In Vitro Percutaneous Absorption of Caffeine from Emulsions for Cosmetic Formulation Development

Sunisa Meesen

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Cosmetic Sciences (International Program)
Prince of Songkla University
2011

Thesis Title	*In Vitro* Percutaneous Absorption of Caffeine from Emulsions for Cosmetic Formulation Development
Author	Miss Sunisa Meesen
Major Program	Cosmetic Sciences (International Program)

Major Advisor:

.....

(Assist. Prof. Dr. Somrutai Jitpukdeebodintra)

Co-advisor:

.....

(Assoc. Prof. Dr. Wantana Reanmongkol)

Examining Committee:

.....Chairperson

(Assoc. Prof. Dr. Ruedeekorn Wiwattanapatapee)

.....

(Assist. Prof. Dr. Somrutai Jitpukdeebodintra)

.....

(Assoc. Prof. Dr. Wantana Reanmongkol)

.....

(Assoc. Prof. Dr. Somlak Kongmuang)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Cosmetic Sciences (International Program)

….....

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์	การดูดซึมตัวยาผ่านผิวหนังนอกกายของคาเฟอีนจากอิมัลชันเพื่อการพัฒนาสูตรตำรับทางเครื่องสำอาง
ผู้เขียน	นางสาวสุนิสา มีเสน
สาขาวิชา	วิทยาศาสตร์เครื่องสำอาง (นานาชาติ)
ปีการศึกษา	2553

บทคัดย่อ

คาเฟอีนเป็นสารเสพติดที่มีฤทธิ์ต่อระบบสำคัญของร่างกาย คาเฟอีนถูกนำมาใช้ในทางเครื่องสำอางอย่างกว้างขวางหลายรูปแบบ เช่น ครีมลดริ้วรอย ครีมลดรอยหมองคล้ำรอบดวงตา ผลิตภัณฑ์ป้องกันผมร่วงและผลิตภัณฑ์กำจัดเซลลูไลต์ วัตถุประสงค์ของงานวิจัยนี้เป็นการศึกษานอกกายของการดูดซึมคาเฟอีนผ่านผิวหนัง แล้วนำผลการศึกษาไปใช้พัฒนาผลิตภัณฑ์ให้คาเฟอีนแทรกซึมเข้าสู่ที่ผิวหนังเท่านั้น ไม่ดูดซึมไปยังอวัยวะอื่นๆ ซึ่งส่งผลต่อร่างกายได้ การศึกษาทดลองการซึมผ่านหนังของลูกหมูที่เสียชีวิตหลังคลอดทันทีแทนผิวหนังมนุษย์เป็นเวลา 24 ชั่วโมง เพื่อเปรียบเทียบการซึมผ่านของคาเฟอีนในอิมัลชันชนิดน้ำในน้ำมันกับชนิดน้ำมันในน้ำ ผลการทดลองพบว่า คาเฟอีนจากอิมัลชันชนิดน้ำมันในน้ำซึมผ่านผิวหนัง (12.05 ± 2.76 mg) มากกว่าคาเฟอีนจากอิมัลชันน้ำในน้ำมัน (5.64 ± 2.47 mg) และพบคาเฟอีนค้างอยู่ในผิวหนังจากอิมัลชันชนิดน้ำในน้ำมันปริมาณมากกว่าจากอิมัลชันชนิดน้ำมันในน้ำคือ 1.32 ± 0.01 mg และ และ 0.16 ± 0.04 mg mg ตามลำดับ จึงพัฒนาสูตรตำรับเครื่องสำอางที่มีคาเฟอีนความเข้มข้น 3% โดยน้ำหนักในอิมัลชันชนิดน้ำในน้ำมันเพื่อให้คาเฟอีนคงอยู่ในผิวหนัง และศึกษาเปรียบเทียบคาเฟอีนในผิวหนังจากอิมัลชันชนิดน้ำในน้ำมันที่พัฒนากับคาเฟอีนครีมทางการค้า พบว่าอัตราส่วนของคาเฟอีนในผิวหนังต่อปริมาณคาเฟอีนที่ซึมผ่านผิวหนังจากอิมัลชันที่พัฒนามีค่าสูงกว่าผลิตภัณฑ์ทางการค้าที่นำมาศึกษาเปรียบเทียบ ดังนั้นอิมัลชันชนิดน้ำในน้ำมันจึงเหมาะสมต่อการนำไปใช้ในการพัฒนาเป็นเครื่องสำอางเพื่อให้ปริมาณคาเฟอีนยังคงอยู่ในผิวหนังได้มากที่สุด

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Academic Year	2010

ABSTRACT

Caffeine is a narcotic drug that affects the important part of body. It is extensively used in a variety of cosmetic forms such as anti-wrinkle cream, anti-periobital hyperpigmentation products, hair loss preventing products and cellulite removal products. The purpose of this research is *ex-vivo* study on caffeine absorption through the skin. Then applied study on product development was done to obtain caffeine product that penetrates only into the skin, not absorbed into other organs that affect the health. The skin permeation study of caffeine on newborn pig that died shortly after birth was done to compare the absorption of caffeine from both of water in oil and oil in water emulsion. The results shown that caffeine permeated through the skin from oil in water emulsion (12.05 ± 2.76 mg) was more than from the water in oil emulsion (5.64 ± 2.47 mg), and the amount of remained caffeine on the skin from water in oil emulsion was more than from oil in water emulsion (1.32 ± 0.01 mg and 0.16 ± 0.04 mg, respectively). The cosmetic formulation containing caffeine in concentration of 3% by weight was developed in the form of water in oil emulsion. The comparison of caffeine in the skin was studied between the penetrated caffeine from developed water in oil emulsion and the commercial caffeine cream. It is shown

that the ratio of caffeine in the skin to caffeine in the receptor compartment from developed formulation higher than from the compared commercial product. Therefore water in oil emulsion is suitable for application in the development of cosmetics to caffeine remains in the skin as possible.

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LIST OF ABBREVIATIONS AND SYMBOLS

cAMP	cyclic adenosine monophosphate
CNS	central nervous system
ºC	degree Celsius
cm^2	square centimeter
g	gram
h	hour
HLB	hydrophilic lipophilic balance
HPLC	high performance liquid chromatography
kg	kilogram
LD50	median lethal dose
µs	microsiemens
µg	microgram
mg	milligram
ml	milliliter
O/W	oil on water

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

W/O	water in oil
W/O/W	water in oil in water
%	percentage
rpm	revolutions per minute
RSD	relative standard deviation
SAC	siloxanetriol alginate caffeine
S.D	standard deviation
TEWL	transepidermal water loss
UVB	ultraviolet B

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Nowadays caffeine consumption in cosmetic becomes widespread, such as anti-wrinkles and anti-periobital hyperpigmentation products, hair lost prevention, alopecia areate treatment (Adolf and Kurt, 2005), cellulite treatment products (Sainio *et al*., 2000) etc. A variety of cosmetic products exist in today's marketplace, fulfill a variety of functions by either acting direct on the skin or being a cosmetically elegant vehicle for the delivery of specific active ingredients. Most common forms of cosmetic products are emulsions (Andre *et al.,* 2001).

Emulsions are mixtures of two insoluble materials that are stabilized against separation. An example is oil and water, which will not mix unless an intermediate emulsifier is incorporated into the mixture and the famous commonly formulations are oil in water (O/W) and water in oil (W/O) emulsion forms (Andre *et al.,* 2001).

The cosmetic anti-cellulite treatment products which contain caffeine as active ingredient are usually launched in emulsion form. Now caffeine is obviously topical used and involved a massaging action and the direct physical stimulus of rubbing a cream which may contribute to an improvement of caffeine permeation in the condition with time (Collins *et al.,* 1998). Nevertheless the permeation of caffeine

is strongly affected by the vehicle chosen (Dias *et al*., 2007). The cosmetic formulation has to be optimized so that caffeine reaches the active site in the adipocytes located in the hypodermis because of its slimming effect. Emulsions are vehicles for percutaneous administration that modify drug permeation into the skin with several mechanisms: modification of skin permeability, penetration enhancer action of high amounts of surfactant or co-surfactant, modification of bioavailability of caffeine because of partitioning between aqueous and oil phase and caffeine mobility in the vehicle. Caffeine is a central nervous system (CNS) and cardiac stimulant, so it is considerably more toxic to some other humans due to much poorer ability to metabolize this compound, and to much sensitive to caffeine. Too much caffeine, especially over an extended period of time, can lead to a number of physical and mental conditions. An overdose of caffeine can result in a state termed caffeine intoxication or caffeine poisoning (Eskenazi, 1993).

1.2 Objectives

1.2.1 To compare *in vitro* percutaneous absorption of caffeine from O/W and W/O emulsions

1.2.2 To formulate caffeine emulsion for cosmetic application

1.2.3 To study *in vitro* percutaneous absorption of caffeine formulation and compare to commercial caffeine product

1.3 Expected Outcome and Benefits

1.3.1 The knowledge of caffeine for topical application

1.3.2 Background for a continuing study of *in vitro* caffeine absorption and formulation

CHAPTER 2

LITERATURE REVIEWS

2.1 Caffeine and Its Characteristics

Caffeine is a bitter white crystalline xanthine alkaloid that acts as a psychoactive stimulant drug and a mild diuretic (speeds up urine production) in humans and other animals. Caffeine was discovered by a German chemist, Friedrich Ferdinand Runge, in 1819. He coined the term "kaffein", a chemical compound in coffee, which in English became caffeine. Caffeine is also called guaranine when found in guarana, mateine when found in mate, and theine when found in tea; all of these names are synonyms for the same chemical compound. Caffeine structure formula is shown in Figure 1.

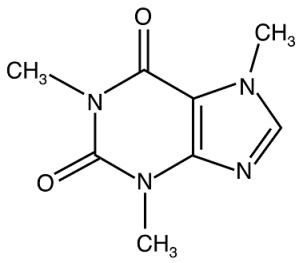


Figure 1 Structural formula of caffeine (Dunnick *et al*., 2007)

Chemical name of caffeine is 1, 3, 7-trimethyl-1*H*-purine-2, 6 (3*H*, 7*H*)-dione. It has other names: 1, 3, 7-trimethylxanthine, trimethylxanthine, theine, matinee, guaranine, methyltheobromine. The chemical formula is $C_8H_{10}N_4O_2$ with

molecular weight is 194.19. It is white solid or long silky crystals. It is odorless with distinctive bitter taste. One gram of caffeine dissolves in 46 ml water, 5.5 ml water at 80°C, 1.5 ml boiling water, 66 ml alcohol at 60°C and 530 ml ether. Melting point is 235.8°C. It is hexagonal prisms by sublimation at 178^{o}C.

Caffeine is found in numerous plant species, such as coffee and tea. Caffeine is one of many xanthine alkaloids. Derivatives of xanthine are a group of alkaloids commonly used for their effects as mild stimulants and as bronchodilators. In contrast to others, more potent stimulants, they only inhibit the actions of sleepiness-inducing adenosine, making them far less effective as stimulants than sympathomimetic amines. Due to widespread effects, the therapeutic range of xanthines is narrow, making them merely a second-line asthma treatment. The therapeutic level is 10-20 μg/ml. The signs of toxicity include tremor, nausea, nervousness, and tachycardia/arrhythmia. Caffeine is quickly absorbed by the body. The human salivary caffeine level, which indicates the extent of absorption, peaks around 45 minutes after caffeine consumption (Liguori *et al.,* 1997). Caffeine is completely absorbed by the stomach and small intestine within 45 minutes of ingestion. After ingestion it is distributed throughout the total body water and is eliminated by first-order kinetics (Bradbrook *et al.,* 1981).

Caffeine is metabolized in the liver by the cytochrome P450 oxidase enzyme system into three metabolic dimethylxanthines, which each has their own effects on the body (Dews *et al*., 1984). Paraxanthine (84%) has the effect of increasing lipolysis, leading to elevate glycerol and free fatty acid levels in the blood plasma. Theobromine (12%) dilates blood vessels and increases urine volume.

Theophylline (4%) relaxes smooth muscles of the bronchi, and is used to treat asthma. Each of these metabolites is further metabolized and then excreted in the urine. Caffeine may, in some people cause tachycardia and gastric symptoms. Caffeine has also been implicated as a cause of food allergy, a non-specific term for a disorder which includes symptoms such as headache, palpitations, vomiting, panic attacks and anxiety. Caffeine interferes with normal sleep patterns and may enhance the absorption of certain drugs. It affects drug-metabolizing enzymes by acting as an inducer of the microsomal system (Mitoma, *et al*., 1968). In cases of extreme overdose, death can result. The median lethal dose (LD_{50}) given orally, is 192 milligrams per kilogram in rats. The LD_{50} of caffeine in humans is dependent on weight and individual sensitivity and estimated to be about 150 to 200 milligrams per kilogram of body weight (Josef and Peters, 1967). That is dependent on half-life so the average half-life of caffeine at 3.5 hours (Timson, 1970).

2.2 Caffeine in Cosmetic Products

Now caffeine is often found in skin-care products with claims that it has anti-wrinkles and anti-periorbital hyperpigmentation, hair loss prevention, alopecia areate treatment (Adolf and Kurt, 2005) and cellulite treatment products etc. Caffeine is in routine use as an active ingredient in cosmetic formulations (Sainio *et al*., 2000) in concentrations up to 3% (Dias *et al.,* 1999) as it is a nontoxic, inexpensive substance which penetrates easily through human skin and the mean maximum absorption rate of caffeine through human skin membranes was 2.24 ± 1.43

μg/cm^2/h, while the amount in the receptor fluid after 24 h was 24.5 ± 11.6% of the dose applied (4.0 mg/ml) (Van de Sandt, *et al*., 2004).

Caffeine is a xanthines mostly used in anticellulite cosmetics due to their lipolytic activity on fatty cells via inhibition of phosphodiesterase, and increasing cyclic adenosine monophosphate (cAMP) levels (Rawlings, 2006). Cellulite is an alteration of the topography of the skin that occurs mainly in women on the pelvic region, lower limbs and abdomen. It is characterized by a padded or orange peel appearance (Rossi and Vergnanini, 2000). Cellulite is a cosmetically unacceptable aesthetic problem; approximately 85% of women over the age of 20 have some degree of this physiological gender-linked condition (Rawlings, 2006). Cellulite presents etiologic plurality of loco-regional character, most frequently developed at the abdomen, buttocks, breasts, upper arms, trochanteric and perimalleolar areas, anterior, posterior, medial and lateral thigh, and knee. The presence of this condition on limited body areas justifies the difference from diffuse adiposity, a common lipodystrophic disease (Draelos, 2005). Among the innumerous possibilities to attenuate the cellulite condition, the use of topical products presents easy access, and the cosmetic approach can be conducted by the consumer. Anti-cellulite cosmetics are composed of active substances whose mechanisms of action are reduction of hypertrophy and hyperplasia of the fatty tissue, inhibition of lipogenesis, reorganization of the connective tissue by cellular regeneration, microcirculation stimuli, and capillary fragility decrease (Rawlings, 2006; Draelos, 2005; Terranova *et al.,* 2006).

Caffeine is an antioxidant used as an ingredient in sunscreen cosmetic products that reduces free radicals. Although caffeine has a sunscreen effect, it also has a biological effect of causing apoptosis programmed cell death, in UVB damaged skin cells, and in tumors but not in normal skin or in areas adjacent to tumors. In addition, it is widely used in an anti-aging product because it has effects for blood circulation enhancement and dilation as well (Rawlings, 2006).

It has been reported by Yao-Ping *et al*. (2007) caffeine sodium benzoate and caffeine are the first examples of compounds that have both a sunscreen effect and enhance UVB induced apoptosis. Topical application of caffeine sodium benzoate or caffeine 0.5 h before irradiation with a high dose of UVB decreased UVB induced thymine dimer formation and sunburn lesions (sunscreen effect). Caffeine sodium benzoate was more active than an equimolar amount of caffeine in exerting a sunscreen effect

There are several studies showing caffeine to have benefit for reducing cellulite. One was conducted by Johnson & Johnson, which owns the RoC® and Neutrogena® brands, both of which sell cellulite creams that contain caffeine. The other was conducted by cosmetics ingredients manufacturers that sell anti-cellulite compounds (Dias, 1999).

The caffeine gets into the fat cell and this makes the fat cell get a little more energized. As caffeine presents a lipolytic affect it blocks enzymes responsible for the destruction of cAMP, which is involved in triglycerides breakage. It also possesses vasodilator properties, increasing blood flow. Therefore, it contributes in both the lipolytic and venotonic effect which reduce puffy eye symptom when applied

topically. However, caffeine does have potential as an antioxidant, so it isn't a wasted ingredient in skin care products. It's found the treatment of periorbital hyperpigmentation in alternative medicine using a warm tea-bag with caffeine place over the closing eye for 10 minutes. This alternative curing said to prevent the eyes from inflammation (Sainio *et al*., 2000).

Over the last few years, studies have suggested that caffeine is capable of staving off baldness. A caffeine extract has proved to be an effective ingredient in a topical hair loss prevention shampoo treatment. Believed to be the only remedy to use this ingredient, the makers claim that huge concentrations can prevent testosterone from damaging hair growth (Otberg, 2007).

German hair care specialist alpecin cosmetic has been selling hair and scalp treatment on the German market since 1930. However, it wasn't until 2000 that the company started marketing after shampoo liquid hair loss remedy with a caffeine derived active complex contained in the ingredients. The study results have found that the active caffeine ingredient helps to regulate the effects of testosterone levels. Male pattern baldness is known to occur on individuals with sensitivity to testosterone, causing damage to hair follicles that eventually leads to baldness. The results showed that using the caffeine treatment average growth was increased by around 46 percents and the life cycle of the hair was extended by 37 percents, when compared to the control study (Adolf and Kurt, 2005).

A cosmetic that comes in contact with human skin will be absorbed. The components of the cosmetic will respond to the chemical and physical laws of nature which direct the absorption process. Cosmetic components will transverse from

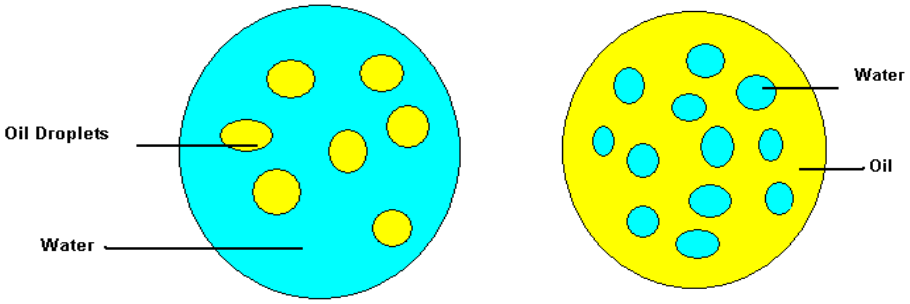
a lipophilic stratum corneum to a more progressively hydrophilic epidermis, dermis and blood microcirculation (Andre, *et al*., 2001). Skin absorption by the vehicle contains the chemical of interest. There is a partitioning of the chemical from the vehicle to the skin. This initiates absorption and excretion kinetics that are influenced by a variety of factors, such as regional and individual variation. These factors moderate the absorption and excretion kinetics (Wester and Maibach, 1992).

Caffeine is a good example of this relation between *in vitro* pharmacology, skin pharmacokinetic activity *in vivo* (Weplerre et al., 1997). Like other methylxanthines, it increases lipolysis (breakdown of triglycerides into free fatty acids and glycerol) both *in vitro* and *in vivo*. By inhibition of phosphodiesterase, caffeine is able to raise levels of cycle AMP, which activates triglyceryl lipase and is used topically as a local slimming ingredient. When caffeine is applied topically, its concentration detected after 100 minutes in the dermis is close to 10^{-4}M. The lipolytic effect found *in vitro* on isolated adipocyte, with a caffeine concentration of 10^{-4}M can be obtain *in vivo*, where the repeated application of a 5% caffeine emulsion on rat skin for 3 weeks leads to fall of more than 15% in the concentration of subcutaneous lipids in the treated area. These data confirm that percutaneous pharmacokinetics methodology allow one to link a biologic activity demonstrated *in vitro* with the effect obtained *in vivo* (Robert and Howard, 1991).

2.3 Emulsion as Cosmetic Vehicle

An O/W emulsion typically contains 10 to 35% oil phase and a lower viscosity. Water in external phase of the emulsion helps hydrate the stratum corneum of the skin. This is desirable when one desires to incorporate water soluble active ingredients in the vehicle. Oil droplets in emulsions have a lower density than phase they are suspended in; to have a stable emulsion it is important to adjust the specific gravity of the oil and water phase as closely as possible. Viscosity of the water phase (external phase) may be increased to impede the upward migration of the oil particles. Addition of waxes to the oil phase will increase specific gravity but have a profound effect on the appearance, texture and feel on application to skin of the product. Increasing water phase viscosity is one of the most common approaches. Natural thickeners and cellulosic gums are used for this purpose (Andre, *et al*., 2001).

W/ O emulsions are less popular than O/ W emulsions; these systems may be desirable when greater release of a medicating agent of the perception of greater emolliency is desired. These emulsions typically have total of 45 to 80% oil phase. Viscosity of W/ O emulsions has correlated with molecular weight of emollients used in formulations. High molecular weight co-emulsifiers formulated with high molecular weight emollient gave more stable W/O emulsions. The polarity of emollients use was found to be important as well. Emollients or mixtures of emollients with medium polarity gave test lotions the most desirable stability results (Andre, *et al*., 2001).



(a) (b)

(a): Oil in water emulsion, (b): Water in oil emulsion

Figure 2 Microscopic feature of the two types of emulsion to be compared

The properties of O/W and W/O emulsions are different as shown in Table 1.

Table 1 Difference between O/W and W/O emulsions (Javed *et al.,* 2007)

Oil in water emulsion	**Water in oil emulsion**
Water is the dispersion medium and oil is the dispersed phase	Oil is the dispersion medium and water is the dispersed phase
They are non greasy and easily removable from the skin surface	They are greasy and not water washable
They are used externally to provide cooling effect e.g. vanishing cream	They are used externally to prevent evaporation of moisture from the surface of skin e.g. cold cream
Water soluble drugs are more quickly released from o/w emulsions	Oil soluble drugs are more quickly released from w/o emulsions
They are preferred for formulations meant for internal use as bitter taste of oils can be masked.	They are preferred for formulations meant for external use like creams.
O/W emulsion give a positive conductivity test as water is the external phase which is a good conductor of electricity	W/O emulsion do not give a positive conductivity test as oil is the external phase which is a poor conductor of electricity.

Emulsifier can act as solubilizer as well as spreading or dispersing agents. Correct use of emulsifiers permits the formulation of homogenous mixtures, dispersions or emulsions of oily, waxy substances with water. Solid may be dispersed in liquids or insoluble liquids with other liquids. These types of properties may be the result of appropriate selection of emulsifiers, active ingredient and other compatible ingredients in the vehicle.

The various analytical procedures for determining HLB values of emulsifiers do not account for interaction between the emulsifiers and the aqueous and oil phases. It may be possible to derive more meaningful values from phase inversion temperature determinations on the emulsions (Boyd et al., 2004).

Emulsifying agents, which are surface active agents (surfactants) are available in a wide range of chemical types. These include nonionic, hydrophilic, lipophilic, ethoxylated and nonethoxylated. A recent trend is to lower or even eliminate surfactants in an effect to minimize the already low irritant potential of the formulation. Thousands of emulsifying agents are available on the world market today. Choosing the best agent is the key responsibility of the formulator. Many agents used in the cosmetic and drug industry are classified by a system known as Hydroplilic-Lipophilic Balance (HLB) number. This system, developed in the mid-1950s, is a useful starting point in emulsifier selection. In this system, each surfactant having a specific HLB number is used to emulsify oil phase having HLB required for a stable emulsion. Using an emulsifier or combination of emulsifiers matching the required HLB of the oil phase will form a stable emulsion. Combinations or single

emulsifying agent giving the theoretical HLB may be not the optimal combination for emulsion stability or product formulation with greater efficacy. In addition theoretical HLB numbers of complex mixtures may not follow a linear additive rule specified in the calculation and the range of HLB can identify water solubility which shown in Table 2. (Konish and Gruber, 1998)

It has been reported by Monika et al.(2008) the ranking of the permeation of the hydrocortisone formulations was: solution > W/O emulsion > O/W emulsion, which permitted the elucidation of penetration enhancing effects, which is not possible with drug release studies. Differences in penetration were most obvious with native skin and reconstructed tissues, which exhibited a well-developed penetration barrier. In conclusion, reconstructed human epidermis and skin preparations may be useful in the development of topical dermatics and in the framework of hazard analysis of toxic compounds and their various formulations.

Table 2 HLB range in some conditions of water solubility and emulsions (Andre *et al*., 2001)

Water solubility	**HLB range**
No dispensability	1-4
Poor dispersion	3-6
Milky dispersion after agitation	6-8
Stable milky dispersion	8-10
Translucent to clear dispersion	10-13
Clear solution	13+
W/O emulsions	4-6
Wetting agent	7-9
O/W emulsions	8-18

Doucet *et al*., (1998) concluded that six hours after the application the diffusion rate of caffeine from the W/O/W multiple emulsions was significantly lower than from the traditional O/W emulsion. Whatever the vehicle used, the necessary time to reach a steady state was long, ranging from 16 to 18 h. It did not seem to differ from one vehicle to another. The permeability coefficient of caffeine, calculated

for the W/O/W multiple emulsion, was smaller than the one of the O/W simple emulsion. At the end of the experiment (24 h), the amount of caffeine absorbed through the skin from the W/O/W emulsion was 2.6 times lower than from the O/W emulsion. The formulation of caffeine cream might be in the form of O/W simple emulsion, which would be more absorbed through the skin.

Bolzinger *et al.*, (2008) studied that the microemulsion allowed delivery of a larger fraction of the caffeine in the hypodermis than from the emulsion and gel dosage form, and this amount was stored in hypodermis and diffused to the receptor compartment of the Franz diffusion cell. They also indicated that the transcutaneous transport of caffeine may reach the target and stay there a long enough time. Therefore skin absorption by the formulation containing caffeine may be accumulated in the oil fragment and harm to the body.

2.4 Percutaneous Absorption

Percutaneous absorption is a complex biological process. The skin is a multilayered biomembrane that has certain characteristics. If the skin was a simple membrane and these would be fairly constant provided there was no change in the chemistry of the membrane. However, skin is a dynamic living tissue and as such its absorption parameters are susceptible to constant change. Many factors and skin conditions can rapidly change the absorption parameters. Additionally, skin is a living tissue it will change through its own growth patterns and this change will also be influenced many factors. When dealing with percutaneous absorption the skin should

not be regarded as an inert membrane. Instead, it should be viewed as a dynamic, living biomembrane with unique properties (Robert and Howard, 1991).

Skin absorption of cosmetic products is influenced by a variety of factors such as type of skin, time of use, type of formulation etc. Percutaneous absorption represents the amount of topical applied test substance that is found in the receptor fluid after termination of an *in vitro* experiment.

Caffeine is frequently used in skin penetration experiments as a model for highly water soluble compounds. Occlusion and skin thickness seem to have little influence on the penetration of caffeine. However, percutaneous absorption rates for caffeine exhibit regional skin differences in humans *in vivo*. The concentration of the emulsifier does not have a significant effect on the release of caffeine. In contrast, diffusion of caffeine from concentrated W/O emulsions has been found to be highly dependent on the internal phase volume. The flux of caffeine increases with the percentage internal water phase. The droplet diameter decreases and the apparent viscosity increases with the percentage of the dispersed phase (Clement *et al.,* 2000).

Brandner *et al.*, (2006) studied that 0.5% caffeine in a hydroxylethyl-cellulose improved barrier function in skin, they concluded that caffeine was beneficial for barrier function in male skin more than in female, and found that caffeine significantly reduced transepidermal water loss (TEWL) in male skin compared with female skin.

Van de Sandt, *et al*. (2004) concluded that the mean maximum absorption rate of caffeine through human skin membranes was 2.24 ± 1.43 µg/cm^2/h.

The mean maximum absorption rate of caffeine through rat skin was 6.82 μg/cm^2/h. For both human and rat skin, indicating that only a small amount caffeine remained in the skin membrane after washing the application area.

Velasco *et al.,* (2007) studied effects of caffeine and siloxanetriol alginate caffeine (SAC), as anticellulite agents in emulsion form, on fatty tissue. They found that emulsion with caffeine caused the most reduction on the diameter of the fatty cells compared with the control and other formulations, the emulsion with caffeine and sodium benzoate, emulsion with SAC, caffeine gels. However it was noticed that gel with SAC promoted a much more reduction on the number of fatty cells.

The percutaneous absorption of caffeine from two vehicles, an emulsion and an acetone solution, was quantified by *in vivo* techniques in humans. A surface recovery technique over a 6-h application and a stripping method after a 30-minutes application was performed on the volar aspect of the forearm on 12 volunteers. Caffeine was assessed by HPLC. Two phases were distinguished in the percutaneous absorption of caffeine: a higher filling up of the stratum corneum with the O/W emulsion than with the acetone solution, which was then followed by a steady-state flux corresponding to the penetration in the living tissues. The permeability constants with emulsion and acetone were 1.59×10^{-4} and 9.53×10^{-8} μg/cm^2/h, respectively (Chambin *et al*., 1993).

It has been reported by Justyna *et al.* (2009) the skin permeation of a hydrophilic model penetrant (caffeine) was investigated from a W/O Pickering emulsion and compared to a W/O classical emulsion stabilize d with an emulsifier.

After 24 h exposure, caffeine was mostly in the receptor fluid and in the dermis; cumulated amounts of caffeine were higher for the Pickering emulsion. The transport of caffeine adsorbed on silica particles was also considered relevant since skin stripping showed that aggregates of silica particles entered deeply the stratum corneum

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Formulation compositions

a) Mineral oil (S. Tong Chemicals Co., Ltd., BKK, Thailand)

b) Cetyl alcohol (P. C. Drug center Co., Ltd., BKK, Thailand)

c) Isopropyl myristate (Vidhyasom, BKK, Thailand)

d) Lanolin anhydrous (S. Tong Chemicals Co., Ltd., BKK, Thailand)

e) Silicone oil (P. C. Drug center Co., Ltd., BKK, Thailand)

f) Triethanolamine (S. Tong Chemicals Co., Ltd., BKK, Thailand)

g) Sorbitan monostearate, Sorbitan stearate, Span 60 (S. Tong Chemicals Co., Ltd., BKK, Thailand)

h) Polyoxyethylene sorbitan monostearate, Tween 60 (S. Tong Chemicals Co., Ltd., BKK, Thailand)

i) Sorbitan sesquioleate, Arlacel 83 (P. C. Drug center Co., Ltd., BKK, Thailand)

j) Ceteareth-25, Cremophor A 25 (BASF, Ludwigshafen, USA)

k) Ceteareth-6 Stearyl alcohol, Cremophor A6 (BASF, Ludwigshafen, USA)

l) Methyl paraben (P. C. Drug center Co., Ltd., BKK, Thailand)

m) Propyl paraben (P. C. Drug center Co., Ltd., BKK, Thailand)

3.1.2 Analytical reagents

a) Acetonitrile (Labscan Asia Co., Ltd., BKK, Thailand)

b) Acetic acid (J. T. Baker® Philipsburg, USA)

c) Methanol HPLC grade (Labscan Asia Co., Ltd., BKK, Thailand)

d) Sodium dihydrogen orthophosphate (Sodium phosphate, monobasic), $NaH_2 \cdot PO_4 \cdot 2H_2O$ (Univar: Ajex Finechem Pty., Ltd., Australia)

e) di-Sodium hydrogen orthophosphate, $Na_2HPO_4 \cdot 2H_2O$ (Unilab: Ajex Finechem Pty., Ltd., Australia)

f) Sodium chloride, NaCl (Labscan Asia Co., Ltd., BKK, Thailand)

3.1.3 Commercial caffeine product for a comparative study is O/W emulsion type containing caffeine, methylsilanol mannuronte, garcinia cambogia extract, horsetail extract, centella asiatica extract, horse chestnut seed extract, butcher broom extract and fragrance (local distributor, Songkhla).

3.2 Instruments

a) Modified Franz diffusion cell (Hanson®,model 57-6M, USA)

b) pH meter (Mettler Toledo, model 520-K, USA)

c) Homogenizer (Ystral®, model X10/25, Germany)

d) Viscosmeter (Brookfield RVT, USA)

e) Centrifuge (Hermle®,model Z323K,Germany)

f) HPLC (Shimadzu®, model SCL-10A, USA)

g) Sonicator (Crest®, model 575HT, USA

h) Hot air oven (DIN 12880-KI, Memmert, Germany)

i) Conductivity meter (CM-115, Kyoto electronics, Japan)

3.3 Methods

3.3.1 Preparation of Caffeine Emulsions

The simple formulas of emulsions with similarly compositions were prepared for the experiment as shown in Table 3. The required HLB of O/W emulsion was 12.60 which the calculation shown in Appendix 1. The required HLB of W/O emulsion was 6.11 as shown the calculation in Appendix 1. The emulsifiers in O/W emulsion were mix emulsifiers of tween 60 and span 60 (the same series), while the emulsifier in W/O emulsion was a single arlacel 83.

Table 3 Typical formulations of o/w and w/o emulsion used in the experiments

Ingredients	Content (%w/w)		Categories
	O/W emulsion HLB = 12.60	W/O emulsion HLB = 6.11	
Mineral oil	20	56	Emollient agent
Cetyl alcohol	5	7	Stiffening agent
Tween 60	2.45	-	Emulsifier (o/w)
Span 60	7.55	-	Emulsifier (w/o)
Arlacel 83	-	10	Emulsifier (w/o)
Methyl paraben	0.5	0.5	Preservative
Propyl paraben	0.1	0.1	Preservative
Caffeine	3	3	Active ingredient
Purified water to	100	100	Vehicle

(The formulas were adapted from Leelapornpisit, 1989)

The emulsion was prepared by the following procedures: Separately mixed the composition of water phase and the oil phase, then heated each phase to 70°C, poured the hot aqueous phase to the hot oil phase and mixed together with stirring until it was cool and congealed at room temperature.

3.3.2 *In Vitro* Skin Permeation Study of Caffeine from O/W and W/O emulsion: Franz Cell Diffusion

Three steps of experiment were done as following:

3.3.2.1 Preparation of isotonic phosphate buffer pH 7.4

Isotonic phosphate buffer pH 7.4 was prepared by mixing two stock solutions, 200 ml of a solution containing 10.4 g of monobasic sodium phosphate ($NaH_2PO_4 \cdot 2H_2O$) per liter and 800 ml of a solution containing 9.47 g of dibasic sodium phosphate (Na_2HPO_4) per liter. Then it was adjusted to an isotonic by adding 4.4 g of sodium chloride (NaCl). The obtained solution was filtered through a 0.045 μm nylon membrane and degassed by sonification before use. (Stoll and Blanchard, 1990).

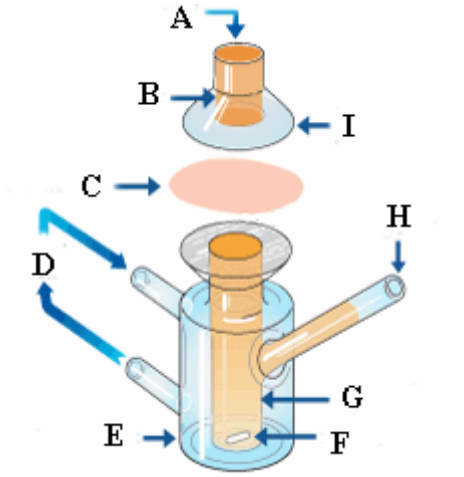
3.3.2.2 Preparation of Full-thickness Newborn Pig Skin

The skin permeation studies were performed in modified Franz's diffusion cells using full-thickness newborn pig skin. Pig skin has also been promoted on the basis of its structural equivalence to human, it is generally accepted that human skin is the most appropriate *in vitro* model (Faith, 2006).

Full-thickness newborn pig skin prepared according to the experiment of Songkro, *et al*. (2003). Newborn pigs that naturally died after birth were freshly obtained from a local pig farm (Songkla, Thailand). Full thickness flank skins of newborn pigs weighing 1.4-1.8 kg were used as membrane. The epidermal hair at the flank area was clipped with a hair clipper as close as possible to the skin without damaging it and excised with a scalpel. The subcutaneous fat and underlying were tissues carefully removed from the dermal surface. The skin was rinsed with phosphate buffer of pH 7.4, blotted with soft household paper, wrapped with aluminum foil and then stored at -20^{o}C for no longer than a month (Songkro *et al.*, 2003).

3.3.2.3 *In Vitro* Percutaneous Absorption Study

The *in vitro* skin absorption study was performed using the modified Franz diffusion cells as shown in Figure 3



A = donor compound
B = donor compartment
C = membrane
D = heater circulation
E = water jacket
F = stirring bar
G = receptor compartment
H = sampling port
I = flat ground joint

Figure 3 The modified Franz diffusion cell (from: www. http://www. Permegear.com/franz.html; accessed on 8 December 2010)

The skin was cut into 4.5x4.5 cm pieces, placed in phosphate buffer of pH 7.4 and hydrated at room temperature for 1 hour before use. Afterward the skin was mounted between the halves of vertical Franz type diffusion cells with a diffusion surface area of 1.77 cm^2, the stratum corneum facing the donor chamber. The receptor compartment of the modified Franz diffusion cell was filled with 11 ml of phosphate buffer (0.1 M, pH 7.4). The dermal side was exposed to phosphate buffered receptor fluid with continuously stirring. The modified Franz diffusion cell was placed in a circulating water bath with magnetically stir at 300 rpm that maintained a constant temperature of the receptor fluid at 37^oC, and provided the skin temperature of 32^oC (Clement *et al.,* 2000).

One gram of each formulation was put in a donor compartment, and spread on the surface of the skin. After 0.5 hour of diffusion test, sample in the receptor compartment was withdrawn and collected in the vial for caffeine content analysis by HPLC method and calibration with standard curve of normal condition.

Then the donor side surface was washed 10 times with 1 ml of fresh receptor liquid and the excess washing liquid was absorbed on cotton swabs. Then all the receptor liquid obtained from the washing collected in the vial and squeezed cotton swab was quantified for caffeine content by HPLC method and calibration with standard curve, normal condition.

The caffeine in skin was extracted by solvent extraction, after the full thickness skin was cut into small pieces with a scalpel and collected in a vial, homogenized with acetonitrile, centrifuged at 15,000 rpm for 15 min at room temperature, and filtered through cellulose acetate filter with pore size of 0.2 μm

(Sartorius,AG, Germany). The filtrate was analyzed by HPLC method and calibration with standard curve, skin condition.

The same technique of quantification of caffeine in the receptor compartment, donor side surface, and the skin after each of 1, 2, 4, 8, 10, 12, and 24 hours diffusion experiment, were done. All experiments were repeated three times.

All the caffeine data in different compartment of the diffusion site were evaluated and discussed. The type of emulsion was then decidedly selected for cosmetic formulation development.

3.3.3 Quantitative Analysis of Caffeine: HPLC Method

The HPLC analysis method modified from the method of Potard *et al*. (1999) was used for caffeine content analyzing. The condition of HPLC analysis was as following

Column:	reverse phase C_{18} column (RP8 250 x 4.6 mm, particle size, 5 μm)
Mobile phase:	water : acetonitrile : acetic acid (85 : 15 : 1 v/v, pH 2.5)
Flow rate:	1.0 ml/ min at 35°C.
Injection volume:	0.1 ml
Detection:	UV working wavelength at 271 nm

3.3.3.1 Standard Curve of Caffeine

There were 2 conditions of standard curve of caffeine were done. The first was a standard curve of caffeine in normal condition, which was the standard caffeine in isotonic phosphate buffer pH 7.4. The second was a standard curve of caffeine in skin condition, which was the standard caffeine in skin and was then extracted by solvent of water: acetonotrile (50: 50). The two conditions of standard curve of caffeine were done by the following:

a. Normal condition: Caffeine concentrations ranging from 2.0 to 50 µg/ml of water: acetonotrile (50: 50) were prepared, stood for 30 min, filtered through Whatman® filter paper No.1, and injected into the HPLC column to read their absorbance. Then a standard curve of caffeine concentration and absorbance were constructed.

b. Skin condition: 100 µg/ml of caffeine in solvent of water: acetonotrile (50: 50) with 4.5x4.5 cm full thickness skin, which cut into small pieces, will be prepared, stood for 24 hours, centrifuged 15,000 g for 15 min room temperature and filtered the rough debris through filter paper (Whatman® No.1). Afterward the filtrate will be two fold diluted for serial procedure with solvent of water: acetonotrile (50: 50) until solution of 2.0 to 50 µg/ml in water: acetonotrile (50: 50) will be obtained, filtered through filter paper; pore size of 0.2 µm (Sartorius,AG, Germany) before injected into the HPLC column to read their absorbance. Then the standard curve for the measurement of caffeine in skin used the supernatant form a skin homogenate that had contained a known amount of caffeine.

3.3.3.2 Validation of Quantitative Analysis of Caffeine

All data were triplicate done and given as mean ± S.D. The calibration curve of standard caffeine performed, as well as the caffeine analysis both intra-day and inter-day for 4 times a day and for consecutive 4 days respectively. It meaned that experiments were conducted both under 4 different environments of the day (in the morning, noon, afternoon, and evening); and for 4 different day.

3.3.4 Caffeine Formulation Development

Cosmetic caffeine products at least 4 formulations were developed in the form of emulsion. The type of emulsion was selected .The chemicals used in formulation development were selected from the following:

A: Oil were mineral oil and palm oil, either one component or mixed component

B: Stiffening agents were beeswax, stearic acid, cetyl alcohol, lanolin

C: Emollients were polysynlane, dimethicone, isopropyl myristate,

D: Moisturizing agents were propylene glycol, glyceryl monostearate

E: Emulsifiers were glyceryl monostearate, tween 80, span 80, cremophor A6, cremophor A25

F: Anti-oxidant was butyl hydroxyl toluene (BHT)

G: Preservative was paraben concentrate.

3.3.4.1 Evaluation of Caffeine Developed Formulations

The developed products as well as the acquired commercial products were comparatively tested on the following topics:

3.3.4.1.1 Type of the Emulsions

The products were identified their emulsion types by the following methods according to Javed *et al.,* (2007)**.**

A. Dilution test: One millilitre of the products was mixed vigorously with 10 ml of water. If the emulsion is w/o type, the emulsion would not be miscible with water, but it would be miscible with oil.

B. Conductivity Test: An assembly consisting of a pair of electrodes connected to a lamp was dipped into the product. If the product was o/w type, the lamp glowed and the value was read.

C. Dye Solubility Test: Some drops of 1% erythrosine solution were mixed to the product, then smeared it on the slide and observed under the microscope. If the product was o/w type, the background of the specimen would be red with circle clear zone of internal oil phase.

3.3.4.1.2 Physical Properties of Products

The following characteristics were evaluated after freshly preparation, two weeks storage, three months storage, and the stability tests:

A. Product texture, color, odor and homogeneity were recorded by visual observation and comparison.

B. pH of the products were measured with pH-meter using 1 g. of emulsion diluted with 9 ml of water and shook for 30 minutes until a homogeneous solution obtained, then recorded the pH value.

C. The viscosity of the products was measured with Brookfield Dial Reading Model RVT at a speed of 5 rpm and spindle number 4 for 60 sec.

3.3.4.2 Stability Study of Caffeine Developed Formulations

The stability tests were done both normal and accelerative conditions.

The normal condition of stability test was done by placing the products in the atmospheric moisture and temperature of 4^{o}C, and 45^{o}C separately.

The accelerative condition of stability test was doing freeze-and-thaw cycles, the products were kept in the refrigerator at 4^{o}C for 48 hours and alternately in the hot air oven at 45^{o}C for 48 hours for 6 cycles. The physical properties of the product after the stability test were evaluated every month for 3 months, and caffeine content were analyzed after extraction from the product.

Caffeine extraction was performed using the method according to Sousa *et al.* (2007). One gram of product was dissolved in 20 ml of water: acetonitrile (50:50) and the mixture was stirred with a magnetic stirrer for 30 minutes until a homogenous solution obtained. The solution was then centrifuged (3000 rpm, 15 minutes). The caffeine was in the water separated phase, and was quantified by HPLC method described in 3.3.3 and was calibrated by standard curve of caffeine in normal condition.

3.3.4.3 Percutaneous Absorption Study of Formulated and Commercial Product

The developed formulations and acquired commercial products were tested percutaneous absorption using the same method as described in 3.3.2

3.3.5 Data Analysis

The amount of caffeine permeation through the skin was presented as arithmetic mean ± S.D. and plot against time. Analysis of variance (ANOVA, single factor) was used in statistical comparisons. A difference was considered significantly at $p \leq 0.05$.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Typical Caffeine Emulsions

Two types of simple formulated emulsions were prepared and tested for their physical properties and types. Some differences were shown in Table 4. Although conductivity of the two products was different, their pH was nearly 5 which were suitable for skin. There was cetyl alcohol which was stiffening agent in the formula of W/O more than O/W, so W/O was more viscos than O/W.

Table 4 Some physical properties of two typical emulsions.

	O/W emulsion	**W/O emulsion**
Dilution	miscible	immiscible
Conductivity test (μs/ cm)*	0.47 ± 0.03	0.12 ± 0.09
Dye solubility test	Red background with clear circle zone of oil droplet	Clear background with red scattered water phase
pH*	5.18 ± 0.28	4.79 ± 0.08
Color	creamy white	creamy white
Texture - smoothness - oily	 +++ +	 ++++ ++++
Viscosity ($\times 10^3$ cP)*	12.40 ± 0.29	19.26 ± 0.41

The values are mean ± S.D. (n = 3)
Note: + = lowest, ++ = low, +++ = medium, ++++= high, +++++ = highest

4.2 Caffeine Standardization

The caffeine standard curves in two conditions, normal condition (caffeine in isotonic phosphate buffer pH 7.4) and skin condition (caffeine extracted from the skin with solvent of 1:1 water: acetonitrile), were firstly done. Although several HPLC methods which had been reported to measure the concentration of caffeine from skin absorption study, such as the method of Van de Sandt *et al.* (2004); Monti *et al*. (2001); Bolzinger *et al*.(2008) and Clement *et al.* (2000). But the HPLC method described in this study was modified from the method of Potard *et al*. (1999), which was simple and rapid.

The typical chromatogram of caffeine in normal conditions is shown in Figure 4. The caffeine concentration range of 2-10 μg/ml were quantified, and plotted between the peak areas under various caffeine chromatograms. The linear curve was obtained as shown in Figure 5, with linear correlations (R^2) of 0.999 and linear equation y = 78185x + 37427. All the data were triplicated and shown in Table 5.

The typical chromatogram of caffeine in skin condition is shown in Figure 6. The caffeine concentration range of 2-10 μg/ml were quantified, and plotted between the peak areas under various caffeine chromatograms. The linear curve was obtained as shown in Figure 7, with linear correlations (R^2) of 0.997 and linear equation y = 6679x + 22504. All the data were triplicated and shown in Table 6.

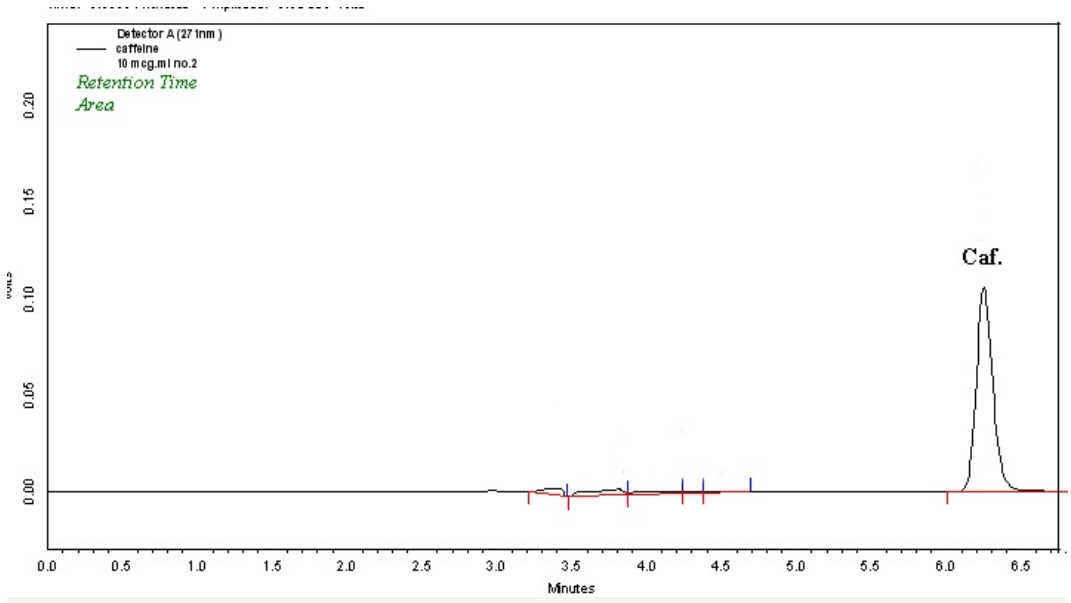


Figure 4 Caffeine peak detected by HPLC spectrophotography in normal condition (caffeine in isotonic phosphate buffer pH 7.4) (λ_{max} 271 at 6.3 min.).

Table 5 The peak area under the chromatogram of the standard caffeine (in the normal condition) concentration range of 2-10 µg/ml.

Concentration (µg/ml)	1st Time	2nd time	3rd time	Average ± S.D.
2	1193230	193151	193097	193159 ± 66.89
4	349312	349135	348948	349131 ± 18.02
6	502615	515262	502125	506667 ± 74.03
8	652811	672879	679225	668305 ± 25.88
10	806972	819018	821279	815423 ± 51.62

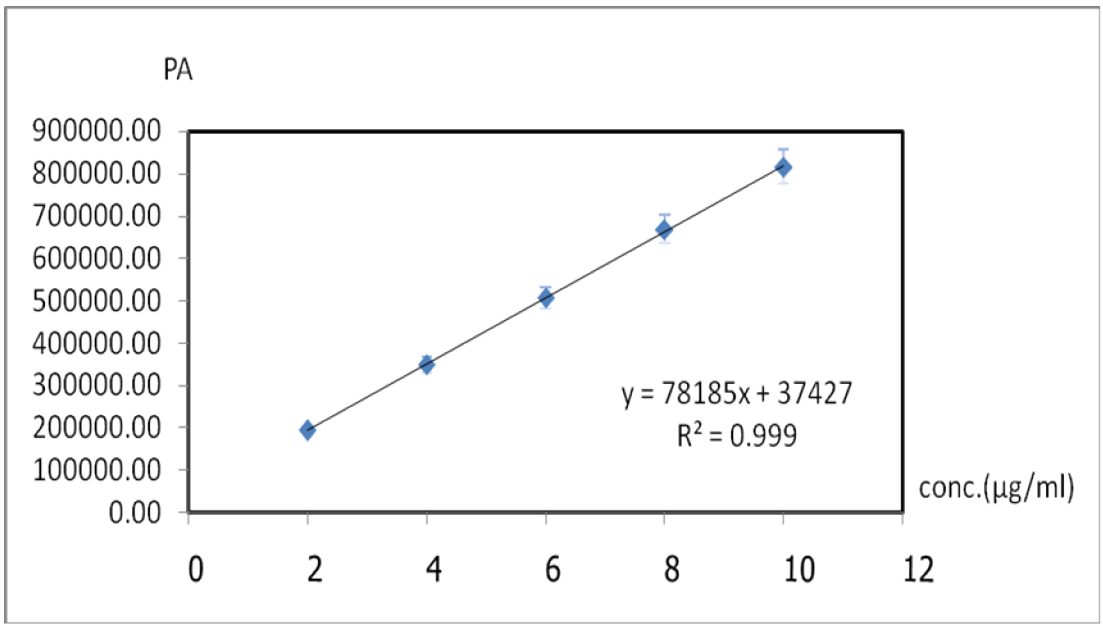


Figure 5 A standard calibration curve of caffeine in normal condition.

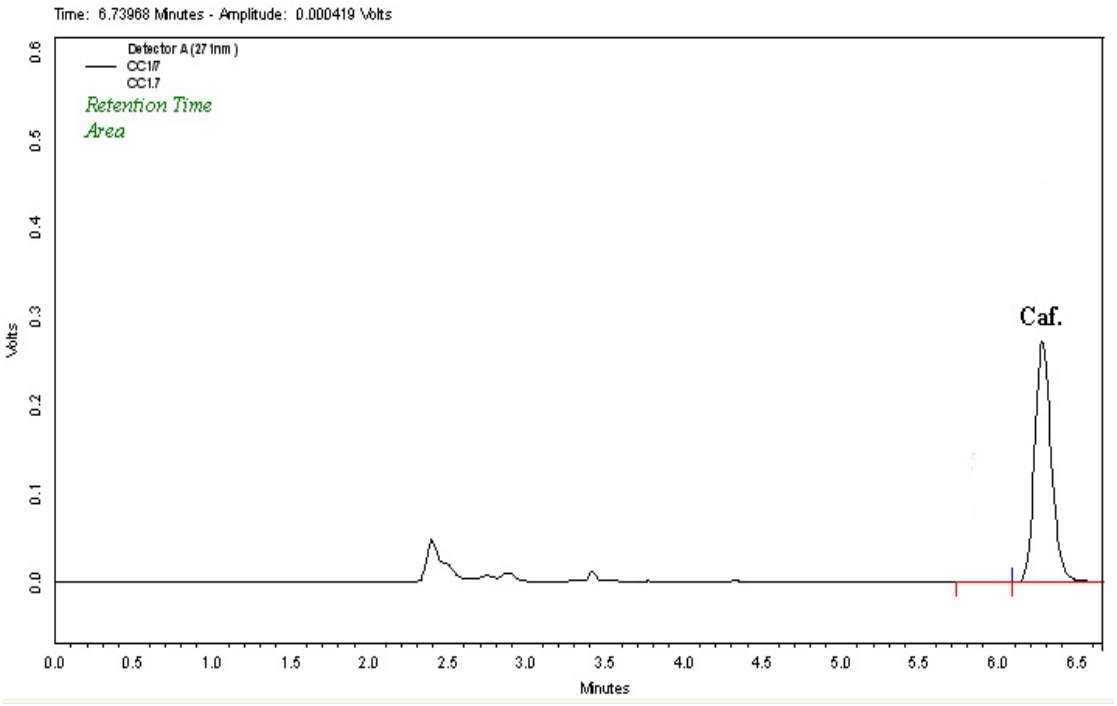


Figure 6 Caffeine peak detected by HPLC spectrophotography in skin condition, (λ_{max} 270 at 6.3 min).

Table 6 The peak area under the chromatogram of the standard caffeine (in skin condition) concentration range of 2-10 µg/ml.

Concentration (µg/ml)	1st Time	2nd time	3rd time	Average ± S.D.
2	35153	35225	35481	35286 ± 39.71
4	54454	50128	47206	50596 ± 59.47
6	60876	62238	64129	62414 ± 65.33
8	70072	75284	77962	74439 ± 25.12
10	90181	90008	90279	90156 ± 22.37

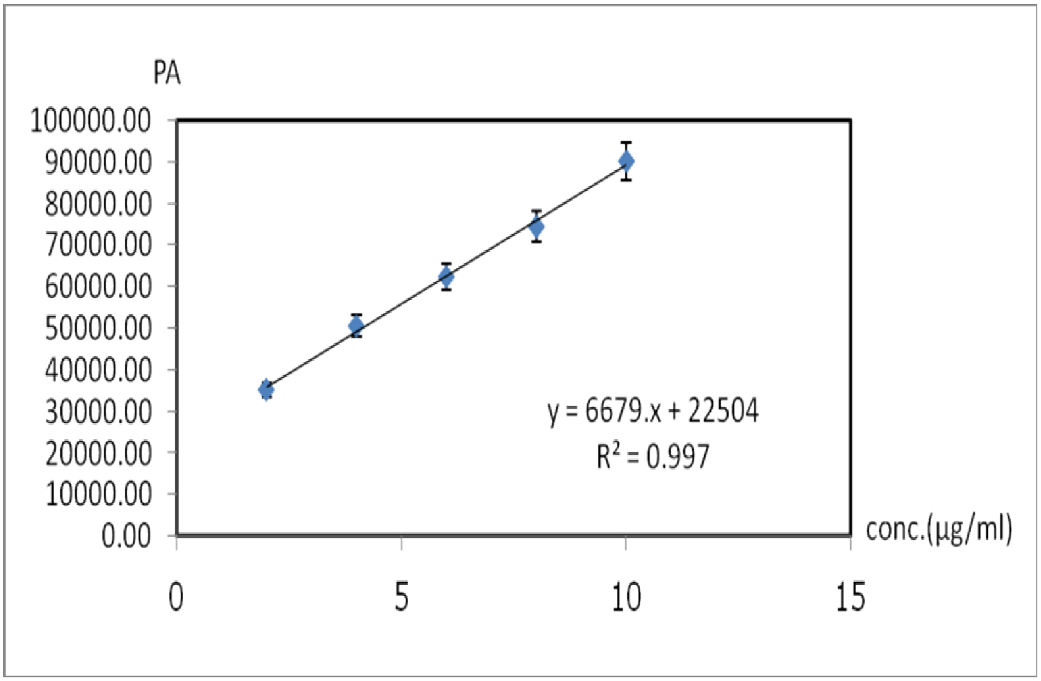


Figure 7 A standard calibration curve of caffeine in skin condition.

4.3 Validation of quantitative analysis of caffeine

The analytical accuracy and precision were evaluated intra-day and inter-day variability of the assay. At each concentration, the intra-day relative standard deviation (RSD) varies between 0.03 - 2.06%. Table 7 shows the intra-day reproducibility of the caffeine assay, the accuracy of the average measured concentration for each day reported in terms of percent recovery is between 99.51 – 100.86%. The inter-day reproducibility of method given in Table 8, shows that the standard deviation varies between 0.01 – 0.13 % and the recovery is in range 95.31 – 109.05%. The precision of the proposed HPLC method was established through repeatability of the responses, expressed as relative standard deviation in pharmaceutical analysis, a RSD less than 2% substantiates the precision of an analytical method (Jenke, 1996; Santoro *et al*., 2000).

Table 7 Intra-day variability of the caffeine analysis

Conc. (µg/ml)	Day	Mean of measured conc. (µg/ml)	Average recovery (%)	%RSD
2	1	1.99 ± 0.00	99.28	0.18
	2	1.98 ± 0.02	98.81	1.08
	3	1.97 ± 0.03	98.41	1.28
6	1	6.55 ± 0.02	109.24	0.29
	2	6.53 ± 0.01	108.89	0.20
	3	6.55 ± 0.01	109.15	0.15
10	1	10.35 ± 0.00	103.54	0.04
	2	10.31 ± 0.06	103.15	0.62
	3	10.34 ± 0.03	103.39	0.29

The values are mean ± S.D. (n = 3)

Table 8 Inter-day variability of caffeine analysis

Conc. (μg/ml)	Mean of measures conc. (μg/ml)	Average recovery (%)	%RSD
2	1.99 ± 0.48	99.59	0.03
6	6.00 ± 0.38	100.03	1.47
10	9.95 ± 0.37	99.51	1.01

The values are mean ± S.D. (n = 3)

4.4 *In vitro* skin permeation study of caffeine O/W and W/O emulsion: Franz Cell Diffusion

Skin permeation study, also known as percutaneous penetration, the absorption or penetration of a substance passed through the skin barrier and into the skin was measured. The objective of skin permeation studies was to determine how much chemical penetrated the skin and thereby whether it had the potential to be absorbed into the systemic circulation. In the present study, skin permeation studies of caffeine in two typical emulsions were done. The result is shown in Table 9. The concentration of caffeine in various sites of the studied dead fetal pig skin: the surface of the skin, in the skin and in the receptor compartment of modified Franz's diffusion cells, were collected and measured by HPLC technique, at various times. Then the percentage concentrations were calculated and reported. Caffeine both in O/W and W/O did diffused into the receptor compartment of modified Franz's diffusion cells less than 1% by wt. Most of the caffeine (around 97-99% by wt.) still remained on the skin. However, caffeine from W/O diffused into the skin more than O/W.

Table 9 Caffeine diffused in different sites of the dead fetal pig skin at different times comparing between O/W and W/O emulsion.

Time (hour)	Amount of caffeine	Diffused caffeine					
		From O/W emulsion			From W/O emulsion		
		On the surfaces	In the skin	In the receptor	On the surfaces	In the skin	In the receptor
0.5	mg	295.69 ± 0.03	4.30 ± 0.18	0.06 ± 0.01	292.63 ± 0.01	73.66 ± 0.31	0.00
	% w/w	98.55	1.43	0.02	79.89	20.11	0.00
1	mg	297.83 ± 0.01	2.16 ± 0.89	0.12 ± 0.28	294.27 ± 0.01	57.33 ± 0.24	0.02 ± 0.07
	% w/w	99.24	0.72	0.04	83.69	20.11	0.00
2	mg	298.94 ± 0.06	1.03 ± 0.41	0.33 ± 0.06	295.86 ± 0.01	41.32 ± 0.17	0.07 ± 0.03
	% w/w	99.55	0.34	0.11	87.73	12.25	0.02
4	mg	299.39 ± 0.03	0.52 ± 0.19	0.95 ± 0.11	297.04 ± 0.03	29.22 ± 0.12	0.42 ± 0.18
	% w/w	99.51	0.17	0.32	90.93	8.95	0.13
8	mg	299.41 ± 0.03	0.36 ± 0.12	2.30 ± 0.22	298.18 ± 0.02	17.20 ± 0.07	1.05 ± 0.43
	% w/w	99.12	0.12	0.76	94.23	5.43	0.33
10	mg	299.25 ± 0.03	0.26 ± 0.08	4.93 ± 0.28	298.97 ± 0.28	8.44 ± 0.03	1.88 ± 0.81
	% w/w	98.29	0.09	1.62	96.66	2.73	0.61
12	mg	299.05 ± 0.09	0.19 ± 0.05	7.57 ± 2.45	299.24 ± 0.03	4.06 ± 0.01	3.53 ± 1.50
	% w/w	97.47	0.06	2.47	96.66	1.32	1.15
24	mg	298.64 ± 0.65	0.16 ± 0.04	12.05 ± 2.76	299.17 ± 0.77	1.32± 0.01	5.64 ± 2.47
	% w/w	96.07	0.05	3.88	97.31	0.86	1.83

The values are mean ± S.D. (n = 3)

The data of diffused caffeine into the receptor compartment of modified Franz's diffusion cells, from Table 9, could be presented as the graph between diffused caffeine and time as shown in Figure 8. It was shown that caffeine from O/W diffused through the skin into the receptor compartment after 24 hours, was 120.51±27.60 µg/ml or 0.40±27.60 % by wt, while caffeine from W/O diffused through the skin into the receptor compartment after 24 hours, was 56.41±24.65 µg/ml or 0.19 ± 8.74 % by wt. That was O/W diffused through the skin more than W/O did, because it was believed that O/W more moisten the skin than W/O which made the skin more porous.(Bolzinger, *et al*., 2008)

By the way, the data of diffused caffeine in the skin , from Table 9, was shown that caffeine from O/W diffused into the skin after 24 hours was 15.88±0.36 µg/ml or 0.05 ± 0.36 % by wt., whereas caffeine from O/W diffused into the skin after 24 hours was 26.41±0.79 µg/ml or 0.09 ± 0.79 % by wt. It meaned that W/O could be deposited in the skin more than O/W, because the external phase of W/O was similar to the component of the skin which enhanced the miscibility. (Dias, *et al*., 2007)

However, most of the caffeine from both O/W and W/O were still remain on the skin, did not diffuse through the skin. It was supposed that caffeine did not well dissolve in water, so it did slightly penetrate into the hydrated skin. (Faith, 2006)

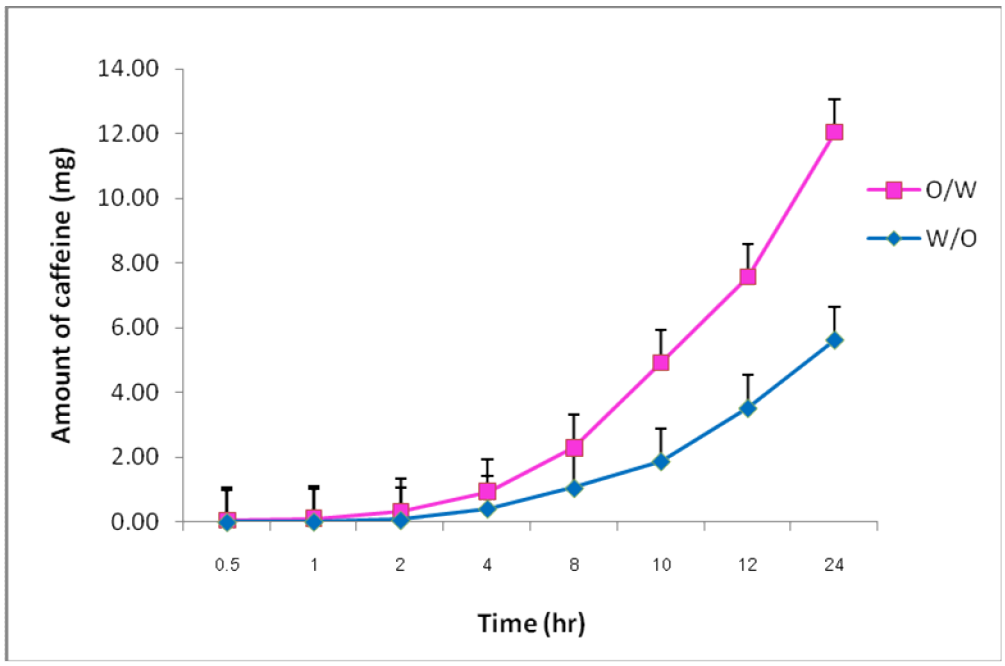


Figure 8 Diffusion of caffeine into the receptor compartment of Franz cell, compared between diffused caffeine from W/O emulsion and O/W emulsion, at various time.

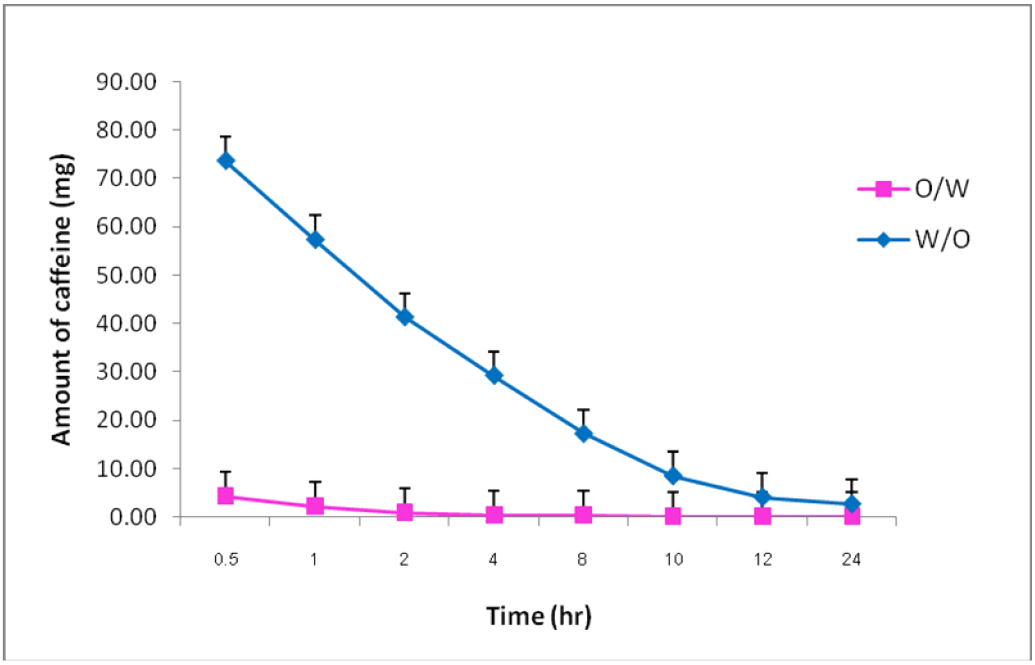


Figure 9 Diffusion of caffeine in the skin, compared between diffused caffeine from W/O emulsion and O/W emulsion, at various time.

Summarizely, the caffeine in both types of tested emulsion did not penetrate through the skin so well, because there were not any enhancers in the formulation of both types of emulsion. Now that caffeine from O/W diffused into the receptor site or circulation more than W/O, so the safety caffeine cream to be formulated might be W/O emulsion, which caffeine was not so much penetrated into blood stream. However, the formulation of cosmetic caffeine must be further developed for a better penetration into cutaneous layer. Moreover, the time to reach a steady state was long, ranging from 16 to 18 hrs., so the vehicle used and massaging time were considered in the caffeine cosmetic products. Conclusively W/O emulsion should be considered for cosmetic formulation and preparation, because caffeine in W/O emulsion was absorbed into the skin more than O/W emulsion.

4.5 Formulation of caffeine emulsions

Four formulated preparations were developed from the typical W/O emulsion as shown in Table 10. Formulas 1 to 3 were composed of two oils with various proportions. Formulas 2 to 4 consisted of one or two enhancers. Formulas 2 and 4 were added one and two stiffening agents respectively. Antioxidant and preservative were used in all 4 formulas. Then some physical properties including dilution, conductivity, dye solubility, pH, texture and viscosity were observed as shown in Table 11. All 4 formulas had similar features and properties, except viscosity. It was found that formula 3 was most viscous. It was very hard to make the decision which formula was the best one. So the stability test was done and used as a criterion to select the best formula to study skin penetration.

Table 10 Four formulated caffeine W/O emulsions.

Compositions	Formulation 1 (g)	Formulation 2 (g)	Formulation 3 (g)	Formulation 4 (g)
Mineral oil	22	15	12	-
Palm oil	12	10	10	14
Bees wax	-	5	-	6
Dimethicone	1	1	-	-
Lanolin	-	2	-	1
BHT	0.2	0.2	0.2	0.2
Cetyl alcohol	3	3	2	1
Stearic acid	-	-	-	4
Polysynlane	5	-	8	12
GMS	2	-	2	-
Tween 80	0.35	0.35	0.35	0.30
Span 80	5.65	4.65	5.65	3.70
Propylene glycol	-	-	10	10
Paraben concentrate	1	1	1	1
Isopropyl myristate	-	4	4	-
Caffeine	3	3	3	3
Water q.s. to	100	100	100	100

Table 11 Some physical properties of 4 formulated W/O emulsions.

Physical properties	**Formulation 1**	**Formulation 2**	**Formulation 3**	**Formulation 4**
Calculated HLB	4.92	4.98	4.92	5.04
Dilution	immiscible	immiscible	immiscible	immiscible
Conductivity test (μs/cm)	0.29 ± 0.15	0.24 ± 0.15	0.30 ± 0.20	0.22 ± 0.06
pH	4.85 ± 0.00	4.25 ± 0.00	4.97 ± 0.00	4.05 ± 0.00
Color	white	white	white	white
Viscosity ($\times 10^3$ cP)	16.0±0.00	18.0±0.00	19.6±0.29	18.9±0.00

The values are mean ± S.D. (n = 3)

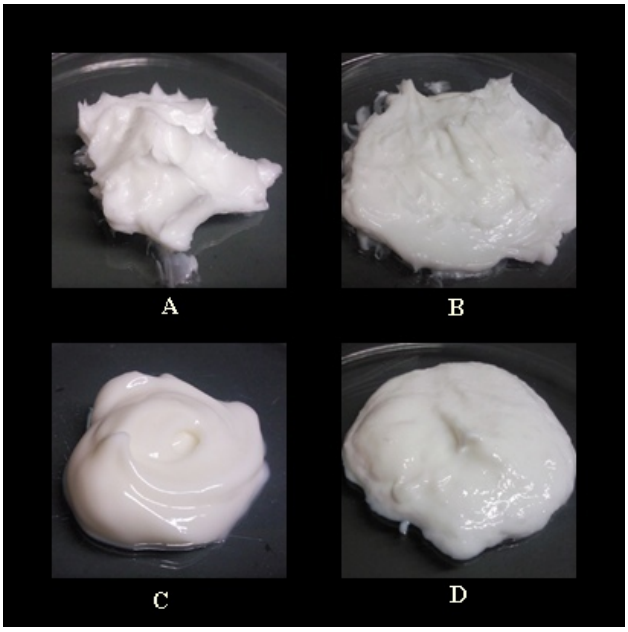


Figure 10 The morphological pictures of 4 formulated caffeine W/O emulsions (A: Formulation 1, B: Formulation 2, C: Formulation 3, D: Formulation 4)

Table 12 Some physical properties of the selected formulated W/O emulsions in the stability test.

Physical properties	**Before freeze thaw test**	**After freeze thaw test**	**After 3 months storage at RT**
Conductivity test	0.29 ± 0.10 µs/cm	0.28 ± 0.02µs/cm	0.28 ± 0.09 µs/cm
pH	4.97 ± 0.27	5.18 ± 0.24	4.86v± 0.34
Color	white	unchanged	unchanged
Texture	smooth cream	unchanged	unchanged
Viscosity ($\times 10^3$ cP)	19.6±0.29	19.5±0.58	19.7±0.29

The values are mean ± S.D. (n = 3)

In the stability test of the 4 formulated W/O emulsions. Formulation 3 emulsion was most stable, because it was most viscous. It showed good appearance, good dispersion, pH was 4.97 which was the most appropriate to the skin of pH 5.5 as shown in Table 11. While the other 3 formulated emulsions were coalescing, poor disperse to the surface of the skin, and had the rough texture. Therefore, the formulation 3 was selected for further skin permeation study.

4.6 Percutaneous absorption study of formulated and commercial product

The commercial caffeine cosmetic product was studied for some physical properties compared to the selected third formulated W/O emulsion as shown in Table 13. The result showed that commercial caffeine product was O/W emulsion type, because the O/W emulsion was the most popularly produced in the industry. It was well soluble in water; light, soft, gentle and not viscous cream. But its pH was a

neutral one, higher than the pH of the skin. The percutaneous absorption of the selected formulated W/O emulsion and commercial product at various times is shown in Table 14. Then the graph was plotted between the caffeine concentration in the receptor compartment of Franz cell and in the skin versus time as shown in Figure 12 and 13, respectively.

Table 13 Some physical properties of selected formulated W/O emulsion and commercial caffeine cosmetic product.

Physical properties	**Formulated W/O emulsion**	**Commercial product**
Dilution	immiscible	miscible
Conductivity test	0.29 ± 010 µs/cm	0.72 ± 0.00 µs/cm
pH	4.97 ± 0.02	6.99 ± 0.01
Color	white	strong pink
Texture	gentle cream	light, soft, gentle cream
Viscosity ($\times 10^3$ cP)	19.6 ± 0.29	2.6519 ± 0.06

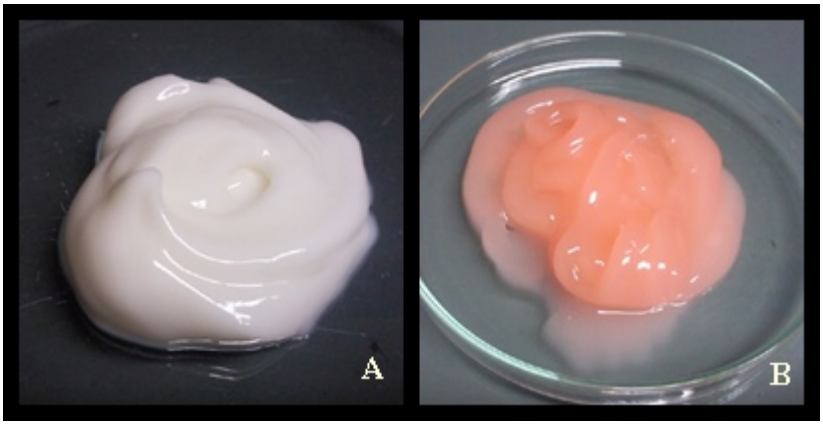


Figure 11 The morphological pictures of formulated W/O caffeine emulsions (A) and commercial caffeine product (B)

Table 14 Caffeine diffused in different sites of the dead fetal pig skin at various times comparing between selected formulated caffeine W/O emulsion and commercial caffeine product.

Time (hour)	Amount of caffeine	Diffused caffeine							
		Selected W/O formulated emulsion				Commercial caffeine product			
		On the surfaces	In the skin	In the receptor	Ratio*	On the surfaces	In the skin	In the receptor	Ratio*
0.5	mg	287.49 ± 0.02	7.60 ± 0.34	0.04 ± 0.03	200.11	17.50 ± 0.00	1.47 ± 0.02	0.08 ± 0.01	17.45
	% w/w	97.41	2.58	0.01		91.86	7.69	0.44	
1	mg	286.94 ± 0.06	7.85 ± 0.04	0.04 ± 0.01	182.52	19.39 ± 0.01	1.65 ± 0.51	0.10 ± 0.00	17.38
	% w/w	97.32	2.66	0.01		91.74	7.81	0.45	
2	mg	284.46 ± 0.09	6.20 ± 0.02	0.06 ± 0.05	103.29	19.73 ± 0.13	1.66 ± 0.05	0.17 ± 0.00	9.91
	% w/w	97.85	2.13	0.02		91.55	7.68	0.77	
4	mg	280.93 ± 0.07	5.78 ± 0.02	0.51 ± 0.24	45.04	18.56 ± 0.02	1.66 ± 0.03	0.38 ± 0.00	4.40
	% w/w	97.81	2.01	0.18		90.09	8.08	1.83	
8	mg	271.23 ± 0.15	4.57 ± 0.06	1.14 ± 0.28	4.01	18.24 ± 0.02	1.40 ± 0.04	1.64 ± 0.00	0.85
	% w/w	97.94	1.65	0.41		85.70	6.58	7.71	
10	mg	253.73 ± 0.02	3.25 ± 0.07	2.10 ± 0.61	1.55	18.64 ± 0.08	1.29 ± 0.06	3.44 ± 0.01	0.38
	% w/w	97.93	1.25	0.81		79.78	5.52	14.70	
12	mg	235.08 ± 0.04	1.60 ± 0.05	1.85 ± 1.75	0.36	18.24 ± 0.01	0.88 ± 0.04	6.74 ± 0.01	0.13
	% w/w	97.48	0.66	19.58		70.53	3.41	26.07	
24	mg	204.95 ± 0.02	1.48 ± 0.04	8.13 ± 1.04	0.18	18.83 ± 0.01	0.28 ± 0.00	10.15 ± 0.01	0.03
	% w/w	95.52	0.69	3.79		64.37	3.41	34.69	

The values are mean ± S.D. (n = 3), * Ratio of % caffeine in the skin: in the receptor compartment

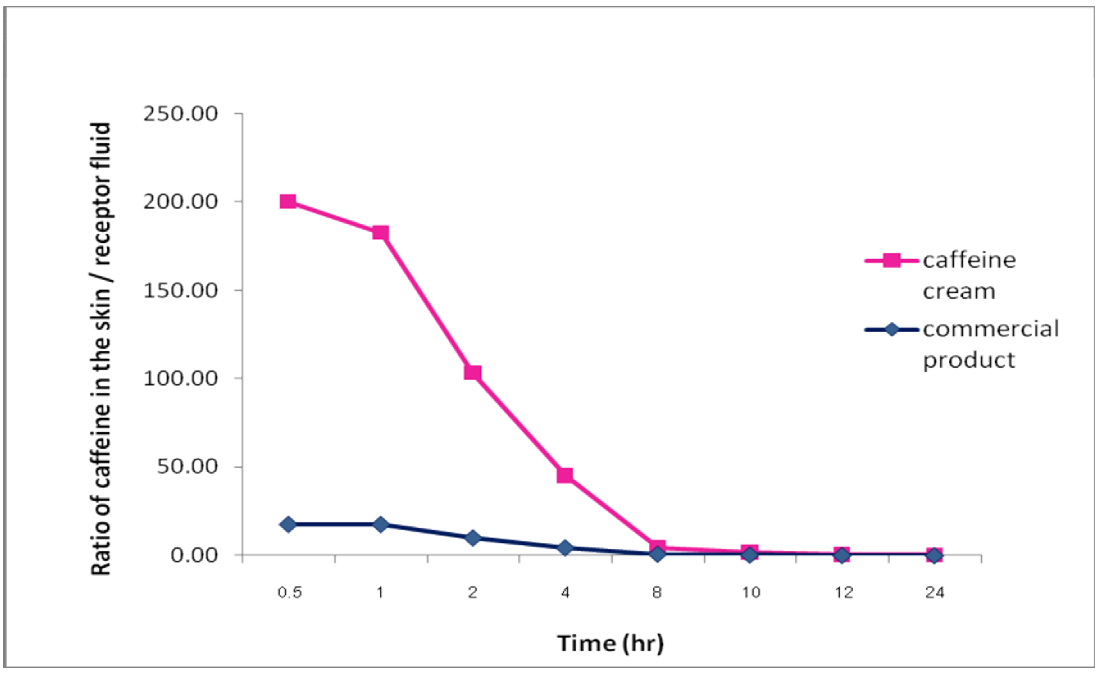


Figure 12 The plotted curve between the ratio of penetrated caffeine (%) in the skin/ in the receptor of Franz cell and penetrating time, compared between the formulated caffeine W/O emulsion with commercial caffeine product.

The results could be concluded that commercial caffeine product did not penetrate through the skin, but the caffeine was still most remained on the skin. So the caffeine did not absorb into the skin and body. The cosmetic effectiveness had to be studied. While the formulated W/O emulsion penetrated into the skin and was still remain in the skin, so it was useful for cosmetic aspect.

It has been reported by Franklin *et al. (*2004) increasing the temperature of the receptor was also found to enhance the transdermal permeation of all the penetrants involved due to unavailability of sufficient human epidermis from the same donor, skin permeation studies for caffeine could not be performed at 23ºC. The amount of caffeine penetrant retained in the epidermis was increasing whilst the transdermal fluxes increased with increasing receptor temperature.

The percutaneous absorption of caffeine from two vehicles, an emulsion and an acetone solution. Caffeine was assessed by HPLC, two phases were distinguished in the percutaneous absorption of caffeine: a higher filling up of the stratum corneum with the O/W emulsion than with the acetone solution (Chambin-Remoussenard, 1993).

Gernerally, the Organization for Economic Cooperation and Development recommends caffeine as a reference substance for in vitro skin absorption tests using Franz diffusion cells so the differentiation of penetration pathways by combining the Franz diffusion cells and the follicle closing technique. Caffeine showed a surprisingly high rate of penetration through the follicular shunts *in vitro* (Trauer *et al.*, 2010).

In addition, the delivery of one compound through the skin from a simple vehicle (one phase) can be considered as a compromise between its thermodynamic activity in the vehicle and the partition coefficient between the vehicle and the stratum corneum. However, with heterogeneous vehicles (emulsions) the global release of the active ingredient from the formulation results from successive exchanges occurring between the continuous and discontinuous phases. It thus appears that the percutaneous penetration of one defined ingredient from a multiple emulsion cannot be predicted simply from its chemical characteristics and from the type of emulsions used. In fact, the physical impact of the application procedures that are likely to dramatically modify the whole structure of the formulation applied to the skin surface would be of major importance (Doucet *et al.,* 1998).

CHAPTER 5

CONCLUSION

Caffeine is a xanthine alkaloid and widely used in cosmetic aspect, so it is interesting to be test which type of cream base is suitable for caffeine penetration into the skin. As consumers and health care professionals had become educated about safety, the percutaneous penetration of cosmetic and fragrance ingredients had gained interest. The newborn pig that died shortly after birth was used in the skin penetration test of the caffeine into the two typical emulsions, W/O and O/W emulsion. The amount of caffeine diffused from O/W emulsion through the full thickness skin was 12.05 ± 2.76 mg (3.88 % w/w) after 24 hrs experiment, while caffeine diffused from W/O emulsion through the full thickness skin was 5.64 ± 2.47 mg (1.83 % w/w). Whereas the caffeine, from O/W emulsion penetration, remained in the skin was 0.16 ± 0.04 mg (0.05 % w/w), the caffeine from W/O emulsion penetration remained in the skin was 1.32 ± 0.01 mg (0.86 % w/w). This shown that caffeine permeated through the skin from O/W emulsion more than from the W/O emulsion and the amount of remained caffeine in the skin from W/O emulsion is more than from O/W emulsion. So it is conclude that the appropriate type of cream for caffeine effectiveness in cosmetic product is W/O emulsion because the caffeine was still in the skin more than penetrated through the skin and in the circulating system. The penetration of caffeine between the formulated caffeine W/O emulsion and the commercial caffeine product was also compared. The results indicated that the ratios of penetrated caffeine in the skin: receptor compartment were less than those of formulated caffeine W/O

emulsion. The formulated caffeine W/O emulsion, containing more than one skin penetration enhancer, such as 10% propylene glycol, 2% cetyl alcohol, 2% glyceryl monostearate, tween 80 around 0.35%, and span 80 around 5.65% by weight, had a higher caffeine penetration into the skin.

The cosmetic active ingredients rarely penetrate the skin. However, many experts today suspect that penetration occurs with most ingredients. The safety and effectiveness was considered. In conclusion, the results shown that caffeine W/O emulsion type of cream can be used to reduce the risk of caffeine absorption into the bloodstream and be save the consumers.

A fast drug delivery of caffeine occurs through shunt routes. Therefore, hair follicles are considerable weak spots in protective sheath against penetration into the body by hydrophilic substances. These findings are of importance for the development and optimization of topically applied drugs and cosmetics. In addition, such properties must be considered in the development of skin protection measures. (Nina *et al*., 2007)

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VITAE

Name Miss Sunisa Meesen

Student ID 4910720040

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Sciences (Aquatic Sciences)	Prince of Songkla University	2006

Scholarship Awards during Enrolment

Academic-Excellence Program in Pharmacuetical Sciences, Prince of Sonkla University, 2009.

List of Publication and Proceedings

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