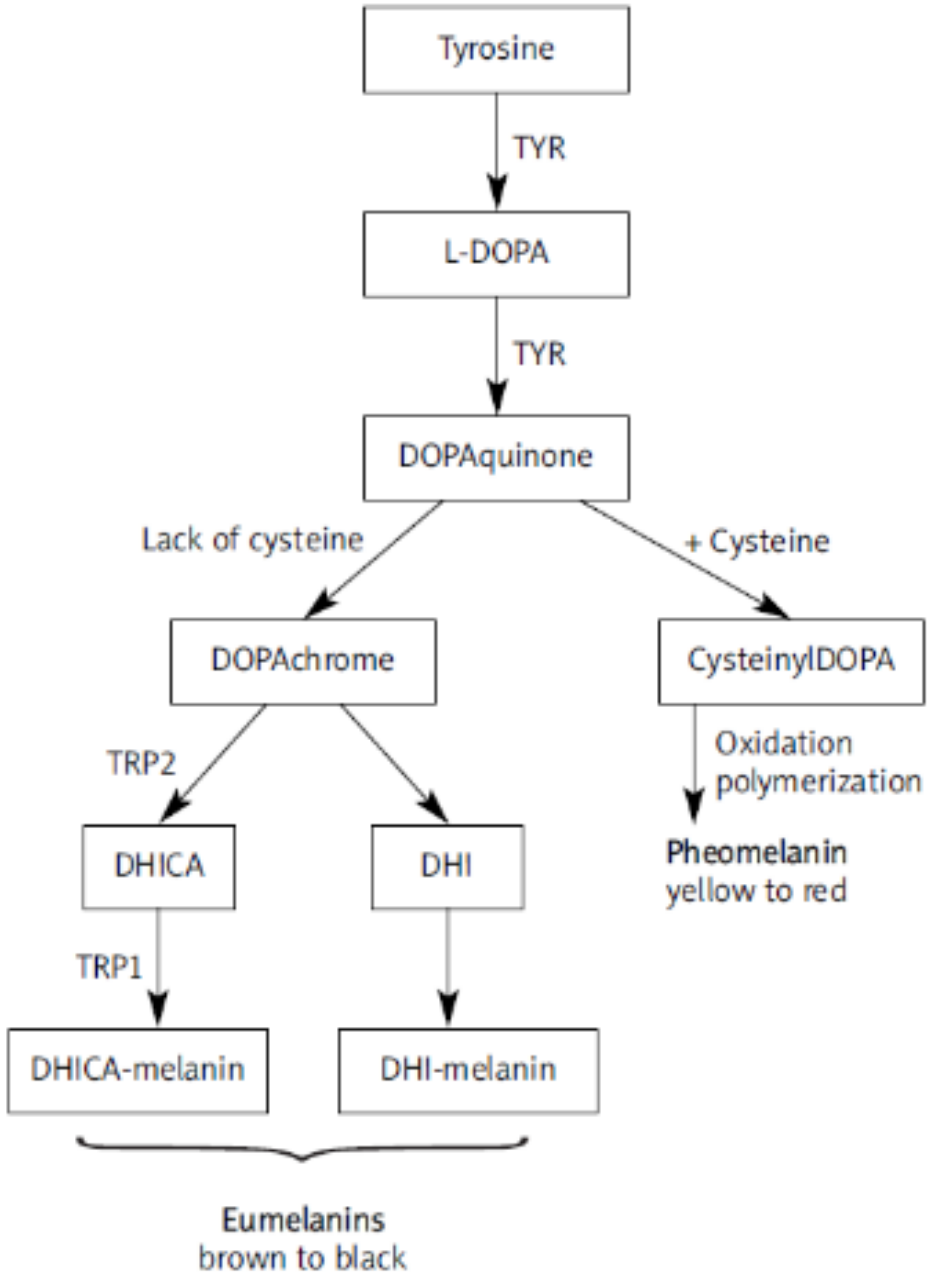


Figure 3. Melanin synthesis pathway in melanocytes during melanogenesis. Tyrosine under influence of the basic enzymes such as tyrosinase (TYR), tyrosine-related protein 1 (TYRP1) and 2 (TYRP2), 5,6- Dihydroxyindole 2- Carboxylic acid (DHIC) and 5,6-Dihydroxyindole (DHI) (Cichorek et al. 2013).

An individual skin color is primarily due to genetic factors and other ethnicity, gender, age, body site and external factors. Melanin production is also

stimulated by ultraviolet light exposure, oxidative stress, melanocyte-stimulating hormone (MSH), injury, inflammation, chronological skin aging and photoaging (Cazorla 2014). Due to the diverse perception on beauty of skin tone and skin pigmentation problems, physical methods (superficial dermabrasion or laser treatment) or skin lightening agents (natural extracts or synthetic compounds) are booming interested. Skin lightening products are popular for both personal preference and dermatological need. Skin lightening agents are used to correct the skin abnormal pigmentation such as melasma, senile lentigo, freckles or vitiligo. Prolong exposure to UV radiation causes skin tanning which takes days to years to recover the uniformity of skin color. Skin lightening agents can be applied as mono- or combination therapy with antiaging or after sun care products (Ortonne and Bissett 2008; Ong and Maibach 2014; Burger et al. 2016).

Skin lightening agents can lessen undesired pigment by acting at one or more steps in melanogenesis by the following manners. They can hinder tyrosinase transcription before melanin synthesis, interfere activity of tyrosinase and peroxidase and scavenge ROS during synthesis. Moreover, some can inhibit the melanosome transfer and accelerate skin turnover after melanin pigment is produced (Ong and Maibach 2014; Burger et al. 2016). For the safety of consumers and regulation concern, some lightening agents such as retinoids, hydroquinone and mercury have been inhibited to be used in cosmetic products (Burger et al. 2016; Grimes et al. 2019). Therefore, continuously studying to approach the optimal skin lightening with safe active ingredients in effective formulation is highly demanded.

2. Nicotinamide

Among several skin whitening agents, nicotinamide is one of the effective and safe agents. It is the biologically active amide of niacin called vitamin B3. It functions as an essential component of the enzyme co-factors, nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP) which are involved in many intracellular oxidation-reduction reactions (Baumann 2014).

Nicotinamide has been used in dermatology to treat acne, rosacea and skin hyperpigmentation. It can reduce transepidermal water loss (TWEL) and protect UVA and UVB radiation (Baumann 2014). Furthermore, nicotinamide could be used to treat acnes induced by *Propionibacterium acnes* (Grange et al. 2009). In human studies of double blind, placebo-controlled, split face study in eye area, nicotinamide showed antiwrinkle effect and 64% of volunteers revealed marked or moderate improvement in fine lines after applying 4% nicotinamide for 8 weeks. Additionally, the average roughness of skin surface also significantly reduced when compared the pre- with post-applications (Kawada et al. 2008). Nicotinamide is also used clinically for some dermatological conditions, such as seborrheic dermatitis, psoriasis, and effective for other inflammatory conditions. It can be utilized as monotherapy or in combination with others for synergistic action (Otte et al. 2005; Grange et al. 2009; Siadat et al. 2013; Fabbrocini et al. 2014).

Its chemical structure is described in Figure 4 and its physicochemical properties are presented in Table 1

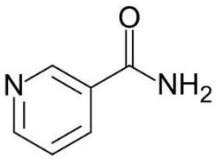


Figure 4. Chemical structure of nicotinamide (https://pubchem.ncbi.nlm.nih.gov/compound/nicotinamide#section=InChI, Accessed date – 11nd February 2019).

Table 1. The physicochemical properties of nicotinamide (https://pubchem.ncbi.nlm.nih.gov/compound/nicotinamide#section=InChI, Accessed date – 11nd February 2019)

IUPAC name	Pyridine-3 carboxamide
CAS number	98-92-0
Formula	$C_6H_6N_2O$
Molar mass	122.13 g/mol
Physical Appearance	White powder
Density	1.40 g/cm^3
Solubility	Very soluble in water, 500000 mg/L (at 25 °C)
pKa	3.35
Log K $_{\text{n-Octanol/Water}}$	-0.37
Melting point	129.5 °C (265.1 °F)
Boiling point	334 °C (633 °F)

In 2002, Hakozaki et al. studied the *in vitro* effects of nicotinamide in a purified mushroom tyrosinase assay, cultured melanocytes, a keratinocyte/melanocyte coculture model, and a pigmented reconstructed epidermis (PREP) model. They found that nicotinamide was no effect on melanogenesis but significantly inhibited melanosome transfer in keratinocyte. For human clinical studies, 5% nicotinamide noticeably decreased hyperpigmentation and increased skin lightness while vehicle alone did not provide these effects after 4 weeks application. In another study, the volunteers were divided into 3 groups and they all applied vehicle, sunscreen and 2% nicotinamide with sunscreen. Without overlapping the products, each group applied 2 different products, i.e. one product for each half face. The 2% nicotinamide with sunscreen could lighten the skin better than the sunscreen alone and vehicle, respectively (Hakozaki et al. 2002).

Greatens et al. studied the effect of nicotinamide in melanosome transfer in 2005. The 2 groups of 39 subjects and 40 subjects were assigned to use 2% and 5% nicotinamide compared to the vehicle moisturizer. Time interval of 8 weeks for treatment and 34 weeks for recovery period was defined. After the study period, it was

observed that 5% nicotinamide improved skin lightening statistically whereas 2% nicotinamide did not. However, no difference in the efficacy of 5% nicotinamide compared to vehicle was observed after 42-week application. Based on the observation, they concluded that the inhibitory activity was reversible, and dose related (Greatens et al. 2005).

It has been reported that the acute toxicity of nicotinamide after oral administration or dermal application was very low. Skin irritation studies indicated that the concentration up to 10% of nicotinamide has no potential to show irritancy. According to the Cosmetic Ingredient Review Expert Panel' review, evidences from human exposure can be concluded that nicotinamide was well tolerated by skin (Panel 2005). The concentration of nicotinamide used for dermatological studies usually ranged from 1.4% to 5% depending on the purpose of usage and combination or monotherapy (Forbat et al. 2017).

Nicotinamide is water-soluble vitamin and therefore effective topical formulations of this compound are required to overcome the barrier function of SC. The extracellular lipid bilayers in SC are predominantly made of ceramides, free fatty acids, and cholesterol. The dense packing of these lipid bilayers is moisture barrier and prevents penetration of many types of chemicals (McGrath et al. 2004). To exert effects on the melanocytes at the basal layer of the epidermis, nicotinamide has to penetrate the SC barrier. The delivery of actives beyond the SC barrier is challenging to cosmetic sciences and a lot of strategies to overcome this problem have been developed. Microemulsion is one of the considerable vehicles to transport active ingredients to the target sites.

3. Microemulsions

The first microemulsion was obtained by mixing oil, water, and an ionic surfactant combined with a cosurfactant and named as special colloidal dispersions by Hoar and Schulman in 1943. Later in 1959, the term "microemulsion" had been introduced. The properties of microemulsions include thermodynamically stability, visually transparency, small particle size usually below 140 nm, ultralow interfacial tension and the capacity of solubilizing both water and oil soluble ingredients. Due to spontaneous formation, microemulsions can be produced without utility of high energy consuming machines (Solans et al. 1997; Lawrence and Rees 2012).

There are two main structures of microemulsion, i.e., discrete and bicontinuous structures. In discrete microemulsion, the lesser proportion of phases (water or oil) is dispersed in other phases of higher proportion in the presence of surfactant or/and cosurfactant mixture. If water phase is dispersed in oil phase, water-in-oil (w/o) microemulsion is obtained. In contrast, if oil phase is dispersed in water phase, oil-in-water (o/w) microemulsion is originated. Similar ratio of oil and water phases is found in bicontinuous microemulsion. However, it usually requires high amount of surfactant to reduce interfacial tension compared to discrete one (Malik et al. 2012).

The microemulsion type can be anticipated from the geometric packing of surfactant molecules which is calculated as critical packing parameter (CPP).

$$\text{CPP} = v/a_o l_c$$

where

a_o = optimal cross-sectional area of hydrophilic head

l_c = length of hydrophobic tail

v = alkyl chain volume

This ratio is influenced by several factors such as salinity, temperature, type and character (hydrophilicity or lipophilicity) of surfactants and volume fraction of each components. When packing ratio CPP is less than 1, the curve of aggregates will bend towards the water phase and o/w type will be preferred. The opposite

curvature with w/o type is formed if CPP is greater than 1 (Mitchell and Ninham 1981). The different formations of structures and shape are corresponded to CPP value as presented in Figure 5.

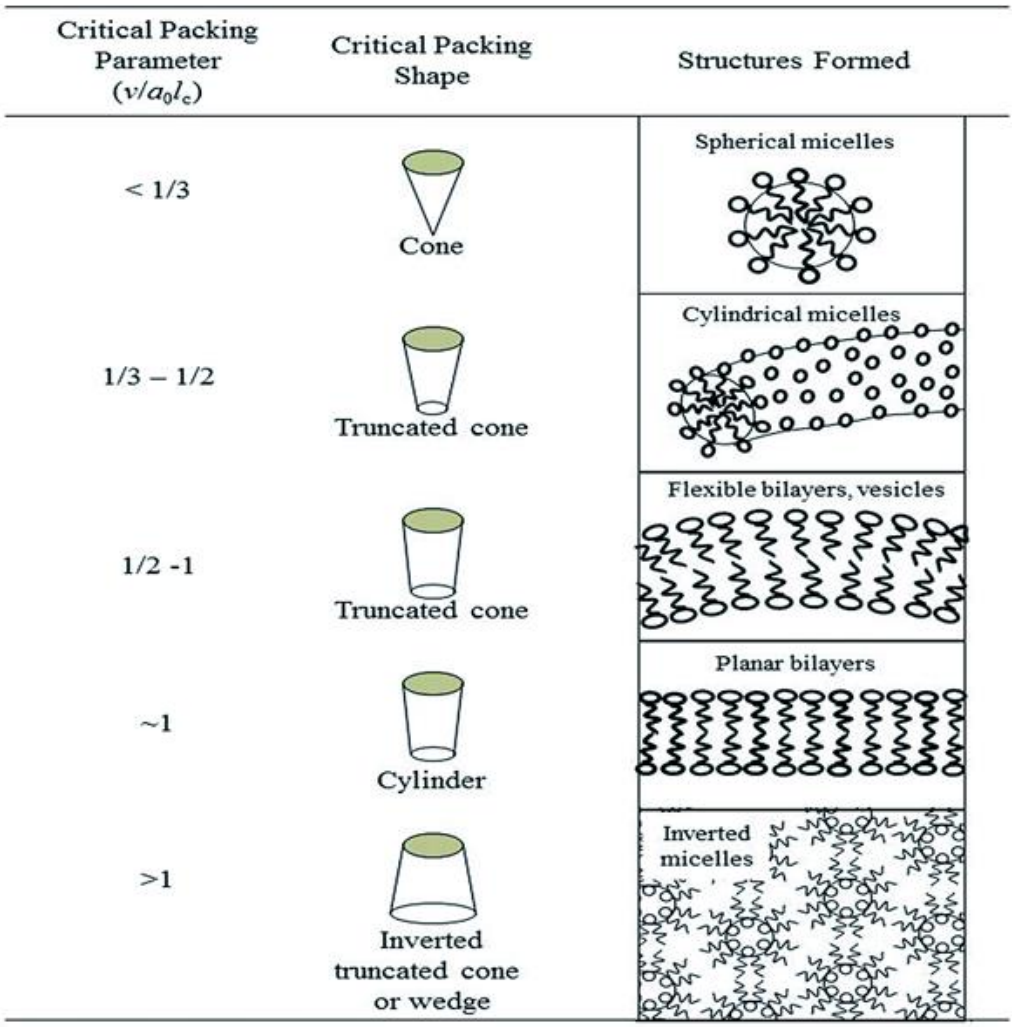


Figure 5. Aggregation type and geometric packing of surfactant molecules (Clint J. H. 1992).

Phase behavior of each phase can be studied by construction of pseudoternary phase diagram. As displayed in Figure 6, various association structures can be found when mixing oil, water, surfactant and cosurfactant together. The volume fraction of each component can affect the surface curvature causing the transformation of geometric packing unit. Consequently, metamorphosis from one phase to another phase occurs (Clint J.H. 1992; Malik et al. 2012).

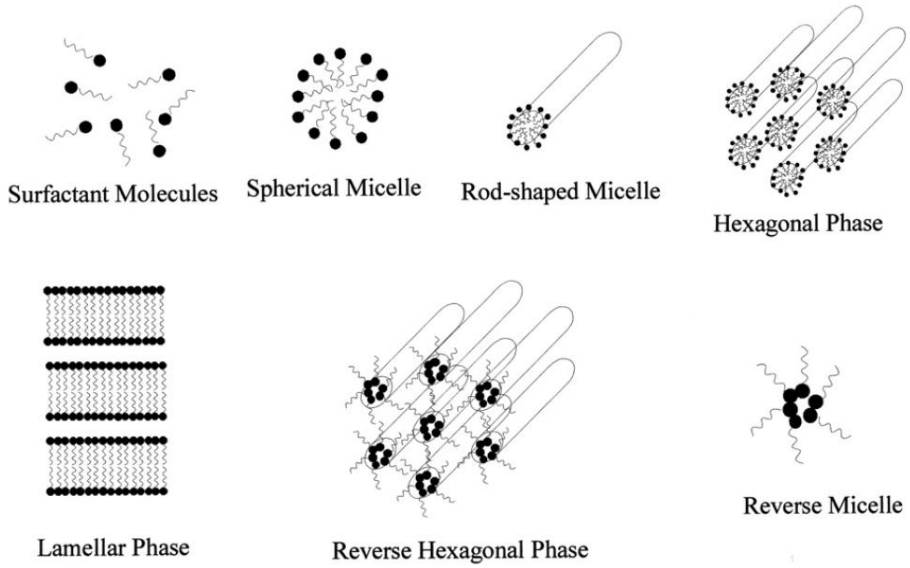


Figure 6. Association structures when mixing oil, water, surfactant and cosurfactant (Lawrence and Rees 2012).

To characterize the microemulsion, both visually and machine aided methods can be used. The physical appearance such as clarity and phase separation can be optically detected. The polarized light microscopy is used to differentiate microemulsion and lamellar or hexagonal liquid crystals. The o/w and w/o type of microemulsion types can be simply examined by drop dilution test and conductivity measurement. Additionally, transmission electron microscope (TEM) can differentiate discrete and bicontinuous microemulsion. Other techniques such as differential scanning calorimetry (DSC), cryo-field emission scanning electron microscopy (cryo-FESEM) and nuclear magnetic resonance (NMR) are also used for further identification of microemulsions (Boonme et al. 2006).

The clear appearance and less sticky texture of microemulsion make it be attractive to use as vehicle for active ingredients in cosmetic applications. Not only for aesthetic purpose but also for the promising delivery, microemulsions become favored in many beauty industries. Skin care, hair care and other personal care products are now available either in microemulsion liquid form or microemulsion-based gel form (Boonme 2009; Souto et al. 2011).

Hesperetin, water-insoluble flavonoid from citrus fruits, was prepared as microemulsions for topical whitening and skin care regiment after UV radiation. Experiments proved that microemulsion systems were auspicious for solubilizing and permeability of hesperetin (Tsai et al. 2010). An active compound 20(S)-Protopanaxadiol, deglycosylated metabolite of ginsenosides, possesses antiwrinkle and skin whitening effects. However, its poor solubility and membrane permeability requires overcoming the SC barrier. Formulating this compound as microemulsion-based gel preferred topical delivery after confirmed by both *in vitro* and *in vivo* (Kim et al. 2018).

Depending on the purpose of usage, microemulsions may be formulated as discrete or bicontinuous type for different targets. The unstable ascorbic acid molecules were well protected in the microemulsion formulation and delivered to the target site by exerting skin whitening effect (Pakpayat et al. 2009). When benzophenone 3, sunscreen agent, was prepared in w/o microemulsion type, it highly retained in the skin membrane, resulting in protection from the harmful ultraviolent radiation (Songkro et al. 2014). Many industrial fields such as pharmaceutical, agrochemical, food, cosmetics, household, etc. are applying the concept of microemulsion for manufacturing the commercial products (Boonme 2007; Lopes 2014).

4. Surfactants

Surfactants (surface active agents) are amphiphilic organic compounds with hydrophilic head and hydrophobic tail and can modify the interface between opposing phases. They are firstly created by William C. Griffin in the 1940s. He introduced Hydrophilie Lipophilie Balance (HLB) system in order to determine the utility and relation between surfactants. Microemulsion usually requires a pair of surfactants for stability. It is important to blend surfactants to get the correct required HLB (Courtney 1997; Xavier-Junior et al. 2016).

Surfactants serve a variety of functions mainly 6 categories as cleansing, emulsifying, solubilizing, suspending agents, foam boosters and hydrotropes in cosmetic products (Rieger 1997; Som et al. 2012). By interacting with SC, surfactants can enhance the skin permeation. The mechanisms of interaction with SC are (1) by modifying SC structure (2) by solubilizing lipids or water-soluble constituents of SC, and (3) by denaturing of epidermal keratin (Zatz and Lee 1997; Jackson et al. 2014).

Surfactants are classified according to their ionic charge. Anionic surfactants owe their water solubility to the negative charge on the molecule and lipid compatibility to a long hydrocarbon chain. Large numbers of anionic surfactants are either sulfates or sulfonates of fatty alcohols or ethoxylated fatty alcohols. Cationic surfactants, usually quaternary ammonium compounds and amine oxide, have water solubility to positively charged nitrogen. Nonionic surfactants, mainly ethoxylated fatty alcohols and phenols groups, have no charge on the molecules but owe hydrophilic character to a polar group and hydrophobic to the long chain hydrocarbon. Amphoteric surfactants possess two oppositely charged ionizable sites on the molecule, cationic charge on a ternary amine group and anionic charge on a carboxylate or sulfonate group. Amphoteric surfactants carry a positive charge at low pH, a negative charge at high pH and may be a zwitterion at intermediate pH (Rieger 1997; Som et al. 2012).

Since surfactants can alter the skin barrier function, the skin irritation potential should be considered. The tendency to enhance skin penetration is related to the concentration and chemical structure (ionic type and hydrocarbon chain length). Anionic or cationic surfactants can form adsorption complexes with protein due to polar

and nonpolar binding. Nonionic or amphoteric ones show no interaction with proteins. In addition, nonionic surfactants have long been recognized as the least irritating to the skin compared to other surfactant types (Jackson et al. 2014; Kaur and Mehta 2017).

In this study, Tween 80 (polyoxyethylene (20) sorbitan monooleate) and Span 80 (sorbitan monooleate) were used as surfactant and cosurfactant respectively. They are nonionic sorbitan ester type, and therefore good compatibility to skin. Tween 80 is the ethoxylation derivative of Span 80 by substitution of 20 oxyethylene groups and its HLB is 15. Span 80 is lipophilic character and its HLB is 4.3. Both Tween 80 and Span 80 possess the same oleate group hydrophobic tail (Rieger 1997; Som et al. 2012). Their general properties are described in Tables 2 and 3.

Table 2. Properties of Tween 80 (https://www.sigmaaldrich.com/catalog/product/sigma/p5188?lang=en&region=US, Accessed date – 24th March 2019)

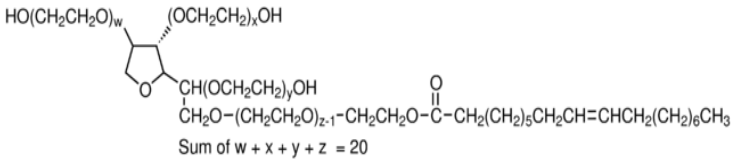
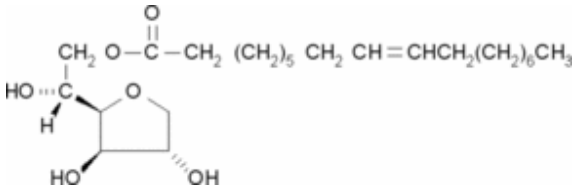
Molecular structure	
Chemical name	Polyoxyethylene (20) sorbitan monooleate
Formula	$C_{64}H_{126}O_6$
Molar mass	1310 g/ mol
HLB	15
CAS Number	9005-65-6
Physical Appearance	Amber colored viscous liquid
Solubility	Very soluble in water
Use	Nonionic surfactant, emulsifier

Table 3. Properties of Span 80 (https://www.sigmaaldrich.com/catalog/product/sigma/s6760?lang=en&region=US, Accessed date – 24th March 2019)

Molecular structure	
Chemical name	Sorbitan monooleate
Formula	$C_{24}H_{44}O_6$
Molar mass	428.61 g/ mol
HLB	4.3
CAS Number	1339-43-8
Physical Appearance	Light yellow colored viscous liquid
Solubility	Insoluble in water
Use	Nonionic surfactant, emulsifier

5. Olive oil and virgin coconut oil

Olive oil is extracted from the flesh of olive fruit (*Olea europaea*) where its origin is the Mediterranean (Gouvinhas et al. 2017). In addition to moisturizing and emollient effects, antiaging, anti-inflammatory and antimicrobial effects have investigated in olive oil (Badiu et al. 2010). Clinical trials of olive oil-based formulations applied on immobilized elder patients suffering pressure ulcers, olive oil could improve the wound healing due to its anti-inflammatory and antioxidant properties (Lupiáñez-Pérez et al. 2013). For advanced biotechnological fields, lecithin-based olive oil microemulsions could be used as microreactors in several enzymatic reactions (Papadimitriou et al. 2007).

Virgin coconut oil is extracted from fresh and mature kernel of coconut (*Cocos nucifera*) by wet method with or without heat (Marina et al. 2009). Virgin coconut oil is the staple edible oil for food processing. This oil has long been used for skin and hair application by people in the tropical region. Furthermore, virgin coconut oil could be used for therapeutic application for skin conditions such as xerosis, atopic dermatitis and eczema (Evangelista et al. 2014).

Not only the fatty acids but also the bioactive compounds are present in both olive oil and virgin coconut oil. These two oils have been utilized as food, medicine and topically on dermal and hair since ancient time. Polyunsaturated free fatty acids are majority components in olive oil while saturated fatty acids are main component in virgin coconut oil. According to the assessment of Cosmetic Ingredient Review Expert Panel, olive oil and virgin coconut oil are not primary dermal irritants and safe to use. Both oils are used in variety of leave on and rinse off products for eye area, dermal, hair, nail, deodorant, bath products. They can also be used in baby products. Normally, the concentrations of olive oil and virgin coconut oil used in cosmetic products are in the range of 0.0005-100% and 0.0001- 80 % respectively (Burnett et al. 2017).

Generally, saturated oil is usually stable to rancidity as their carbon bonds are inactive to react with oxygen (deMan 2013). Although olive oil contains high amounts of unsaturated fatty acids, it possesses simple and complex bioactive phenolic

compounds such as cinnamic and benzoic acids, phenolic alcohols, secoiridoids, lignans, hydroxy-isochromans and flavonoids (Gouvinhas et al. 2017). These compounds are natural antioxidant which can retard the oxidation sequences in olive oil.

In this research, olive oil and cold pressed virgin coconut oil were studied as oil phase in the microemulsions. Both oils provide beneficial effects for skin health, biocompatible, commercially available and reasonable price. Besides, they have low molecular weight and medium chain fatty acids which can be easier to be microemulsified compared to high molecular weight ones. Suitable microemulsion systems can be established by comparing the unsaturated olive oil and saturated virgin coconut oil. Green microemulsions formulated with natural derived oils are biocompatible and eco-friendly.

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Acetonitrile, HPLC grade (Lot No: 18030206, RCI LabScan, Bangkok, Thailand)
2. Brilliant blue, pharmaceutical grade (Winner's, Bangkok, Thailand)
3. Deionized water (Prepared in house)
4. Di-sodium hydrogen orthophosphate, analytical grade (Lot No: 1707055158, Univar, New South Wales, Australia)
5. Methanol, HPLC grade (Lot No: 18020018, RCI LabScan, Bangkok, Thailand)
6. Nicotinamide, pharmaceutical grade (Lot No: 1242072017/A, P.C Drug Center Company, Bangkok, Thailand)
7. Olive oil, pharmaceutical grade (Lot No: 969852, P.C Drug Center Company, Bangkok, Thailand)
8. Potassium dihydrogen orthophosphate, analytical grade (Lot No: 1102146, Univar, New South Wales, Australia)
9. Sodium chloride, analytical grade (Lot No: 1605218703, Univar, New South Wales, Australia)
10. Span 80 (Sorbitan monooleate), pharmaceutical grade (Lot No: 7090E, P.C Drug Center Company, Bangkok, Thailand)
11. Triethylamine, analytical grade (Lot No: 411891, Fluka Chemika, Buchs, Switzerland)
12. Tween 80 (Polyoxyethylene (20) sorbitan monooleate), pharmaceutical grade (Lot No: 36253, P.C Drug Center Company, Bangkok, Thailand)
13. Virgin coconut oil, pharmaceutical grade (Lot No: 20180319, Chemipan Coporation Co., Ltd, Bangkok, Thailand)

Equipment

1. Cellulose acetate synthetic membrane (Spectra Pro® 3, Dialysis membrane, MWCO 3,500Da, USA)
2. Conductivity meter (FiveEasy, Mettler Toledo, Switzerland)
3. Electrical balance (XB220A, Precisa Gravimetrics AG, Dietikon, Switzerland)
4. Gas chromatography (GC-FID; Agilent 7890A, USA)
5. High performance liquid chromatography (HPLC) system (Agilent 1100 series, Palo Alto, USA)
6. Magnetic stirrer (MS 115, Harikul Science, Bangkok, Thailand)
7. Modified Franz diffusion cell (Hanson Model 57-6M, Hanson Research Corporation, California, USA)
8. Nylon filter (Sartolon Polyamide, 0.45 μm, Sartorius Stedim Biotech GmbH, Germany)
9. pH meter (S20-K, Mettler Toledo, Switzerland)
10. Polarized light microscope (Olympus BX61, Japan)
11. Sonicator (Crest Ultrasonics, CP100D, Scientific promotion Co. Ltd., Thailand)
12. Syringe filter (0.45μm Nylon Syringe filter 13 mm, Vertical chromatography Co.,Ltd., Thailand)
13. Transmission electron microscope (TEM; JEM-2010, JEOL, Japan)
14. Viscometer (Brookfield DV III Ultra Programmable Rheometer, Brookfield Engineering Laboratories, Middleboro, USA)
15. Vortex mixer (Vortex Genie-2, G560E, Scientific Industries. Inc, USA)
16. Zeta potential analyzer (Zetasizer Nano series, Nano ZS (Red badge ZeN3600, Malvern Instruments limited, UK)

Methods

1. Determination of types and amounts of fatty acids in natural oils

The fatty acids of olive oil and virgin coconut oil were analysed by gas chromatography equipped with flame ionization detector (GC-FID; Agilent 7890A, USA). Determination was processed by some modifications of previous method (Zhang et al. 2015). Helium, split ratio 50:1 with a flow rate of 1 mL/min was used as carrier gas. The sample injector was thermostatically controlled at 290°C. The 30 m length capillary column, Select Biodiesel for FAME was employed. Oven temperature was set at 210°C held for 12 min and increased 20°C/min until reach to 250°C and detained for 8 min by temperature programming. Hydrogen gas and air were supplied to flame ionization detector with flow rate of 30 mL/min and 300 mL/min, respectively, whereas detection temperature was fixed at 300°C.

2. Preparation of microemulsions

2.1.Selection of surfactant:cosurfactant (S:CoS) ratio by HLD concept

HLD concept compares the influenced factors between different formulations or categorizes the experimental scale of constituents quantitatively (Pakpayat et al. 2009). HLD method accounts the impact of variables such as temperature, salinity, and the addition of alcohols or cosurfactants (Witthayapanyanon et al. 2008). Different formulations were carried out by altering the S:CoS ratio with two different vegetable oils, i.e. olive and virgin coconut oil. According to HLD concept, if S:CoS ratios are suitable, Winsor III microemulsion can be observed as three layers which the middle layer show Tyndall effect under laser light.

This protocol involves two phases mixed in trial tubes: oil phase/water phase ratio, 50% for each. The oil phase consists of a% CoS (Span 80) in oil (either olive oil or virgin coconut oil) and pure oil, and the water phase consists of b% S (Tween 80) in water, 3% NaCl solution and pure water. The total amount of S and CoS (a/100 + b/100) represented in fraction of 1 and the ratio was varied from 1:0 to 0:1

with the interval of 0.1. After mixing 2 phases, the tubes were slowly turned (180°) for 30 times and equilibrated in water bath (70°C). Electrolytes solution and 70°C temperature was used to accelerate phase equilibrium (Pakpayat et al. 2009).

2.2.Construction of pseudoternary phase diagrams

The optimal S:CoS ratios were selected to construct pseudoternary phase diagram by titration method. The combination mixtures of S:CoS and oil at various ratios 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9 were prepared. Each mixture was gradually diluted by the aqueous phase while stirring by magnetic agitator at room temperature (28 ± 2°C). The obtained mixture was classified into the microemulsion zone in pseudoternary phase diagram if it was transparent liquid. Ongoing addition of aqueous phase until becomes turbid to find the exit of microemulsion zone. The shifting points (transparency, turbidity) were noted. Pseudoternary phase diagram was used to observe the desired microemulsion zone with appropriate ratios between oil, water and S:CoS mixture. A cut-and-weigh method was used to calculate the percentage of microemulsion area in the phase diagram.

3. Preparation and characterization of blank microemulsions

3.1.Preparation of blank microemulsions

Among the obtained pseudoternary phase diagrams, two pairs of S:CoS ratios were selected to formulate four microemulsions with either olive oil or virgin coconut oil. The diagrams should provide acceptably large microemulsion region in order to ease select a point to formulate. Concerning the stability and phase inversion possibilities, microemulsion should not be prepared from the point nearby the microemulsion boundary. Therefore, one identical point within each microemulsion region was selected. The amount of S:CoS mixture was calculated and mixed with magnetic agitator. After that, S:CoS mixture, oil and water were thoroughly mixed by magnetic agitator again and the microemulsion was found when the formulation become transparency.

3.2.Appearance observation

All preparations were visually observed to determine the transparency and phase separation and microscopically observed under polarized light microscope (Olympus BX61, Japan) to differentiate between microemulsion and liquid crystal. Birefringence is observed only in liquid crystal as it is mesophase while it is not found in isotropic microemulsion.

3.3.Determination of microemulsion type

Drop dilution test was used to characterize the type of microemulsion. Theoretically, if the external phase of formulation mixes well with brilliant blue aqueous solution, it is o/w microemulsion. In contrast, w/o type is miscible with either olive oil or virgin coconut oil. The observation was performed in triplicate. For confirmation of microemulsion type, conductivity measurement was carried out using conductivity meter (FiveEasy, Mettler Toledo, Switzerland) at 25°C for triplicate. The conductivity of o/w type is higher than that of w/o type.

3.4. pH measurement

The pH values were determined by digital pH meter (S20-K, Mettler Toledo, Switzerland) at 25°C in triplicate.

3.5.Viscosity and flow property measurement

For rheological property, a bob-cup viscometer with a small adapter chamber and a spindle number SC4-31 (Brookfield DV III Ultra Programmable Rheometer, Brookfield Engineering Laboratories, Middleboro, USA) was used to measure the viscosity at skin surface temperature (32 ± 1°C), with five different speeds (20, 40, 60, 80 and 100 rpm). The experiment was done in triplicate.

3.6.Determination of droplet size, polydispersity index (PI) and zeta potential

For determination of mean particle size and PI of formulations, each sample was analyzed without dilution by zeta potential analyzer (Zetasizer Nano series, Nano ZS Red badge ZeN3600, Malvern Instruments limited, UK). Zetasizer uses the dynamic light scattering (DLS) and measures the scattering intensity of droplets. The PI represents the width of particle size distribution of a sample. It ranges from 0 (monodisperse) to 1 (very broad distribution). The zeta potential characterizes the particle surface charge. Measurement of zeta potential predicts the physical stability of colloidal system. Prior to the measurement, samples were not diluted in order to prevent phase transition or phase separation. All measurements were done in triplicate at 25°C by running ten times for each measurement.

3.7.Physical stability study

Stress conditions were employed for evaluating the stability of blank microemulsions by 5 times cycling freeze-thaw testing. Microemulsions were kept at very low temperature (-4°C) for 24 h and then placed in an oven at 40°C for 24 h. Afterward, they were examined physical changes.

4. Preparation and characterization of nicotinamide-loaded microemulsions

Blank microemulsions which passed the accelerating stability test were chosen to load nicotinamide. Nicotinamide was added to each blank microemulsion while stirring by magnetic stirrer until completely dissolved to obtain 3% w/w nicotinamide-loaded microemulsion. The obtained nicotinamide-loaded microemulsions were characterized as aforementioned. Furthermore, transmission electron microscope (TEM; JEM-2010, JEOL, Japan) was used for observation of the microstructure. One drop of sample was applied on a Formvar carbon film on 200-mesh copper grid and dried at room temperature. The dried sample was observed under TEM at magnification of x100,000.

5. Stability study of nicotinamide-loaded microemulsions

Nicotinamide-loaded microemulsions were studied for stability. The samples were stored at 4°C, room temperature (28 ± 2°C) and 45°C for three months. They were investigated for physical changes i.e., transparency, phase separation, conductivity, rheological property and pH every month compared with their blank counterparts which were kept in the same conditions. The amounts of active remaining in physical stable nicotinamide-loaded microemulsions were evaluated by high performance liquid chromatography (HPLC) technique. After storage for three months, nicotinamide-loaded microemulsions were detected for the remaining nicotinamide concentration. Briefly, 0.05 g of nicotinamide-loaded microemulsions was diluted with isotonic phosphate buffer pH 7.4 to obtain nicotinamide concentration around 15 µg/mL and filtered by syringe filter. The obtained solution was analyzed by HPLC technique.

6. *In vitro* release study

Physicochemically stable nicotinamide-loaded microemulsions were evaluated for release characteristics compared with simple 3% w/w of nicotinamide solution (NCT sol) using modified Franz diffusion cells as illustrated in Figure 7. Additionally, the nicotinamide-loaded microemulsions were freshly prepared and studied for release characteristics. Their release parameters were compared with those obtained from physicochemically stable nicotinamide-loaded microemulsions.

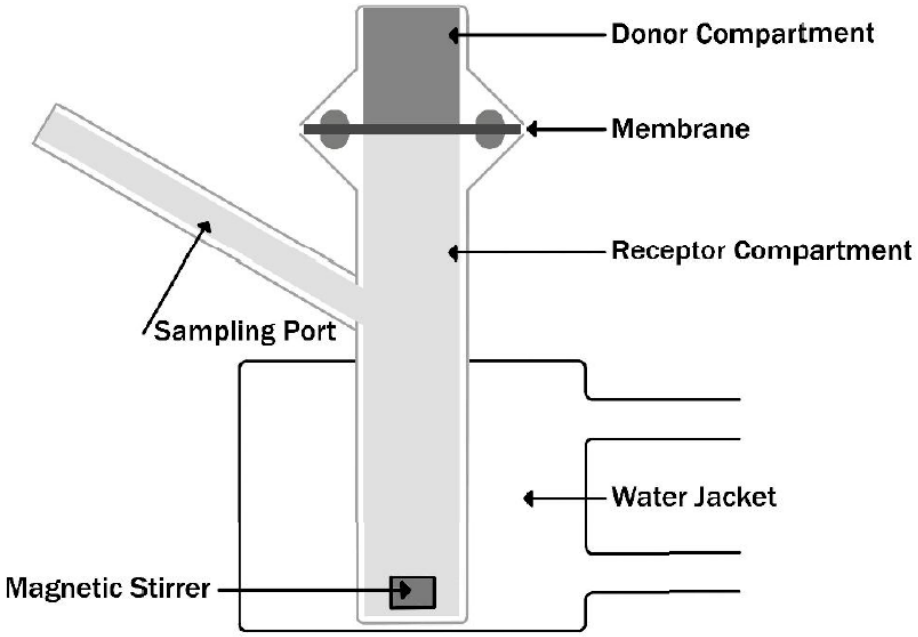


Figure 7. Franz diffusion cell (Bartosova and Bajgar 2012).

In this release study, the dialysis membrane (cellulose acetate synthetic membrane with molecular weight-cutoff (MWCO) 3,500 Da) was used. The method followed the previous studies with some modifications (Bartosova and Bajgar 2012; Boonme et al. 2012). The membrane was soaked in distilled water for 30 min to remove the wax and rinsed before used. It was placed between donor and receptor compartments of the diffusion cells with effective diffusion area of 1.77 cm^2. The receptor medium of 12 mL degassed isotonic phosphate buffer solution (pH 7.4) was filled in the receptor chamber. Nicotinamide is weak base and the optimal pH for unionized form is above pH 7. According to OECD guidelines, receptor fluid resembles to physiologically fluid is favored (OECD 2004). The circulating water bath (37°C) connecting the diffusion cell provided the temperature of surface membrane at 32 ± 1°C liked the human skin. The receptor medium was unceasingly stirred by the magnetic bar at a speed of 200 rpm. The tested sample was applied upon the membrane in the donor chamber.

At specified time intervals (0.5, 1, 2, 4, 8, 10, 12 and 24 h), 500 µL of receptor fluid was withdrawn from receptor chamber and replaced the fresh receptor fluid with the equivalent volume instantly. The withdrawal fluid was examined for

nicotinamide concentration by HPLC. Experiments were done triplicate for each formulation. The cumulative amount of nicotinamide release through the synthetic membrane into the receptor fluid (Q_t) was calculated by the equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i$$

where

C_t = the nicotinamide concentration of the receptor fluid at each sampling time

C_i = the nicotinamide concentration of the i^{th} sample

V_r = the volume of the receptor fluid

V_s = the sampling volume

The release profiles were constructed by plotting the cumulative amount of nicotinamide released per unit area of the membrane against time (t). The release data were further analyzed by using three different kinetics models: zero order, first order and Higuchi model as follows (Costa et al. 2013):

Model	Equations
Zero order	$Q_t = Q_o + k_0 t$
First order	$\ln Q_t = \ln Q_0 + k_f t$
Higuchi	$Q_t = k_H t^{1/2}$

where

Q_t = cumulative amount of nicotinamide release in time t

Q_0 = initial amounts of nicotinamide in the preparations

k_0, k_f, k_H = release rate constants of zero order, first order and Higuchi, respectively

7. Quantitative determination of nicotinamide

The HPLC technique was modified from previous study (Xu and Trissel 2003). The method was validated according to International Conference of Harmonization (ICH) guidelines (ICH 2005).

Chromatographic Conditions and Instrumental Setting:

Analytical column	:Reversed phase Phenomenex® Luna 5µm C_{18}100A column (5 µm particle size, 150 x 4.6mm, C_{18} with TMS endcapping)
Mobile phase	:0.1% triethylamine in 0.067M monobasic potassium phosphate buffer (pH 6.7) and acetonitrile (96.15:3.85, v/v)
Detector wavelength	:260 nm
Flow rate	:1.0 mL/min
Injection volume	:20 µL
HPLC System	: Agilent 1100 series Pumping Systems Agilent 1100 series UV-Visible detector Agilent 1100 series Autosampler

The nicotinamide concentrations of the samples were determined from the standard curve by plotting the peak area of nicotinamide against the nicotinamide concentrations.

7.1.Preparation of standard solutions

The 125 mg of nicotinamide was accurately weighed into a 25 mL volumetric flask and dissolved with isotonic phosphate buffer pH 7.4 and adjusted to volume 25 mL with the solvent to obtain the final concentration of 5 mg/mL nicotinamide solution. It was then pipetted 1.0 mL and placed to stock solution to a 10 mL volumetric flask and diluted with isotonic phosphate buffer pH 7.4 to volume of 10 mL to obtain 500 µg/mL stock solution. Afterward, a series of working standard nicotinamide solutions were prepared by diluting the stock solution with the same solvent to desired concentrations of 1.25, 2.5, 5, 10, 20 and 40 µg/mL

7.2.Validation of HPLC Method

Specificity

Under the selected chromatographic conditions, the peak of nicotinamide (active ingredient) must separate from the peak of other ingredients in the formulations. Chromatogram of the standard solution of nicotinamide was compared with chromatograms of the vehicles and the blank microemulsions.

Linearity

Nicotinamide standard solutions in the concentration range of 1.25, 2.5, 5, 10, 20 and 40 μg/mL were analyzed for three consecutive days (3 sets). Each of standard solution was injected three times. Linearity was evaluated by visual inspection of a plot of signals as a function of analyte concentration. The y-intercept, slope of the regression line and residual sum of squares were calculated. The linear regression coefficient (r^2) ≥ 0.999 is acceptable value.

Accuracy

Accuracy was assessed using a minimum of 9 determinations (3 concentrations in 3 replicates) over a minimum of 3 concentration levels, i.e., 5, 10 and 20 μg/mL covering the specified range. Accuracy was reported as percent recovery by comparing the amounts of drug found and amount of drug added. The percent recovery should be 98-102%.

$$\%\ \text{Recovery} = \frac{C_{measure}}{C_{actual}} \times 100$$

Where; $C_{measure}$ is the concentration of nicotinamide detected by HPLC

C_{actual} is actual concentration of nicotinamide

Precision

Precision was achieved at two levels: repeatability (intra-assay precision) and reproducibility (inter-assay precision) as follows.

The intra-day precision

Three sets of three standard nicotinamide solutions at 5, 10 and 20 µg/mL were analyzed within one day. Each concentration was determined in 5 replicates. Data were used to calculate percent of relative standard deviation (% RSD). The %RSD should not be more than 2.0%.

$$\%\text{RSD} = \frac{\text{SD}}{\bar{\text{X}}} \times 100$$

where SD = the standard deviation of the calibration curve

$\bar{\text{X}}$ = the average peak area

The inter-day precision

Three sets of three standard nicotinamide solutions at 5, 10 and 20 µg/mL were analyzed on different days for three consecutive days. Each concentration was determined in 5 replicates. Data were used to calculate %RSD. The % RSD should not be more than 2.0%.

Limit of Detection (LOD) and Quantification (LOQ)

Nicotinamide standard solution was diluted in the range of 0.1 to 1.00 µg/mL and analyzed. The quantitation limit is the lowest quantity which can be quantitatively determined with suitable precision and accuracy. A calibration curve was plotted, and the slope of the curve and the standard deviation are used to calculate detection limit and quantification limit. LOD and LOQ are calculated using the following equations:

$$LOD = \frac{3.3\ \sigma}{S}$$

$$LOQ = \frac{10\ \sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

8. Statistical Analysis

Data were expressed as mean ± standard deviation (SD) or mean ± standard error of the mean. Statistical comparison was made using paired t-test or one-way ANOVA. Paired t-test was used to study the conditions before and after the stability tests. The t-test was employed to compare pH, conductivity, and viscosity of different formulations. The one-way ANOVA was for the contrast *in vitro* release study of the selected microemulsions and simple nicotinamide solution. The p-value of less than 0.05 was significant.

CHAPTER 4

RESULTS AND DISCUSSION

1. Types and amounts of fatty acids in the studied oils

The types and quantity of fatty acids in olive oil and virgin coconut oil determined by GC-FID are displayed in Table 4. This result was compliance with the earlier report, only some variables in percentage of composition were observed and it might be because of the different geographic source and extraction technique of olive and coconut fruits (deMan 2013).

Table 4. Fatty acids composition in olive and virgin coconut oil analysed by GC-FID.

Fatty acid (%)	Lipid numbers [a]C:D	Olive oil	Virgin Coconut oil
Saturated type			
Caprylric	08:00	0.009	5.897
Nonanoic	09:00	0.013	0.014
Capric	10:00	0	5.814
Undecanoic	11:00	0	0.024
Lauric	12:00	0	48.102
Tridecanoic	13:00	0	0.032
Myristic	14:00	0.025	19.14
Pentadecanoic	15:00	0.007	0.01
Palmitic	16:00	11.32	8.978
Heptadecanoic	17:00	0.069	0
Stearic	18:00	2.786	3.082
Behenic	22:00	0.122	0
Lignoceric	24:00:00	0.03	0
Unsaturated type			
Palmitoleic	16:01	1.07	0
Oleic	18:01	71.53	5.775
Linoleic	18:02	10.968	1.026
Linolenic	18:03	0.633	0
Gondoic	20:01	0.275	0
Erucic	22:01	0.022	0

[a]C and D represent the numbers of carbon atoms and double bonds in the fatty acid respectively.

Polyunsaturated free fatty acids such as oleic acid and linoleic acid are majority components in olive oil, therefore, it is regarded as soft and unsaturated oil. Virgin coconut oil can be classified as saturated oil as it predominantly contains saturated fatty acids lauric acid and myristic acid. GC result confirmed that all unsaturated fatty acids found in both oils were *cis* form which agreed with the previous report (Marina et al. 2009; deMan 2013; Gouvinhas et al. 2017).

2. Microemulsion formation

The serial trial tubes of various S:CoS ratios equilibrated in water bath (70°C) were observed the Winsor system after one week. The formulation scans were recorded continuously for 15 days. The scans were different for each day. Phase transitions between Winsor types (I, II, III or IV) could occur due to variables changes such as temperature, pH and salinity (Salager et al. 2017). In this study, Winsor type III system was not observed in all systems composed both oils by Tyndall effect, probably due to the fact that middle layer was too thin.

All the ratios of surfactant mixtures were studied by titration procedure. Figures 8 and 9 depicted the ratios of surfactant mixtures and percentages of microemulsion regions of olive oil and virgin coconut oil.

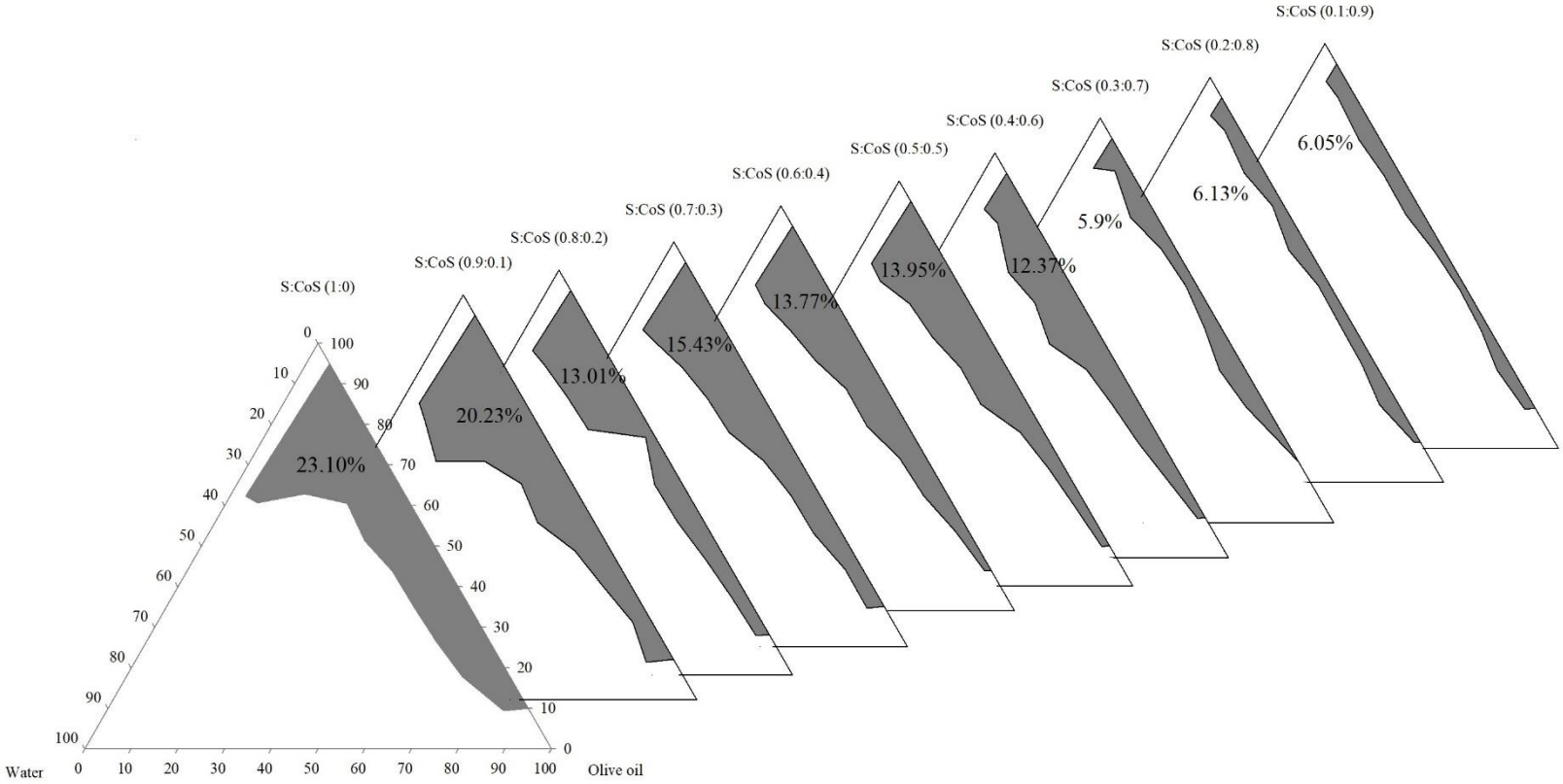


Figure 8. The pseudoternary phase diagram of Tween 80:Span 80:olive oil:water systems at different S:CoS ratios, the shaded area indicated microemulsion region with the its size.

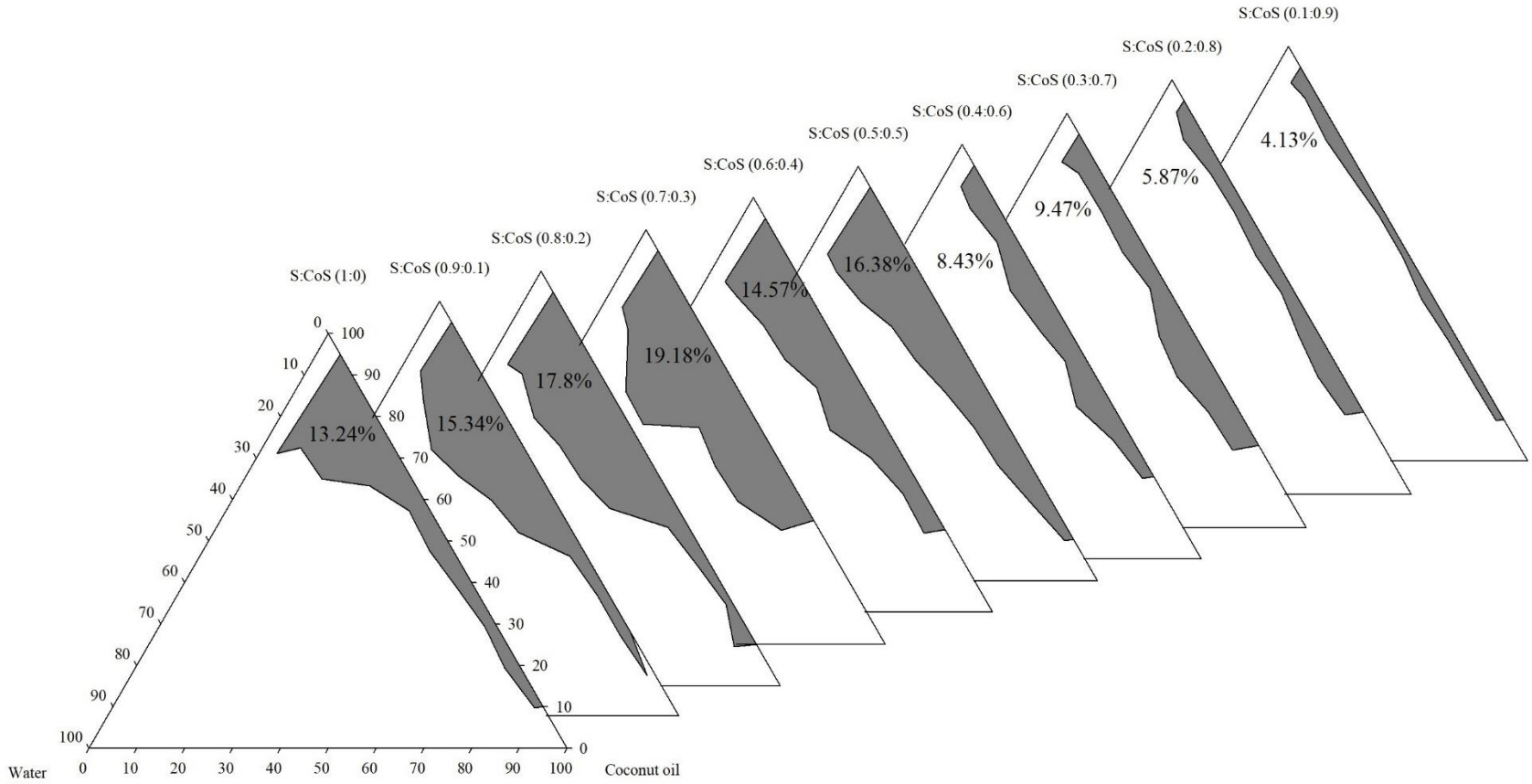


Figure 9. The pseudoternary phase diagram of Tween 80:Span 80:virgin coconut oil:water systems at different S:CoS ratios, the shaded area indicated microemulsion region with its size.

The sizes of ME regions prepared from both oils tended to decrease when S:CoS ratios decreased. The percentage of microemulsion region tended to decrease with the lesser ratio of Tween 80 in S_{mix}. It might be explained that inadequate quantity of surfactant or unsuitable S:CoS ratio in the systems could not provide a strong interfacial film between aqueous and oil phases. A prior study with 4 types of various oils and surfactants combinations by ternary phase diagram revealed that surfactants having opposite characters, i.e., hydrophilic and hydrophobic, could work consistently to reduce interfacial tension (Syed and Peh 2014).

Virgin coconut oil could form MEs since its medium chain fatty acids could easily penetrate the interfacial film (Roohinejad et al. 2015). Although olive oil contained high amount of oleic acid, a long chain fatty acid, it could form MEs due to the structural similarity between its fatty acid chain and oleate hydrophobic tails of Tween 80 as well as Span 80 (Mahdi et al. 2011). Both composition of S_{mix} and fatty acids of the oils affected the ME formation.

3. Characteristics of blank microemulsions

3.1. Preparation of blank microemulsions

Among the different S_{mix}, S:CoS ratios of 0.7:0.3 and 0.6:0.4 were selected and one identical point within the microemulsion boundary was chosen to prepare microemulsions as illustrated in Figures 10 and 11. Their compositions were described in Table 5. The larger amount of Tween 80 in S_{mix}, the better solubilization of ME according to the pseudoternary phase diagrams. Even though S:CoS (1:0 and 0.9:0.1) could form microemulsions, the composition between surfactant and cosurfactant were quite unbalanced. An appropriate pair with sufficient quantity of surfactants and cosurfactant is important to maintain a long-term stability (Courtney 1997; Syed and Peh 2014). In fact, Tween 80:Span 80 (0.5:0.5) had been previously studied in the previous nicotinamide loaded microemulsions (Boonme et al. 2012; Boonme et al. 2016) and clotrimazole microemulsion-based gel (Kaewbanjong et al. 2017) . Therefore, the phase behavior and stability of ME system with S:CoS ratios of 0.7:0.3 and 0.6:0.4 were interested to be investigated.

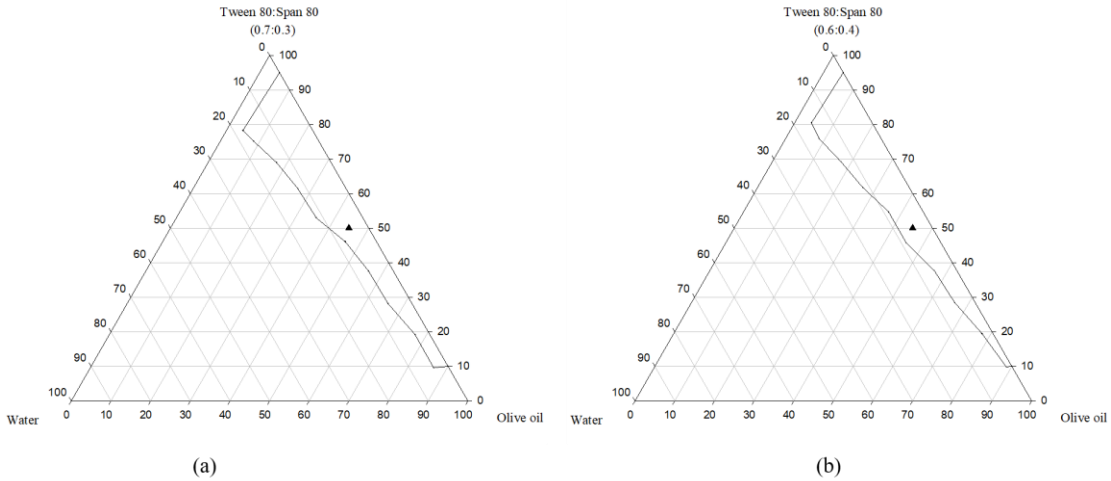


Figure 10. The pseudoternary phase diagram of Tween 80:Span 80:olive oil:water systems (a) S:CoS (0.7:0.3) (b) S:CoS (0.6:0.4), the point represented the selected formulation.

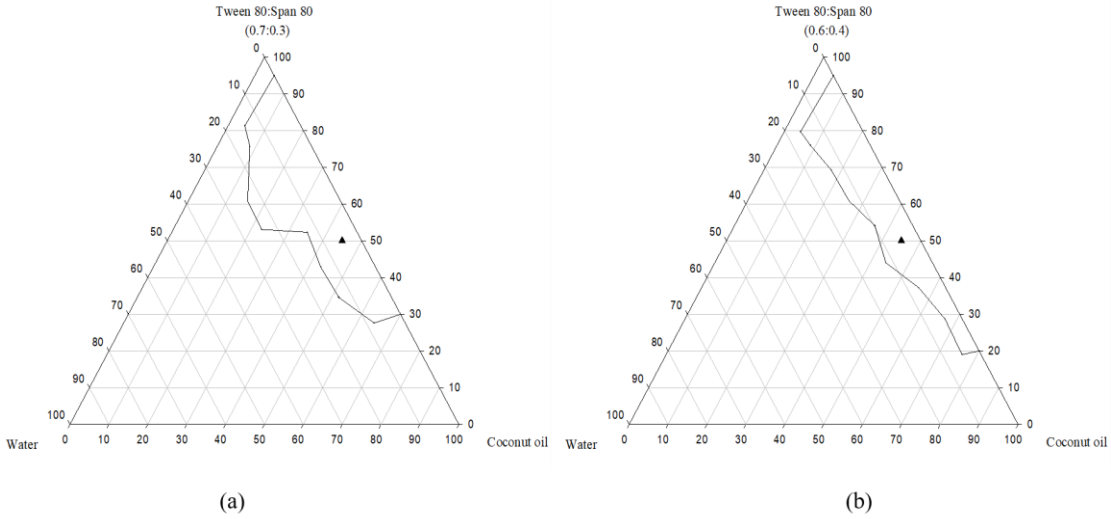


Figure 11. The pseudoternary phase diagram of Tween 80:Span 80:virgin coconut oil:water systems (a) S:CoS (0.7:0.3) (b) S:CoS (0.6:0.4), the point represented the selected formulation.

Table 5. Composition of olive and virgin coconut oil microemulsions.

	Composition				
Formulation	S:CoS ratio	Oil	S:CoS (% w/w)	Oil (% w/w)	Water (% w/w)
MEO1	0.6:0.4	Olive	50	45	5
MEO2	0.7:0.3	Olive	50	45	5
MEC1	0.6:0.4	Virgin Coconut	50	45	5
MEC2	0.7:0.3	Virgin Coconut	50	45	5

3.2. Appearance of blank microemulsions

After preparing the blank microemulsions, they were equilibrated at room temperature for 24 h and checked for visual apperance as shown in Figure 12.

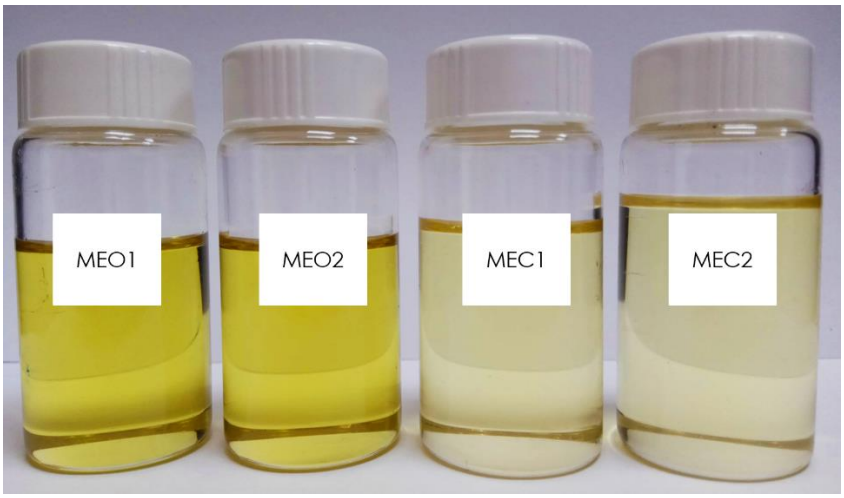


Figure 12. Visual appearance of freshly prepared blank MEs.

Under polarized light microscope as displayed in Figure 13, all olive and virgin coconut oil microemulsions showed no birefringence. Microemulsions are isotropic nature as they do not refract the polarized light (Boonme et al. 2016). All the formulations were determined as microemulsions.

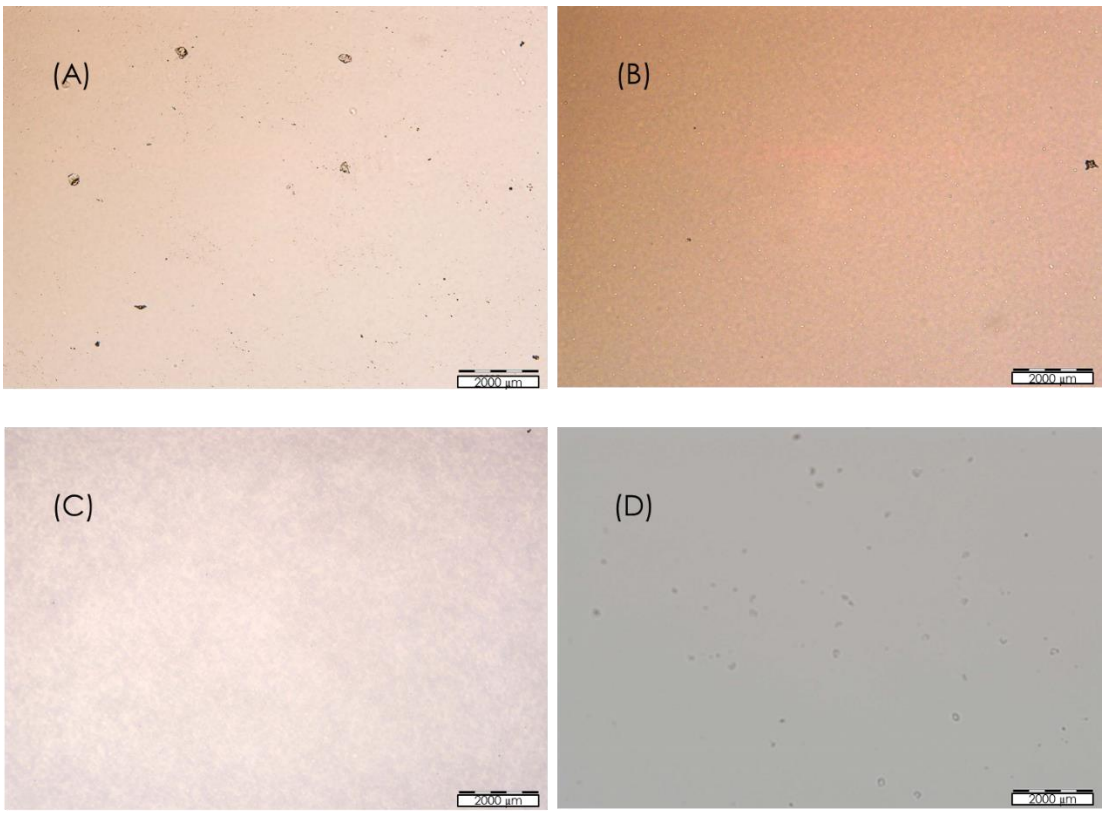


Figure 13. Appearance under polarized light microscopy (magnification x10) of (A) MEO1 (B) MEO2 (C) MEC1 and (D) MEC2.

3.3. Type of microemulsions

The external phase of all studied microemulsions was immiscible well with water-soluble brilliant blue aqueous solution but miscible with olive oil or virgin coconut oil. Therefore, they were visually identified as (w/o) microemulsions as depicted in Figures 14 and 15. Conductivity values of all microemulsions were low as described in Table 6 confirming for w/o microemulsions.

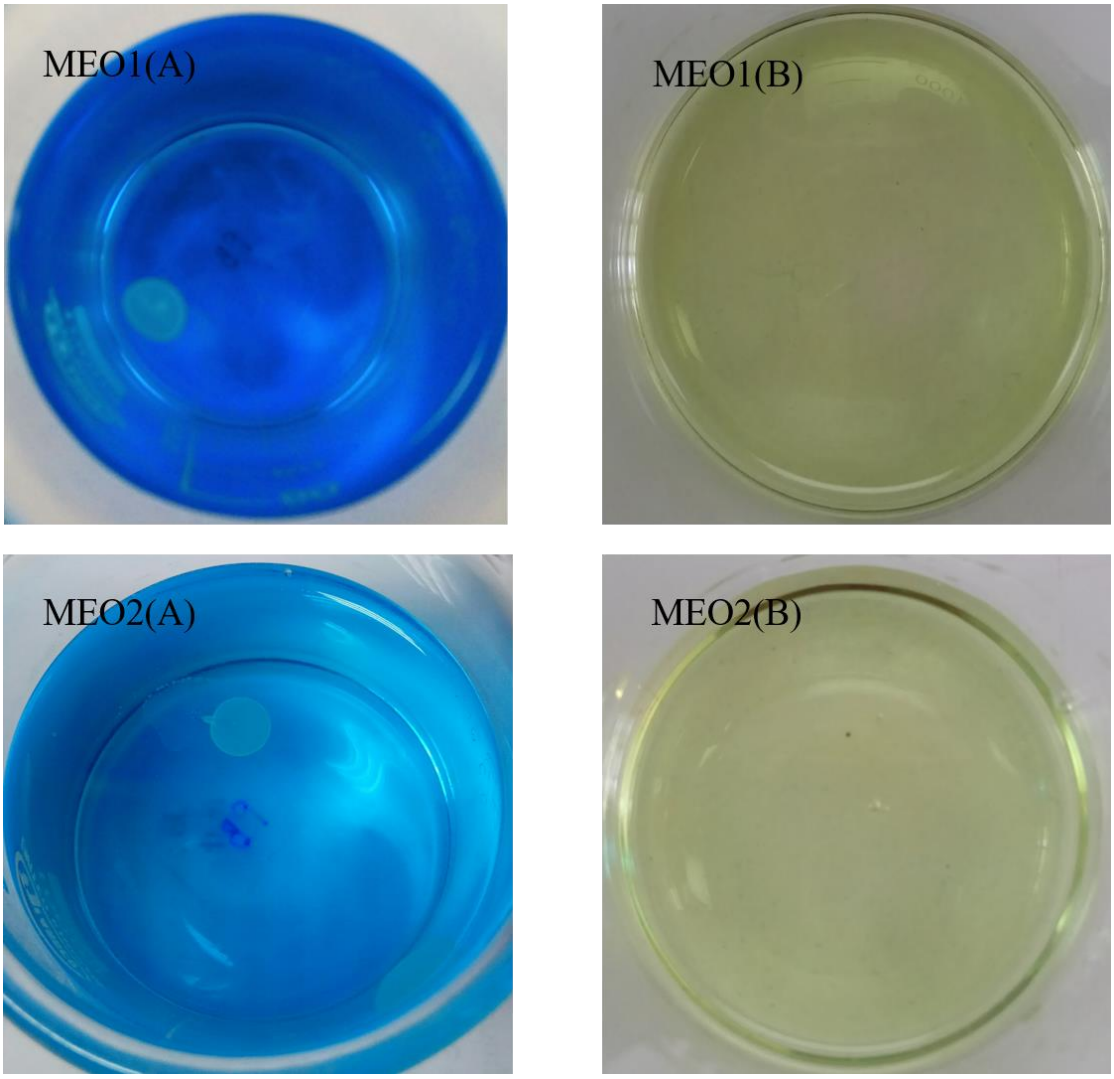


Figure 14. Drop dilution test of MEO1 and MEO2 with (A) brilliant blue aqueous solution and (B) olive oil.

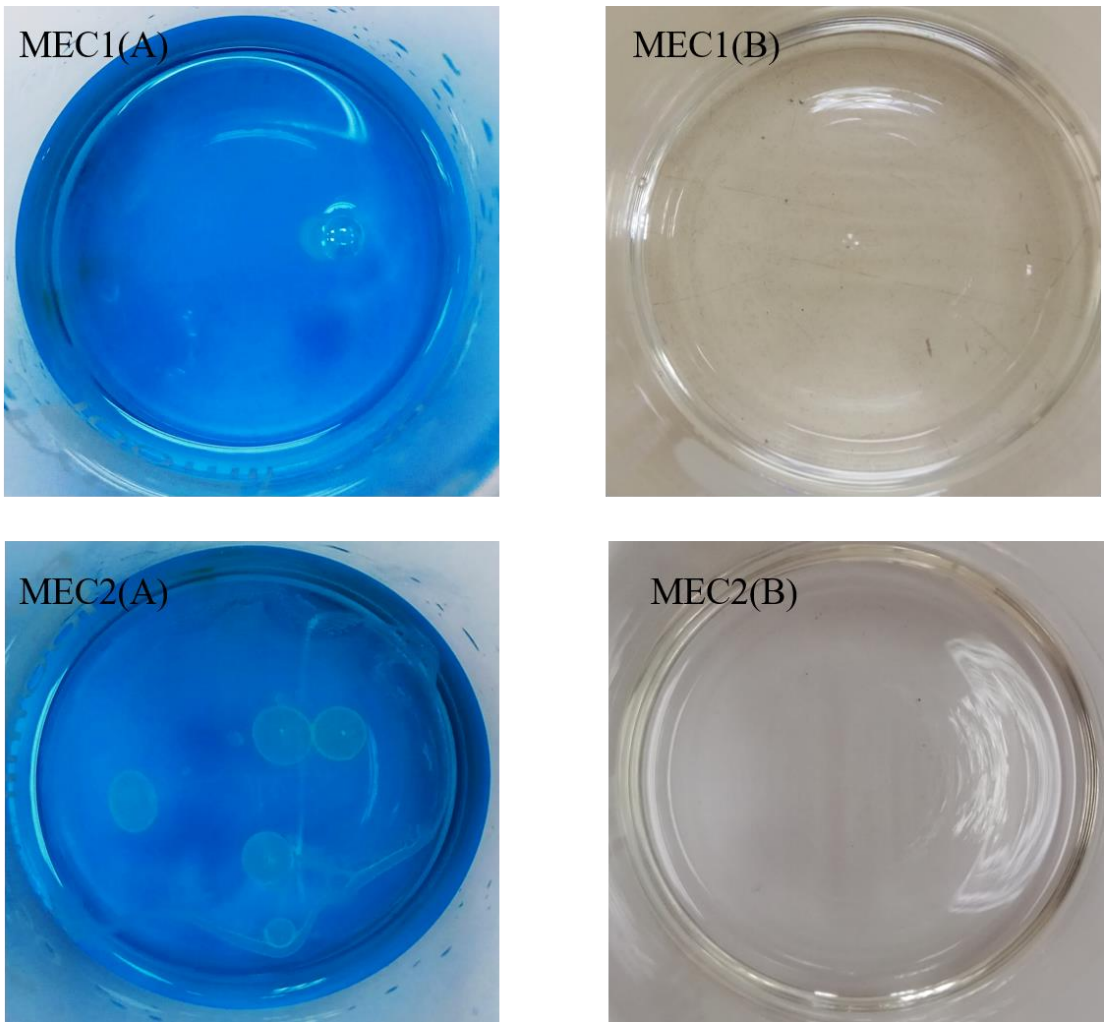


Figure 15. Drop dilution test of MEC1 and MEC2 with (A) brilliant blue aqueous solution and (B) virgin coconut oil.

Table 6. Conductivity of blank microemulsions (mean ± SD, n=3).

Formulation	Conductivity (μS/cm)
MEO1	0.93 ± 0.01
MEO2	1.67 ± 0.03
MEC1	0.71 ± 0.01
MEC2	1.56 ± 0.02

Microemulsion compositions can modify the microenvironment of the surfactants and subsequently affect geometric packing of surfactant molecules

(Lawrence and Rees 2012). Water content at minimum water-to-lipid ratios could form reverse microstructure (Lehtinen et al. 2017). In this study, the amount of oil phase was 45% w/w while that of aqueous phase was only 5% w/w. Hence, the aqueous phase was not enough to behave as continuous phase and could not significantly hydrate the head groups of surfactants, resulting in small optimal head group area (Mitchell and Ninham 1981; Clint 1992). Additionally, greater oil penetration between hydrophobic tails containing unsaturated *cis* double bond carbon chains of Tween 80 and Span 80 leads to large volume of hydrophobic portion. This phenomenon caused negative curvature of the interfacial film, resulting in w/o type (Mitchell and Ninham 1981).

3.4. pH property

Determination of pH was collected in Table 7 and the pH range was compatible to the skin (Songkro et al. 2014).

Table 7. pH values of blank microemulsions (mean ± SD, n=3).

Formulation	pH
MEO1	7.37 ± 0.01
MEO2	7.38 ± 0.01
MEC1	7.43 ± 0.01
MEC2	7.63 ± 0.01

3.5. Viscosity and flow property

In the rheological studies, all formulations behaved Newtonian flow with constant viscosity values under different shear rates. The formulation with S:CoS ratio 0.6:0.4 has lower viscosity than 0.7:0.3 ratio for both olive and virgin coconut oil microemulsions. It seemed that increasing the amount of Tween 80 could increase the viscosity of microemulsion, this agreed with another report (Roohinejad et al. 2015). Microemulsion exhibits Newtonian flow with constant viscosity values under different

shear rate (Lawrence and Rees 2012). The rheogram in Figures 16 described these formulations behaved Newtonian flow.

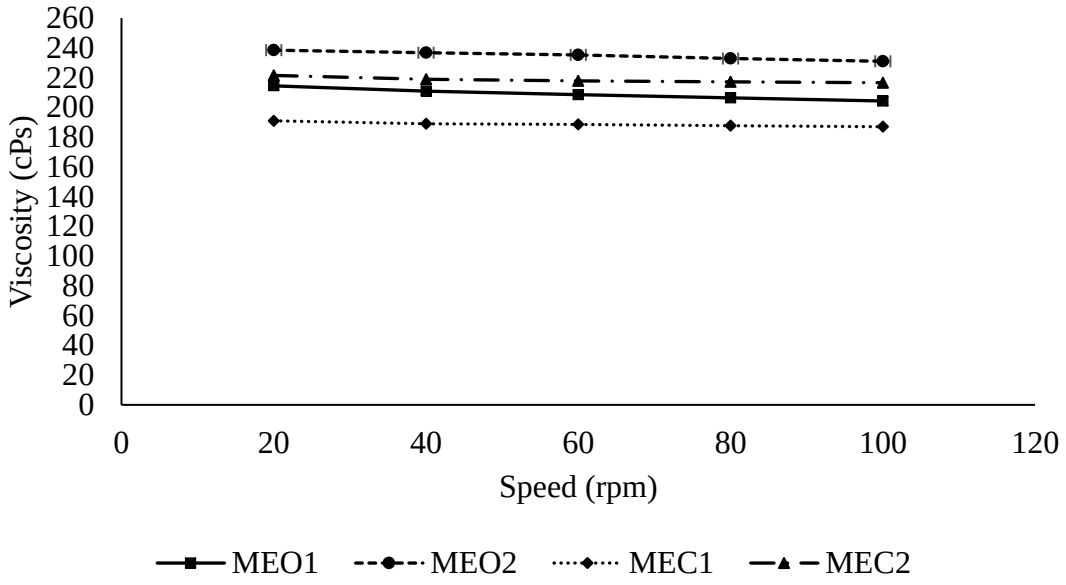


Figure 16. Rheogram of blank microemulsions, each point represents mean ± SD, n=3.

3.6. Droplet size, polydispersity index (PI) and zeta potential

According to Table 8, microemulsions had tiny droplets. MEO1 and MEO2 were monodispersed size distribution while MEC1 and MEC2 were polydispersed ones. Figure 17 displayed bimodal size distribution of MEC2.

Table 8. Droplet size (Z-Ave), polydispersity index (PI) and zeta potential of blank microemulsions (mean ± SD, n=3).

Formulation	Z-Ave (nm)	PI	Zeta potential(mV)
MEO1	27.26 ± 3.04	0.14 ± 0.09	0.20 ± 0.19
MEO2	31.13 ± 1.06	0.27 ± 0.06	0.15 ± 0.41
MEC1	34.30 ± 2.11	1.00 ± 0.00	0.26 ± 0.29
MEC2	25.30 ± 5.85	0.99 ± 0.00	0.01 ± 0.49

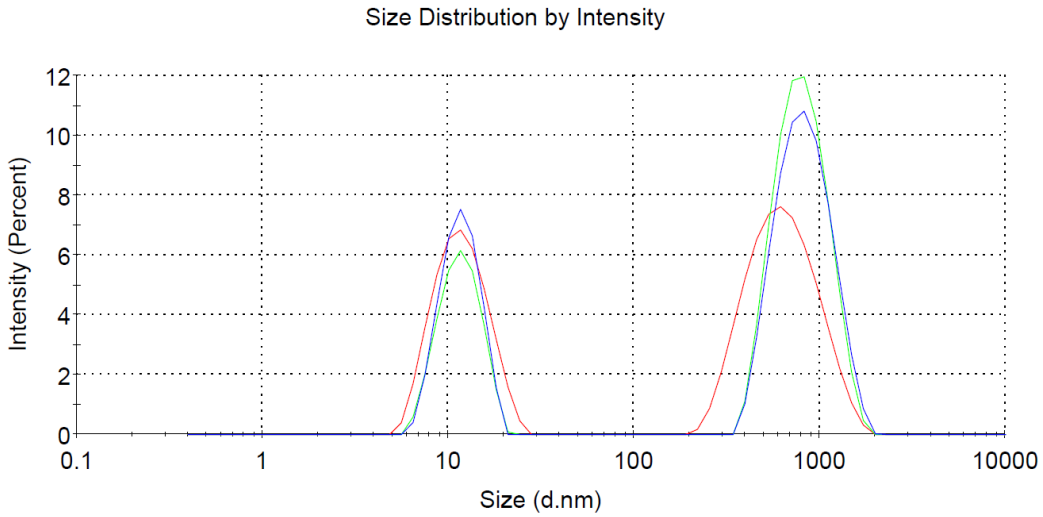


Figure 17. The bimodal size distribution of MEC2.

Generally, microemulsions do not contain uniform sizes and the system is dynamic nature equilibrated by the rate of adsorption and desorption of surfactant molecules at the interface. The rate of surfactant orientation is very rapidly changed and therefore the particle size distribution is continuous (Clint 1992; Khan et al.2016). The droplet radii of w/o type using nonionic surfactants mainly depend on the geometry packing of surfactants (Clint 1992; Koneva et al. 2017). The rate of surfactant orientation can be varied by temperature, pressure, surfactants ratio and oil type (Clint 1992; Schelly 1997; Djekic et al. 2012).

Dispersity of such ME system can be wide-ranging due to temperature, pressure, water to surfactants ratio and oil type (Ricka et al. 1991; Djekic et al. 2012) and influenced by diluent, dilution factor and storage (Constantinides and Yiv 1995). In a research of microemulsion with isopropyl myristate, polyoxyethylene (4) lauryl ether (Brij-30), isopropyl alcohol and water, high PI value was observed when the volume fraction of oil phase increased, and water phase decreased (Acharya et al. 2001). Fluctuation in particle size, PI and zeta potential were observed when the composition in both blank and beta carotene MEs varied (Roohinejad et al. 2015). Different refractive indices of surfactants and the penetration of oil chain length by surfactant layer could affect the data of DLS. Furthermore, the accuracy of size distribution from

DLS is limited by the different refractive indices of surfactants and the penetration of oil chain length to surfactant layer which in term alter the optical matching point (OMP) from the scattering intensity (Ricka et al. 1991; Khan et al.2016). The technical artifacts can result heterogenous PI value since concentrated MEs were analyzed at fixed scattered angle 90° in this research.

Since nonionic surfactants could not provide electrostatic repulsion, zeta potential value was low as displayed in Table 8. In the previous study, the surface charges of ME formulated with a mixture of nonionic surfactants Labrasol and Tween 20 were closed to neutral (Kim et al. 2018). In this aspect, the steric barrier of polyoxyethylene groups in Tween 80 stabilized the ME system.

3.7. Physical stability study

After characterizing the blank MEs, accelerating stability test was done to check physical properties. All blank MEs showed no turbidity, phase separation and color changes. Therefore, these MEs were continued to prepare nicotinamide-loaded microemulsions.

4. Characteristics of nicotinamide-loaded microemulsions

The nicotinamide-loaded microemulsions were characterized and summarized in Table 9. Determined pH range were compatible to the skin. Measurement by zetasizer resulted nano-size droplets with fluctuated size distribution. Zeta potential was almost neutral. Drop dilution test showed in Figures 18 and 19 confirmed that nicotinamide-loaded MEs were w/o type. No birefringence was observed under polarized light microscope as illustrated in Figure 20. TEM photograph in Figure 21 demonstrated the nano-size spherical globules in MEC2-N. Rheological property in Figure 22 showed Newtonian flow. The resulting data presented that incorporation of nicotinamide into the blank MEs did not affect ME type.

Table 9. Characteristics of nicotinamide-loaded microemulsions (mean ± SD, n=3).

Formulation	Conductivity (μS/cm)	pH	Z-Ave (nm)	PI	Zeta potential(mV)
MEO1-N	1.00 ± 0.08	7.31 ± 0.04	27.59 ± 6.04	1.00 ± 0.00	0.01 ± 0.49
MEO2-N	0.94 ± 0.20	7.51 ± 0.01	78.54 ± 41.47	1.00 ± 0.00	- 0.04 ± 0.22
MEC1-N	0.93 ± 0.01	7.49 ± 0.01	34.30 ± 2.11	0.29 ± 0.07	- 0.03 ± 0.46
MEC2-N	1.92 ± 0.01	7.64 ± 0.01	37.87 ± 1.63	0.34 ± 0.04	0.07 ± 0.18

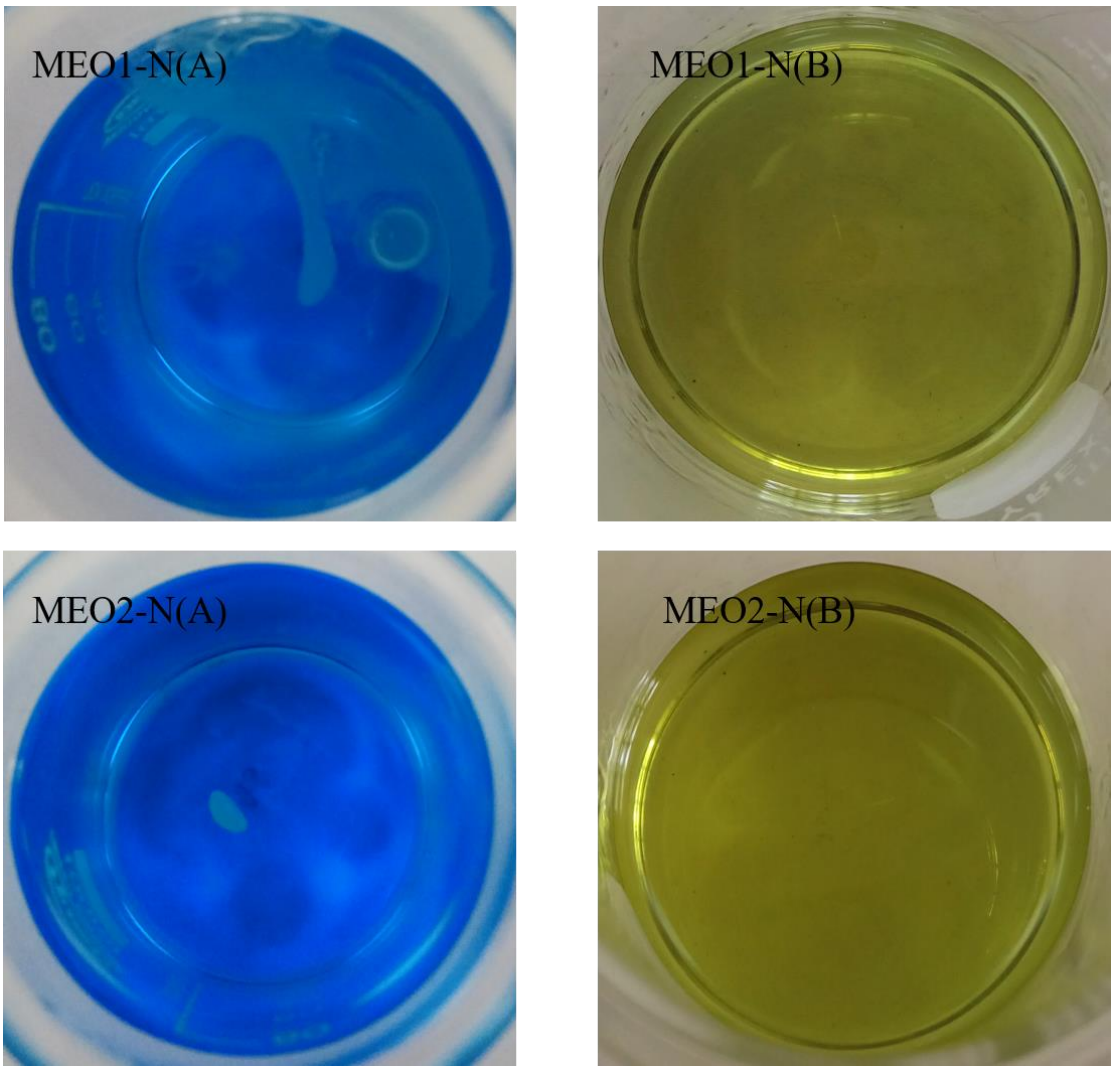


Figure 18. Drop dilution test of MEO1-N and MEO2-N with (A) brilliant blue aqueous solution and (B) olive oil.

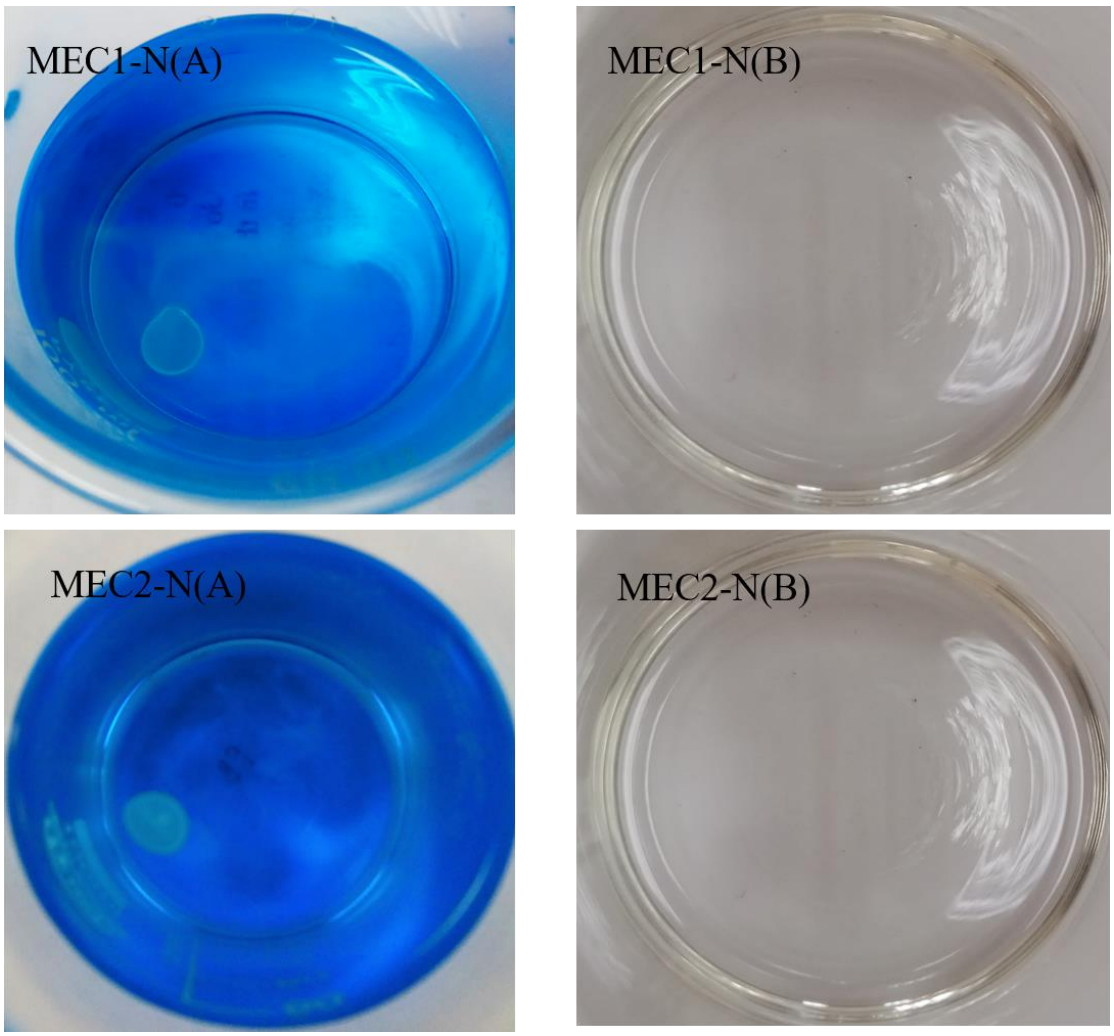


Figure 19. Drop dilution test of MEC1-N and MEC2-N with (A) brilliant blue aqueous solution and (B) virgin coconut oil.

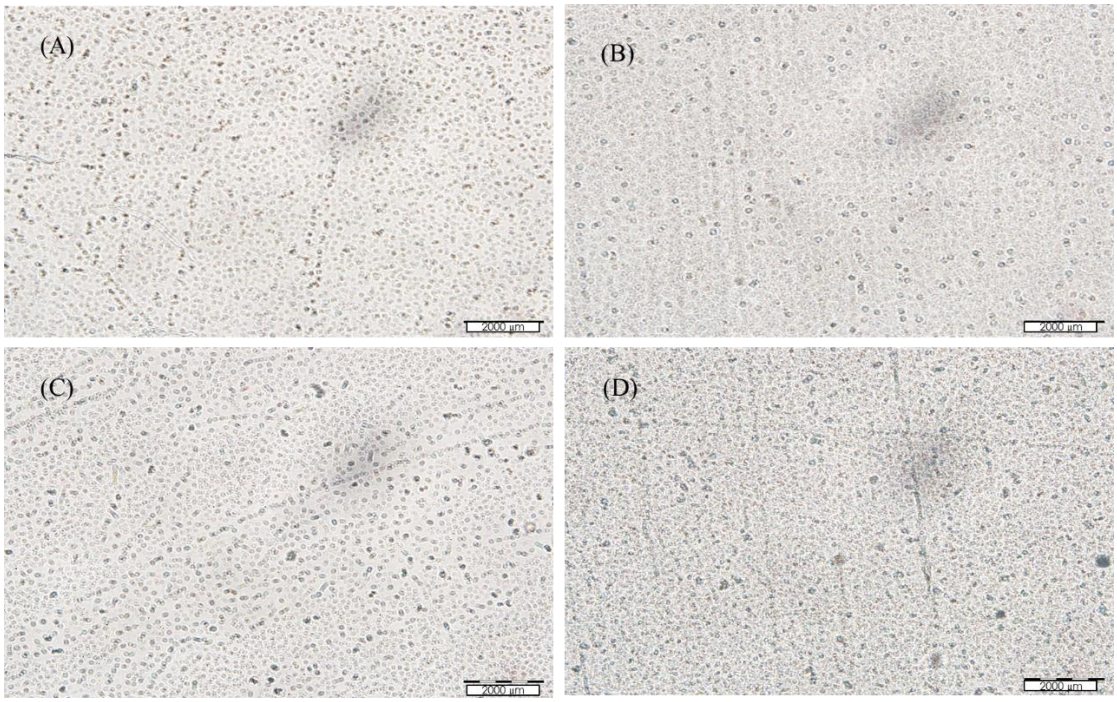


Figure 20. Appearance under polarized light microscopy (magnification x10) of (A) MEO1-N (B) MEO2-N (C) MEC1-N and (D) MEC2-N.

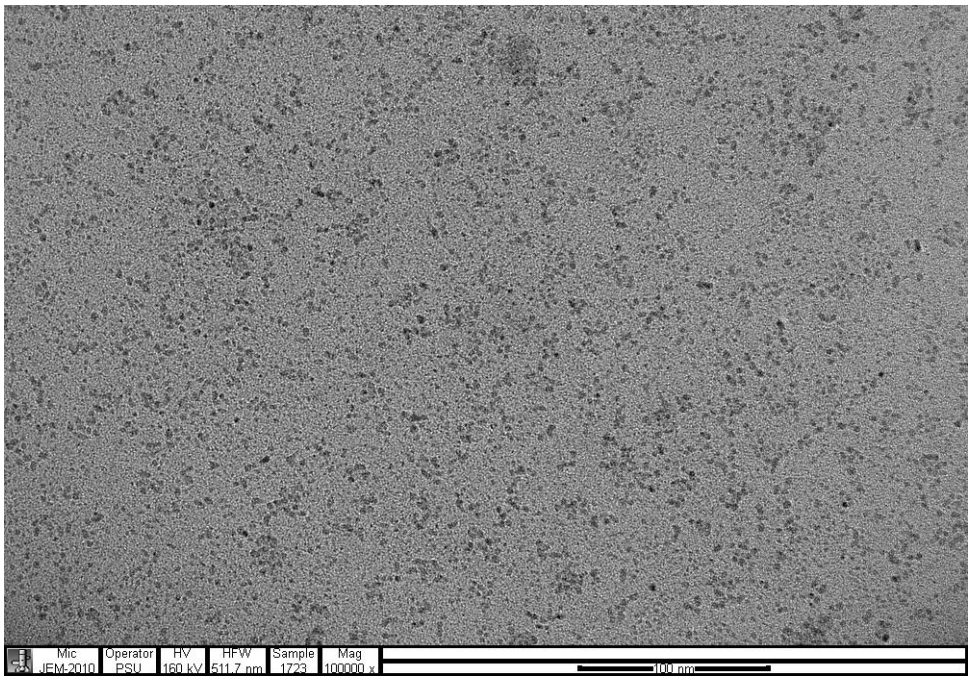


Figure 21. TEM photograph of MEC2-N at magnification of x100,000.

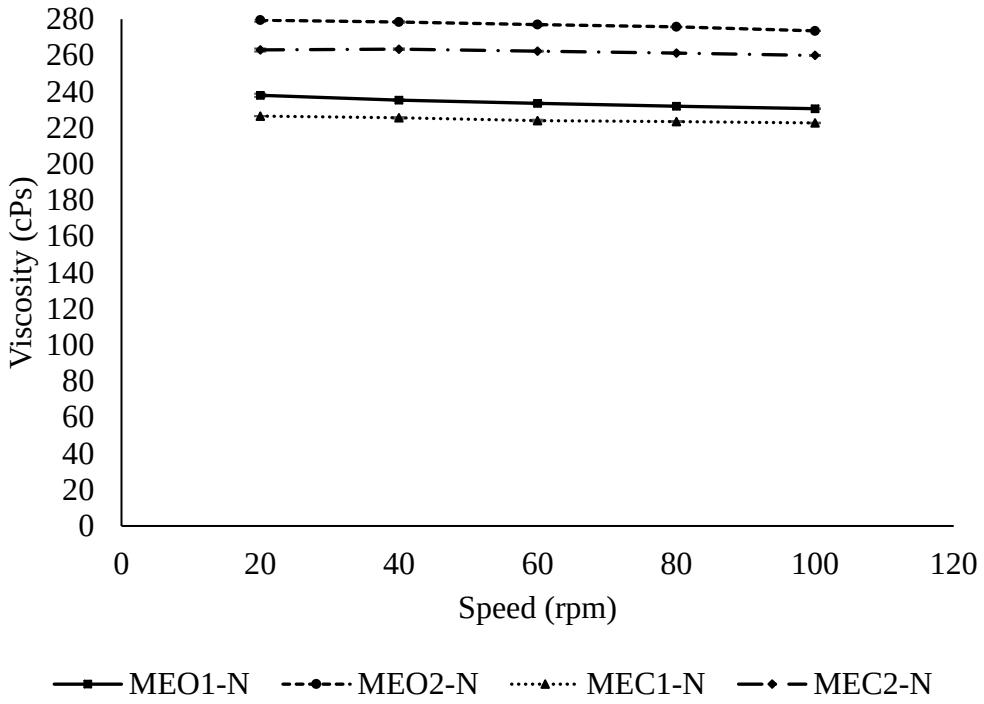


Figure 22. Rheogram of nicotinamide-loaded microemulsions, each point represents mean ± SD, n=3.

5. Stability of nicotinamide-loaded microemulsions

Nicotinamide-loaded MEs kept at different temperatures were visually observed for clarity, phase separation and color changes compared with their counterparts every month. Table 10 and Figure 23 exhibited the visual appearance after 3 months of storage at various temperatures. Color of olive oil microemulsions kept at 45°C changed from yellowish to brownish while that of virgin coconut oil microemulsion changed from light yellow to dark yellow. Those at 4°C and room temperature did not exhibit discoloration. This color darkening was due to oxidation of oil phase. Similarities were found in previous report of nicotinamide-microemulsion-based gel containing soybean oil stored at 60°C (Boonme et al. 2012), nicotinamide microemulsion containing isopropyl palmitate at 45°C (Boonme et al. 2016) and benzophenone-3 microemulsion containing Eutanol G (Songkro et al. 2014).

Table 10. Physical appearance of microemulsions after 3 months of storage at various temperatures, n = 3 (n = number of samples).

Formulation	Physical Appearance		
	4°C	Room Temp (28 ± 2°C)	45°C
MEO1	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.
MEO1-N	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.
MEO2	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.
MEO2-N	Phase separation.	Phase separation.	Phase separation.
MEC1	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.
MEC1-N	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.
MEC2	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.
MEC2-N	Clear. No color changes.	Clear. No color changes.	Clear. Darkening color.

(A)

(B)

(C)

Figure 23. Physical appearance of (A) MEO1-N (B) MEC1-N and (C) MEC2-N at initial and after storage of 3 months at 4°C, room temperature (28 ± 2°C) and 45°C.

Phase separation was observed in MEO2-N at all temperatures in the first month as presented in Figure 24. Selection of surfactants required lipophilic nature of low HLB (i.e. < 10) when hydrophilic active was loaded in lipophilic continuous phase (Comelles and Trullas 1997). HLB value of S_{mix} of 0.7:0.3 was 11.79 while the required HLB of olive oil was 7 and HLB gap was large compared to S_{mix} of 0.6:0.4. The choice of surfactants pair could tailor the phase behaviour which reflected the stability (Americas 1984).

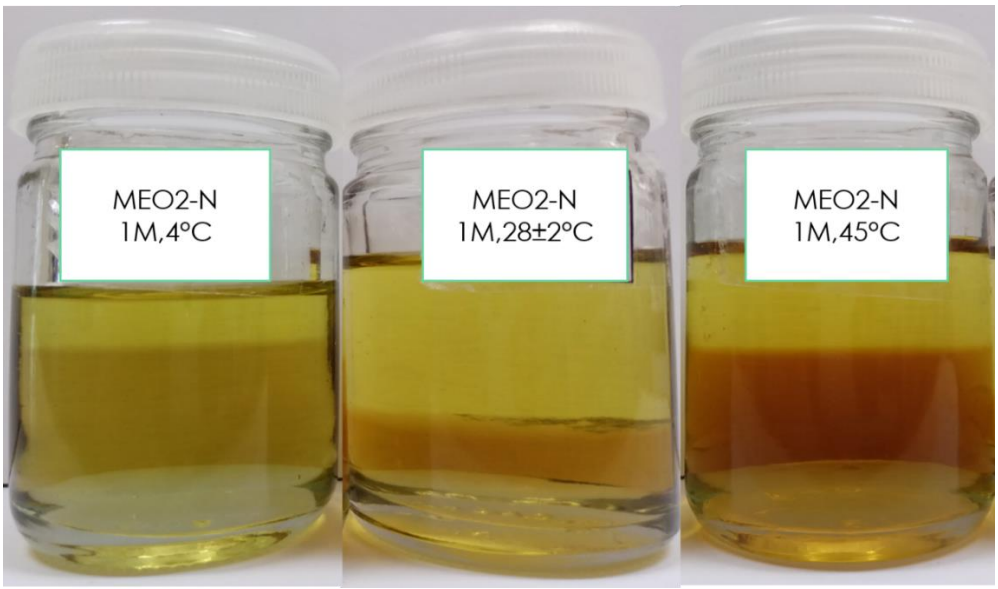
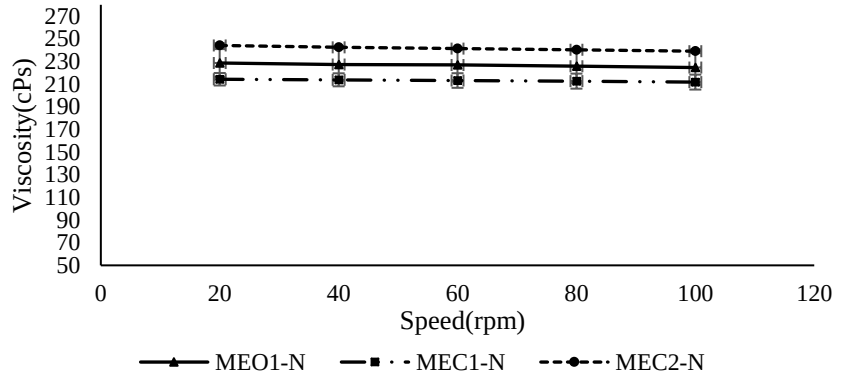


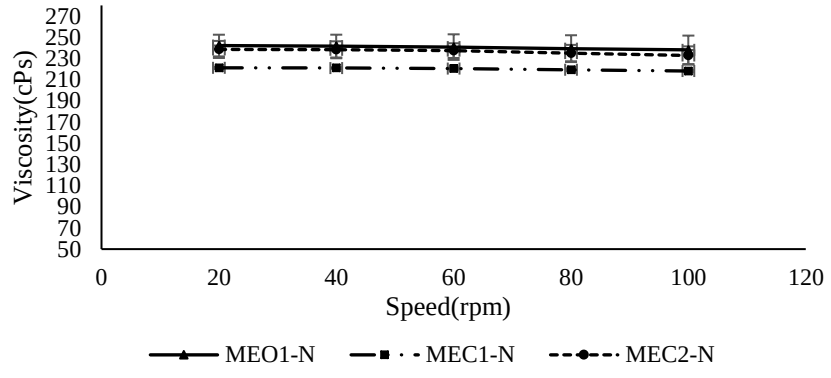
Figure 24. Physical appearance of MEO2-N at the first month stored at 4°C, room temperature (28 ± 2°C) and 45°C.

Nicotinamide is hydrophilic agent and it can attract water molecules through hydrogen bond. In MEO2-N, it probably disturbed the water molecules solvating polyoxyethylene groups and led to the configuration loss and increased entropy. The changed in entropy could approach phase separation (Clint 1992; Lindman et al. 2016). Previous studies reported that diluting a self-assembly system with polar phase could change the association structure (Comelles et al. 1992). The instability happened in MEO2-N might be affected by larger gap between required HLB and total HLB value and the weight fraction of polar phase.

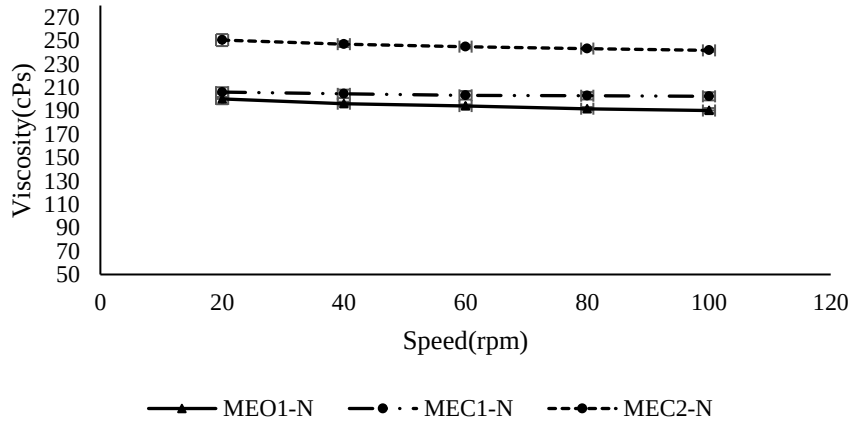
The rheological studies of all formulations behaved Newtonian flow as shown in Figure 25 and the viscosity values were not significantly changed ($p > 0.05$, one way ANOVA).



(A)



(B)



(C)

Figure 25. Rheograms of MEO1-N, MEC1-N and MEC2-N after storage of 3 months at (A) 4°C (B) room temperature (28 ± 2°C) and (C) 45°C, each point represents mean ± SD, n=3.

The percentage of nicotinamide remaining in microemulsions was analysed by HPLC and summarized in Table 11. It was observed that more than 90% of active ingredient was detected at 4°C and room temperature (28 ± 2°C).

Table 11. Active remaining in studied microemulsions after 3 months of storage at 4°C, room temperature (RT, 28 ± 2°C) and 45°C.

Formulation	Temperature	% w/w of active remaining	% of active remaining
MEO1-N	4°C	2.83 ± 0.33	94.42 ± 1.71
	RT	2.75 ± 0.04	91.59 ± 1.36
	45°C	2.69 ± 0.06	89.49 ± 2.11
MEC1-N	4°C	2.70 ± 0.04	90.10 ± 1.31
	RT	2.80 ± 0.07	93.44 ± 2.40
	45°C	2.69 ± 0.01	88.22 ± 0.41
MEC2-N	4°C	2.75 ± 0.08	91.53 ± 2.50
	RT	2.72 ± 0.06	90.76 ± 1.89
	45°C	2.71 ± 0.02	90.46 ± 0.60

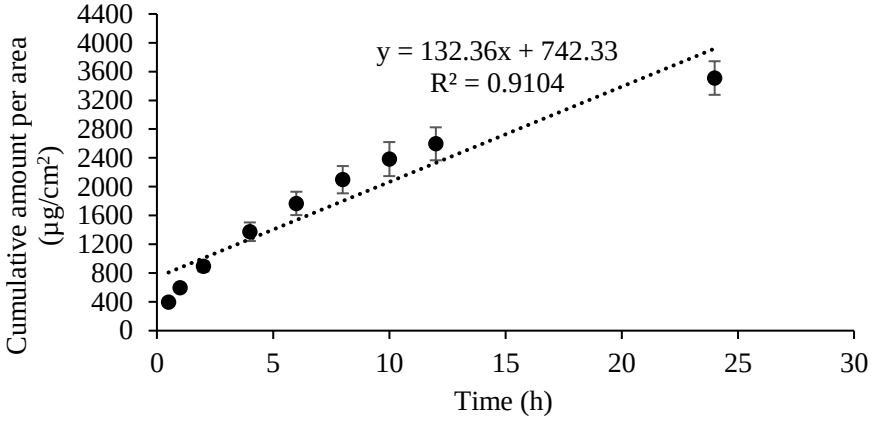
The active remaining in MEO1-N and MEC1-N kept at 45°C was less than 90% of initial concentration which could be impacted by the oxidation of oil. The presence of oxygen, degree of unsaturation of fatty acids and temperature of storage can accelerate autoxidation of oil. Oxidation products such as peroxide and aldehdyde can be formed because of the oxidation reaction (deMan 2013). The aging effect of oleic acid and linoleic acid acids in in soybean lecithin system caused phase transition and migration in the system (Godoy et al. 2015). The concentration of active declined significantly after storage at 40 ± 2°C for two months in quercetin loaded w/o ME (Vicentini et al. 2011).

Factors affecting the stability of active ingredient required to consider in ME system. The structure of nicotinamide contains pyridine nitrogen which can undergo reversible oxidation or reduction (Combs Jr and McClung 2016). It could be assumed that the chemical reaction between the by-products of oil phase and nicotinamide might reduce the active remaining in MEO1-N and MEC1-N kept at 45°C.

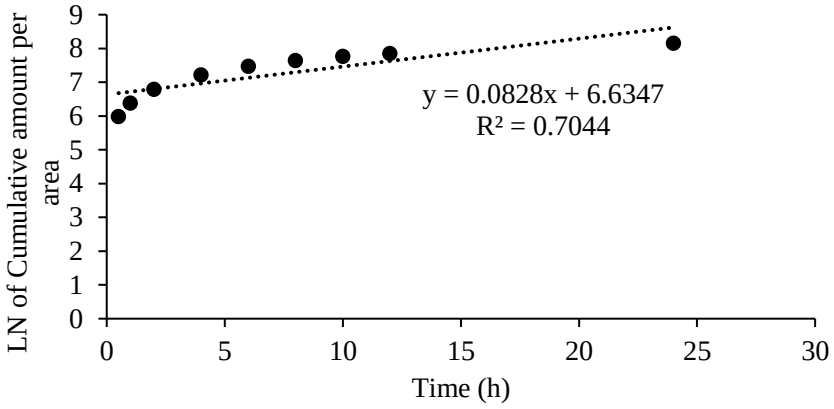
6. *In vitro* release profiles and kinetics of nicotinamide-loaded microemulsions

Among the stable formulations, MEO1-N and MEC1-N kept at room temperature were selected to study *in vitro* release behaviour compared with 3% w/w nicotinamide aqueous solution (NCT sol). These two formulations had the same S_{mix} and their active remaining was more than 90% after three months storage. In addition, MEO1-N and MEC1-N were freshly prepared to study *in vitro* release behaviour. Amount of released nicotinamide was analysed by HPLC method from the withdrawal receptor fluid taken at determined time interval.

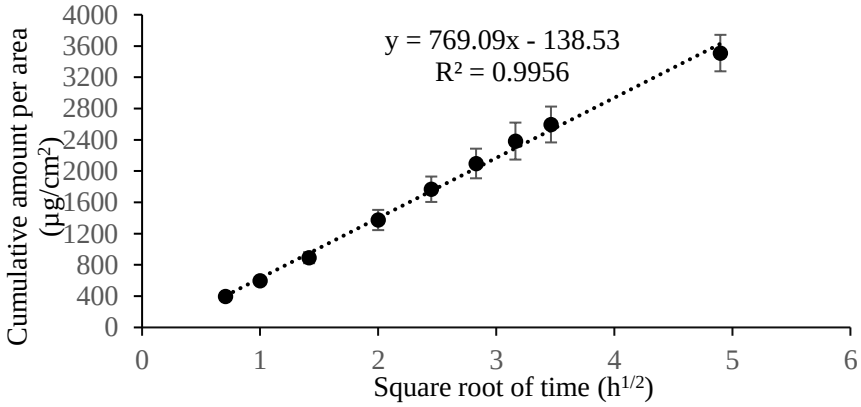
Analysis of data exhibited that the release kinetics of all studied formulations were best fitted to Higuchi model as the coefficient of determinations were close to 1 as demonstrated in Figures 26-29. The release of nicotinamide from microemulsions was depended on diffusion mechanism and controlled by the formulation itself (Siepmann and Peppas 2011). The studied MEs provided the sustained release of nicotinamide from formulations. The attained coefficient of determinations was summarized in Table 12.



(A)

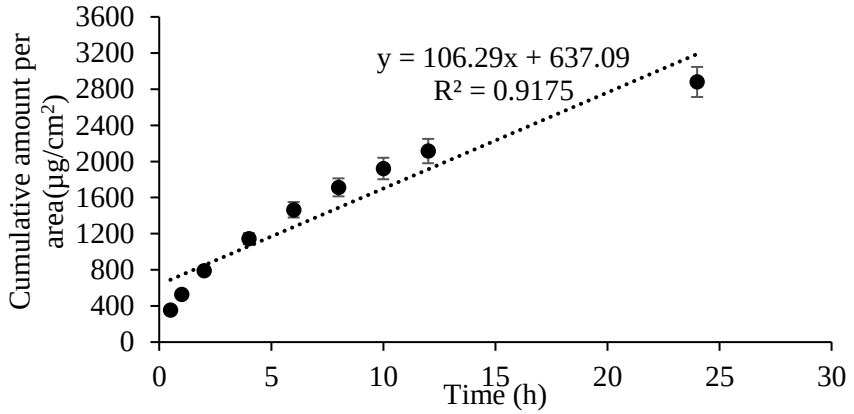


(B)

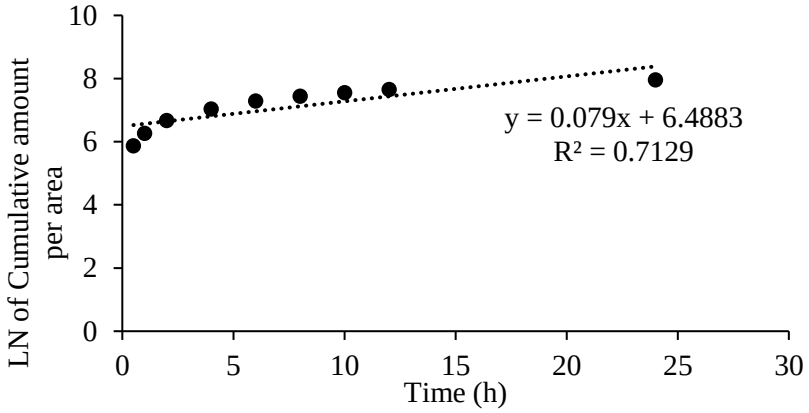


(C)

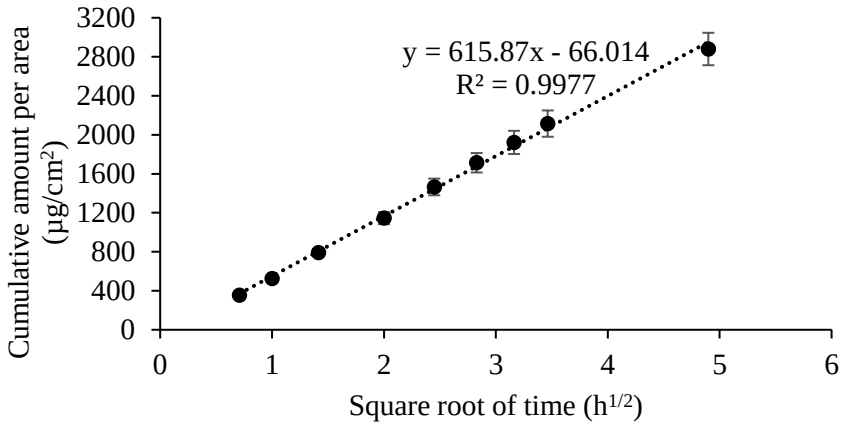
Figure 26. *In vitro* release profiles of nicotinamide from freshly prepared MEO1-N with different mathematical models: Zero order (A), First order (B) and Higuchi (C). Each point represents mean ± SEM, n ≥ 3.



(A)

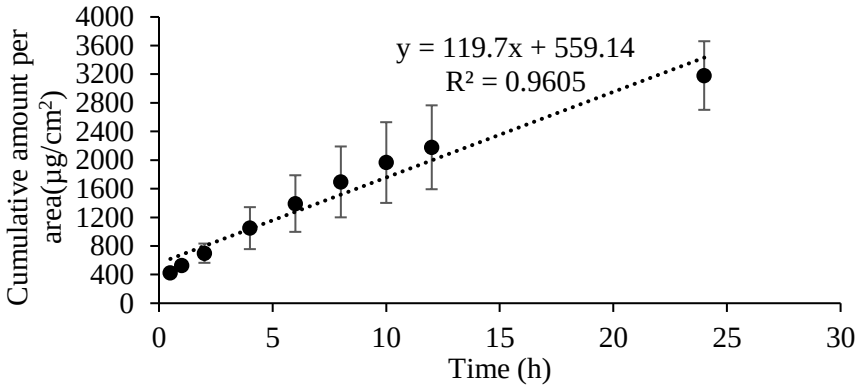


(B)

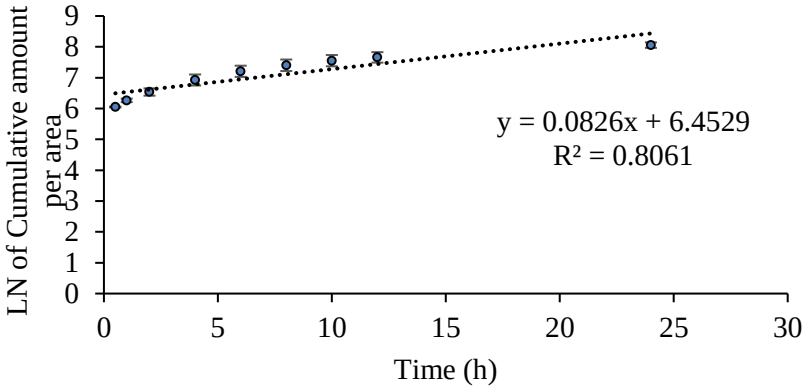


(C)

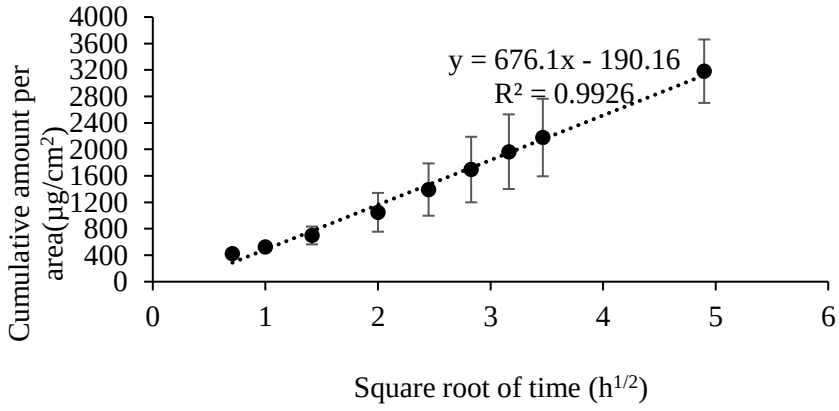
Figure 27. *In vitro* release profiles of nicotinamide from freshly prepared MEC1-N with different mathematical models: Zero order (A), First order (B) and Higuchi (C). Each point represents mean ± SEM, n ≥ 3.



(A)

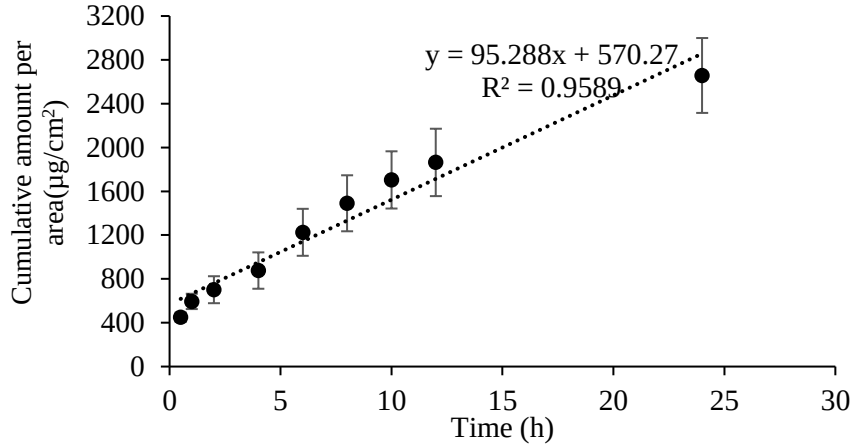


(B)

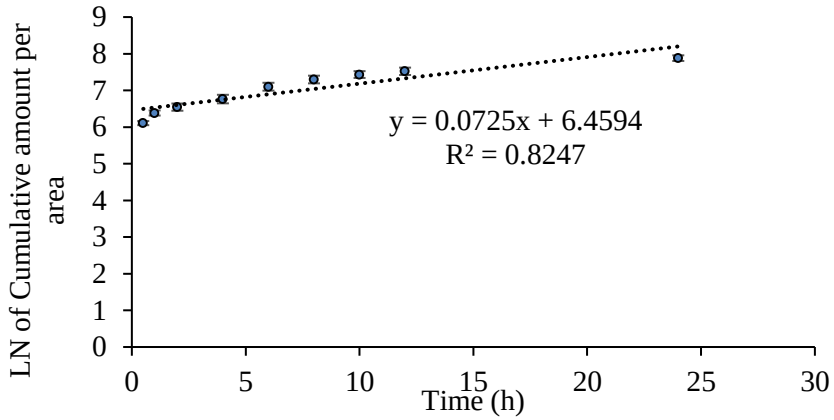


(C)

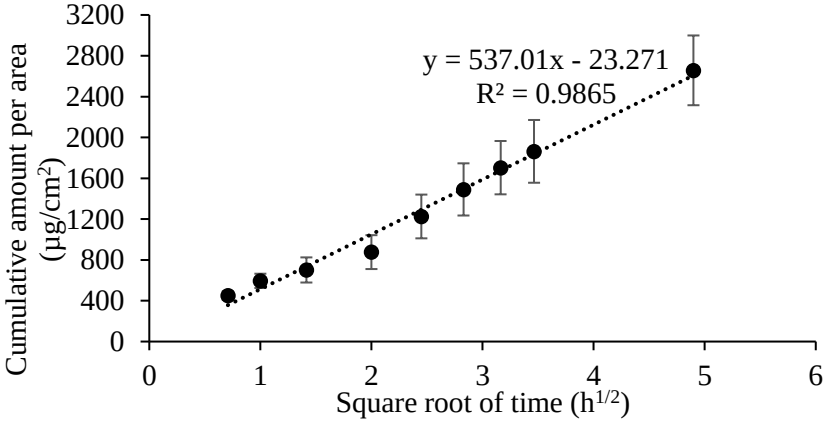
Figure 28. *In vitro* release profile of nicotinamide from 3 months storage at (28 ± 2°C) MEO1-N with different mathematical models: Zero order (A), First order (B) and Higuchi (C). Each point represents mean ± SEM, n ≥ 3.



(A)



(B)



(C)

Figure 29. *In vitro* release profile of nicotinamide from after 3 months storage at (28 ± 2°C) MEC1-N with different mathematical models: Zero order (A), First order (B) and Higuchi (C). Each point represents mean ± SEM, n ≥ 3.

Table 12. Coefficient of determination and release rate constants of kinetic models from the studied formulations. Data are shown in mean ± SEM, n ≥ 3.

Formulation	Zero order		First order		Higuchi order	
	r^2	k_o ($\mu g/cm^2/h$)	r^2	k_f ($ln(Q)/h^{-1}$)	r^2	k_h ($\mu g/cm^2/h^{1/2}$)
Freshly prepared						
MEO1-N	0.9104	132.36 ± 4.97	0.7044	0.08 ± 0.00	0.9956	769.09 ± 31.71
MEC1-N	0.9175	106.29 ± 6.94	0.7129	0.08 ± 0.00	0.9977	615.87 ± 41.04
NCT sol:	0.4630	82.28 ± 35.94	0.4005	0.02 ± 0.02	0.6965	560.73 ± 226.17
After 3 months (28 ± 2°C)						
MEO1-N	0.9605	119.70 ± 12.85	0.8061	0.08 ± 0.00	0.9926	676.10 ± 85.23
MEC1-N	0.9589	95.29 ± 7.56	0.8247	0.07 ± 0.00	0.9865	537.01 ± 45.98

k_o, k_f, k_h are release rate constants of zero order, first order and Higuchi kinetic models, respectively

The cumulative amount of nicotinamide released from NCT sol was notably higher than that from MEs because of the solution dosage form as shown in Figure 30. However, the release rate of NCT sol reached a plateau after 12 h. The studied MEs did not show either burst release or plateau.

The amount of released nicotinamide from both freshly prepared and three months storage of MEO1-N was that higher than of MEC1-N. Since MEO1-N and MEC1-N employed the same S_{mix}, the possible reason of variation in release rate may be due to different oil type. The studied formulations were w/o ME and hydrophilic nicotinamide was entrapped in internal aqueous phase. Olive oil has elongated fatty acid chain length, oleic acid and linoleic acid (C18) while lauric acid (C12) was found in virgin coconut oil. Unsaturated fatty acids have wide intermolecular area whereas saturated ones possess tight area and closely packed (Fameau et al. 2014). Prolong diffusion across the external oil phase lead to slower release rate. Thus, fatty acids in virgin coconut oil might delay the release of nicotinamide from ME.

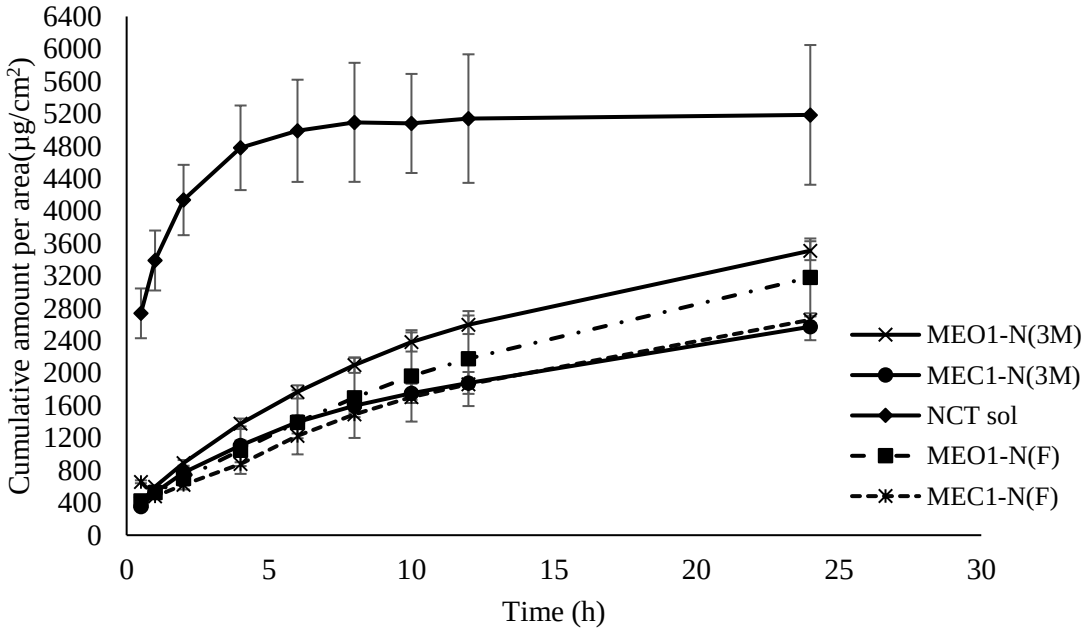


Figure 30. *In vitro* release profiles of nicotinamide from freshly prepared MEO1-N (F), MEC1-N (F), after 3 months of storage MEO1-N (3M), MEC1-N (3M) and NCT solution. Each point represents mean ± SEM, $n \geq 3$.

The cumulative amount of nicotinamide released from freshly prepared and after three months storage was significantly different ($p < 0.05$, *t*-test). This was due to the concentration of nicotinamide in stored MEs was lower than freshly prepared MEs. NCT sol could not provide sustained release and it was not suitable for delivery as the skin is hydrophobic in nature. This was agreed with previous *in vitro* release study of nicotinamide microemulsion-based gels (MBGs) and nicotinamide solution whereas MBGs followed the Higuchi model and the solution reached plateau (Boonme et al. 2012).

The *in vitro* release rate could be affected by several factors such as the structural transformation during contacted with receptor medium and geometry packing of surfactants at the interface film (Trotta 1999). Additionally, the microemulsion type, additives and the partitioning of active between the oil and water phase also influenced the diffusion rate (Jurkovič et al. 2003; Špiclin et al. 2003; Djekic et al. 2012; Panapisal et al. 2012).

The release amount of ascorbyl palmitate (AP) from w/o and o/w MEs compared with their thickener-added counterparts indicated that AP released better in both w/o non thickener and thickener-added MEs. This was due to lipophilic AP was dissolved in external oil phase of w/o which in turn lead to ease of diffusion (Jurkovič et al. 2003). Similarly, hydrophilic sodium ascorbyl phosphate which is cooperated in the external phase of o/w showed higher release from o/w than w/o non thickener and thickener added MEs (Špiclin et al. 2003). The interaction of active and composition played an important role in the *in vitro* release study of 2% w/w silymarin prepared by four different oil type and surfactant mixture (Panapisal et al. 2012).

7. Validation data of analysis method for nicotinamide

Specificity

With the selected chromatographic conditions, no interfere peak was observed nearby the nicotinamide peak at specific retention time as illustrated in Figures 31-33.

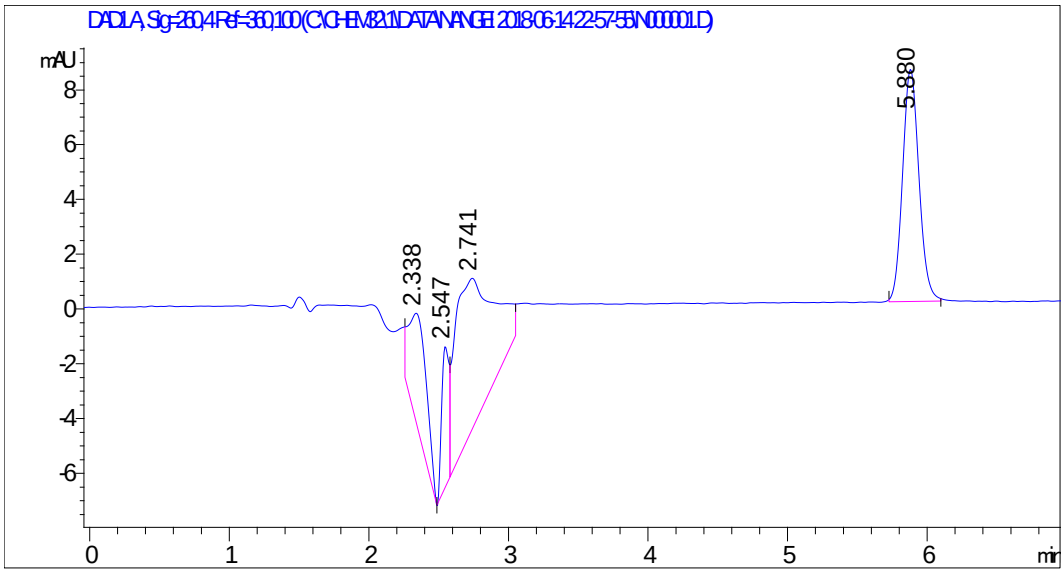


Figure 31. Chromatograph of nicotinamide standard solution at concentration of 1.25 µg/mL.

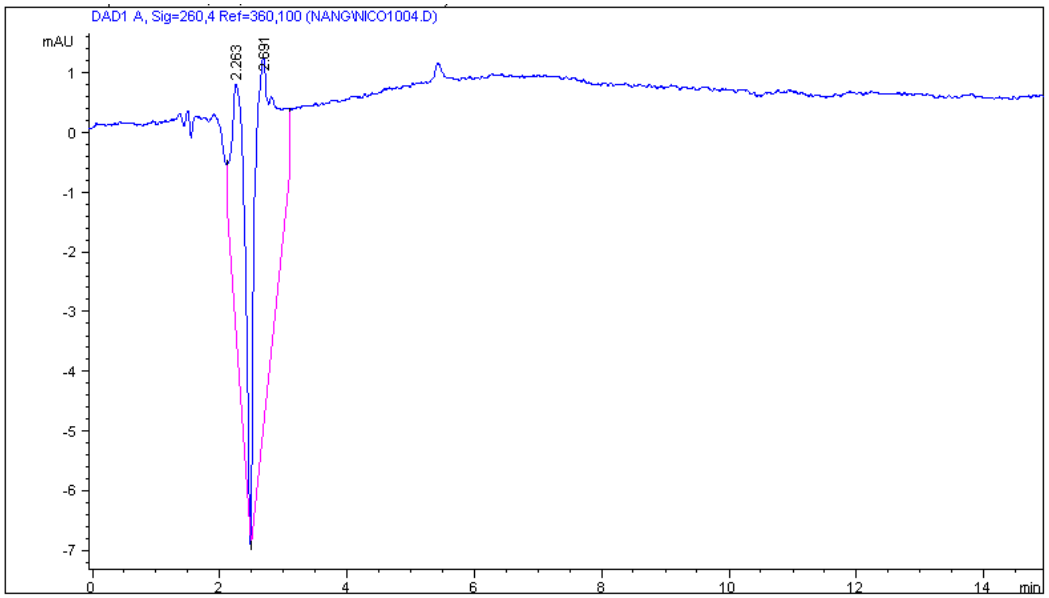


Figure 32. Chromatograph of blank microemulsion, MEO1.

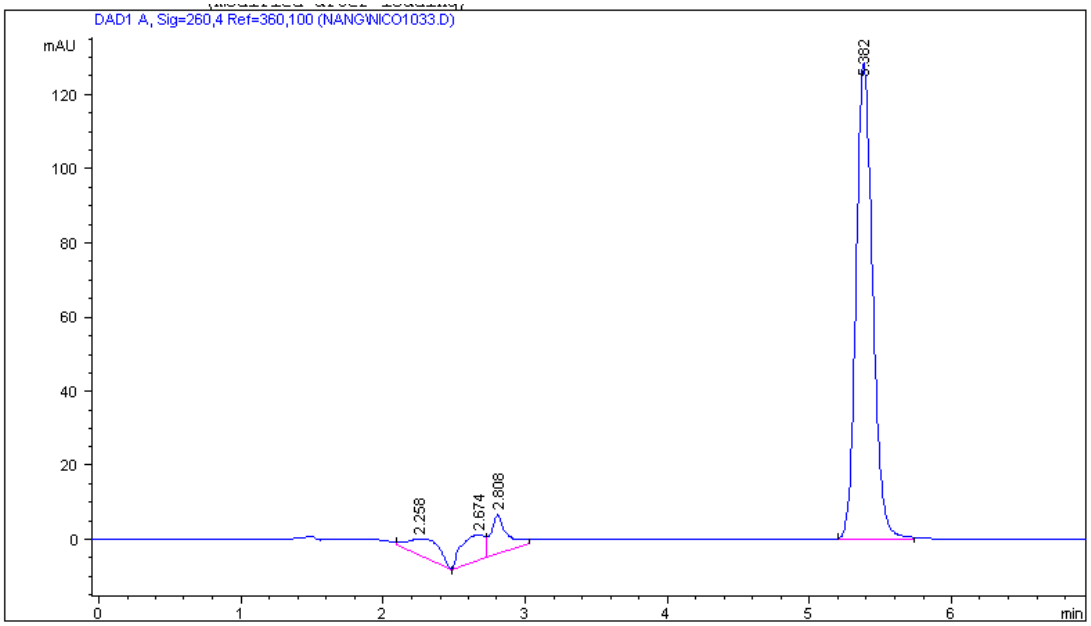


Figure 33. Chromatograph of nicotinamide-loaded microemulsion at concentration of 30 µg/mL, MEO1-N.

Linearity

The calibration curve between 1.25 to 40 µg/mL got linearity of $r^2 > 0.9999$ as shown in Figure 34.

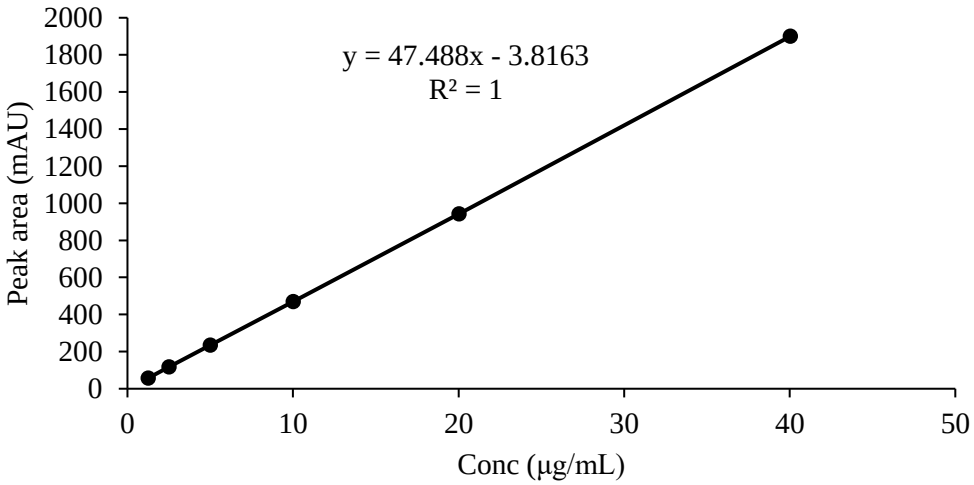


Figure 34. A standard calibration curve of nicotinamide concentrations of 1.25, 2.5, 5, 10, 20 and 40 µg/mL.

Accuracy

The percent recovery was within 98-102% as displayed in Table 13.

Table 13. Accuracy of nicotinamide standard solutions (n=3).

Day	Added Concentration (μg/mL)	Set	Measured Concentration (μg/mL)	% Recovery	Average % of Recovery (mean ± SD)
1	5.01	1	4.99	99.61	99.70 ± 0.10
		2	5.00	99.82	
		3	5.00	99.70	
	10.02	1	9.97	99.48	99.50 ± 0.08
		2	9.96	99.42	
		3	9.98	99.58	
	20.04	1	20.23	100.97	100.94 ± 0.07
		2	20.21	100.86	
		3	20.23	100.99	
2	5.005	1	5.05	100.92	100.93 ± 0.13
		2	5.06	101.09	
		3	5.05	100.83	
	10.01	1	9.99	99.76	99.78 ± 0.06
		2	9.99	99.84	
		3	9.98	99.73	
	20.019	1	19.66	98.21	98.26 ± 0.05
		2	19.68	98.32	
		3	19.67	98.24	
3	5.036	1	5.05	100.33	100.32 ± 0.05
		2	5.05	100.27	
		3	5.05	100.35	

Day	Added Concentration (µg/mL)	Set	Measured Concentration (µg/mL)	% Recovery	Average % of Recovery (mean ± SD)
	10.072	1	10.14	100.65	100.62 ± 0.04
		2	10.13	100.57	
		3	10.14	100.62	
	20.144	1	20.14	99.99	100.04 ± 0.06
		2	20.17	100.10	
		3	20.15	100.03	

Precision

The intra-day precision

The relative standard deviations (RSD) of intra- and inter-day precision were presented in Tables 14 and 15. All obtained %RSD were less than 2%.

Table 14. The intra-day precision of nicotinamide standard solutions (n=3).

Added Concentration (µg/mL)	Set	Measured Concentration (µg/mL)	Measured Concentration (µg/mL) (mean ± SD)	%RSD
5.036	1	5.02	5.02 ± 0.01	0.19
	2	5.01		
	3	5.03		
10.072	1	10.10	10.10 ± 0.01	0.14
	2	10.11		
	3	10.08		
20.144	1	20.14	20.14 ± 0.01	0.03
	2	20.13		
	3	20.14		

The inter-day precision

Table 15. The inter-day precision of nicotinamide standard solutions (n=3).

Day	Added Concentration (μg/mL)	Set	Measured Concentration (μg/mL)	Measured Concentration (μg/mL) (mean ± SD)	%RSD
1	5	1	5.05	5.02 ± 0.04	0.74
		2	5.02		
		3	4.98		
	10.01	1	9.98	9.97 ± 0.04	0.37
		2	10.00		
		3	9.93		
	20.02	1	19.67	19.90 ± 0.22	1.10
		2	19.93		
		3	20.11		
2	5.036	1	5.02	5.02 ± 0.01	0.19
		2	5.01		
		3	5.03		
	10.072	1	10.10	10.10 ± 0.01	0.14
		2	10.11		
		3	10.08		
	20.144	1	20.14	20.14 ± 0.01	0.03
		2	20.13		
		3	20.14		
3	5.036	1	5.00	5.01 ± 0.03	0.62
		2	5.05		
		3	4.99		

Day	Added Concentration (µg/mL)	Set	Measured Concentration (µg/mL)	Measured Concentration (µg/mL) (mean ± SD)	%RSD
	10.072	1	10.12	10.11 ± 0.05	0.46
		2	10.05		
		3	10.14		
	20.144	1	20.13	20.13 ± 0.02	0.08
		2	20.15		
		3	20.12		

Limit of Detection (LOD) and Quantification (LOQ)

The analysed data for LOD and LOQ were presented in Table 16.

Table 16. Limit of detection and quantification of nicotinamide standard solutions (n=3).

Day	LOD Conc (µg/mL) (mean ± SD)	LOQ Conc (µg/mL) (mean ± SD)
1	0.02 ± 0.01	0.04 ± 0.03
2	0.02 ± 0.02	0.05 ± 0.06
3	0.02 ± 0.02	0.05 ± 0.07

The validation of nicotinamide was presented and summarized in Table 17. The results were within the range according to ICH guidelines (ICH 2005).

Table 17. Validation of nicotinamide by HPLC assay

Validation by HPLC	Nicotinamide
Linearity (r^2)	y = 47.488x - 3.8163, $r^2 = 1$
Accuracy (%recovery)	98.26 ± 0.06 to 100.95 ± 0.13
Intra-day Precision (%RSD)	0.03 – 0.19
Inter-day Precision (%RSD)	0.03 – 1.10
LOD (µg/mL)	0.02 ± 0.00
LOQ (µg/mL)	0.05 ± 0.00

CHAPTER 5

CONCLUSIONS

In this research, microemulsion formation was observed by HLD followed by constructing pseudoternary phase diagrams using titration methods for finding microemulsion region. It was found that both fatty acids in studied oils (olive oil and virgin coconut oil) and total HLB value of various S:CoS ratios of Tween 80 and Span 80 affected microemulsion formation.

Four blank formulations (MEO1, MEO2, MEC1 and MEC2) were selected from four pseudoternary phase diagrams. They were prepared from different S:CoS ratios of 0.6:0.4 and 0.7:0.3 and either olive oil or virgin coconut oil.

Characterization of these formulations indicated w/o type despite HLB value of S_{mix} 0.6:0.4 was 10.72 and that of 0.7:0.3 was 11.79. The composition of water was too low to form external phase which in turn influenced the phase behavior as w/o microemulsions. They were physically stable after accelerating test.

Therefore, four nicotinamide-loaded microemulsions were formulated from these four blank microemulsions. Nicotinamide powder was directly added into each blank microemulsion to obtain the active concentration of 3% w/w. Different characterization results confirmed that the addition of nicotinamide did not affect the type of microemulsion.

Stability test was performed at 4°C, room temperature (28 ± 2°C) and 45°C for three months. Visual inspection indicated that storage at 45°C caused darken color. Additionally, phase separation was found in MEO2-N at all conditions since the first month. The phase separation was affected by increased entropy which disturbed the equilibrium of monophasic system in MEO2-N. Other three formulations (MEO1-N, MEC1-N and MEC2-N) were physically stable at 4ºC and room temperature for three months. The amounts of nicotinamide remaining in MEO1-N, MEC1-N and MEC2-N possessed the averaged nicotinamide remaining in the range of 90.10% to 94.42% after kept in clear-glass containers at 4ºC and room temperature for three months.

In vitro release profile of MEO1-N and MEC1-N showed release kinetics of Higuchi model. Therefore, MEO1-N and MEC1-N was stable enough to load nicotinamide and provide sustained release of nicotinamide. The current observation revealed that green microemulsion were possibly promising nano-carriers for topical delivery of nicotinamide.

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Proceeding

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