ผลของอนุภาคแคลเซียมคาร์บอเนตขนาดนาโนเมตรต่อประสิทธิภาพการป้องกันแสงแดดในหลอด
ทดลองของสารกันแดดทางกายภาพ

นางสาววชิราภรณ์ ยืนสุข

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EFFECT OF CALCIUM CARBONATE NANOPARTICLES ON *IN VITRO* PHOTOPROTECTION

EFFICIENCY OF PHYSICAL SUNSCREENS

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Department of Pharmaceutics and Industrial Pharmacy

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วชิราภรณ์ ยืนสุข : ผลของอนุภาคแคลเซียมคาร์บอเนตขนาดนาโนเมตรต่อประสิทธิภาพการป้องกันแสงแดดในหลอดทดลองของสารกันแดดทางกายภาพ (EFFECT OF CALCIUM CARBONATE NANOPARTICLES ON *IN VITRO* PHOTOPROTECTION EFFICIENCY OF PHYSICAL SUNSCREENS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภญ. ดร.นฤพร สุตัณฑวิบูลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ภญ. ดร.จิตติมา ชัชวาลย์สายสินธ์, หน้า.

สารกันแดดทางกายภาพมีความปลอดภัย  คงตัวต่อแสงและสามารถป้องกันรังสีอัลตราไวโอเลตได้ครอบคลุมมากกว่า แต่ยังมีค่าเอสพีเอฟน้อยกว่าสารกันแดดทางเคมี การศึกษานี้จึงนำแคลเซียมคาร์บอเนตขนาดอนุภาคนาโนเมตรผสมกับไทเทเนียมไดออกไซด์และซิงค์ออกไซด์เพื่อเพิ่มประสิทธิภาพการป้องกันแสงแดดแก่สารกันแดดทางกายภาพ  โดยความเร็วรอบและระยะเวลาการเตรียมตัวอย่างด้วยเครื่องกวนความเร็วสูงถูกเปลี่ยนแปลงเพื่อศึกษาสภาวะที่เหมาะสมในน้ำมันไดเมทิโคน ผลแสดงว่าความเร็ว 2000 รอบต่อนาทีและเวลา 10 นาทีเป็นวิธีการเตรียมที่เหมาะสม

ความเข้มข้นของสารกันแดดทางกายภาพและแคลเซียมคาร์บอเนตถูกเปลี่ยนแปลงเพื่อศึกษาการเพิ่มประสิทธิภาพการป้องกันแสงแดดโดยใช้เครื่องวัดการดูดกลืนแสง  ผลพบว่าการผสมแคลเซียมคาร์บอเนตสามารถเพิ่มประสิทธิภาพการป้องกันแสงแดดในทุกความเข้มข้นของสารกันแดดทางกายภาพ ซึ่งผลการเพิ่มประสิทธิภาพการป้องกันแสงแดดนี้ถูกยืนยันด้วยการวัดค่าเอสพีเอฟและค่ายูวีเอพีเอฟจากเครื่องทดสอบค่าการป้องกันแสง  ไทเทเนียมไดออกไซด์ผสมแคลเซียมคาร์บอเนตเกิดการเสริมฤทธิ์ของประสิทธิภาพการป้องกันแสงแดด   ในขณะที่ซิงค์ออกไซด์ผสมแคลเซียมคาร์บอเนตแสดงผลการรวมฤทธิ์เท่านั้น ดังนั้นไทเทเนียมไดออกไซด์ผสมแคลเซียมคาร์บอเนตจึงถูกนำไปเตรียมในสูตรอิมัลชันทั้งชนิดน้ำในน้ำมันและชนิดน้ำมันในน้ำ   เพื่อศึกษาชนิดของอิมัลชันที่เหมาะสม ผลพบว่าอิมัลชันชนิดน้ำในน้ำมันมีค่าเอสพีเอฟและค่ายูวีเอพีเอฟที่มากกว่า ($p < 0.05$) จึงสรุปได้ว่าแคลเซียมคาร์บอเนตขนาดอนุภาคนาโนเมตรสามารถนำไปใช้เป็นสารเพิ่มประสิทธิภาพการป้องกันแสงแดดแก่สารกันแดดทางกายภาพทั้งสองชนิด   โดยเฉพาะไทเทเนียมไดออกไซด์ซึ่งจะสามารถลดความเข้มข้นของไทเทเนียมไดออกไซด์ในสูตรตำรับได้

| ภาควิชา | วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม | ลายมือชื่อนิสิต | ............................................. |
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# # 5776254733 : MAJOR COSMETIC SCIENCE

WACHIRAPHON YUENSOOK: EFFECT OF CALCIUM CARBONATE NANOPARTICLES ON *IN VITRO* PHOTOPROTECTION EFFICIENCY OF PHYSICAL SUNSCREENS. ADVISOR: NARUEPORN SUTANTHAVIBUL, Ph.D., CO-ADVISOR: JITTIMA CHATCHAWALSAISIN, Ph.D., pp.

Physical sunscreens are safer, more photostable and broader spectral protection but exhibit lower SPF than chemical sunscreens. In this study, calcium carbonate nanoparticles were combined with both titanium dioxide and zinc oxide to improve UV protection efficiency. Speed and duration of high speed disperser were varied to investigate suitable dispersing condition in dimethicone oil. The results showed that speed of 2000 rpm and duration of 10 minutes was the optimal condition.

Concentrations of physical sunscreens and calcium carbonate were varied to study the UV improving protection using UV-Visible spectrophotometer. It was found that addition of calcium carbonate can improve UV absorption in every physical sunscreen concentrations. The enhancing results were confirmed by evaluating the sun protection factor SPF and UVA protection factor UVA-PF values using SPF analyzer. Titanium dioxide combined with calcium carbonate resulted in synergistic effect on UV protection whereas the combination of zinc oxide and calcium carbonate exhibited only additive effect. Therefore, titanium dioxide combined with calcium carbonate was chosen to be formulated in water in oil (w/o) and oil in water (o/w) emulsions to investigate for suitable emulsion type. The results showed that w/o had better SPF and UVA-PF values than o/w ($p<0.05$). In conclusion, calcium carbonate nanoparticles can be used as photoprotection enhancer for both physical sunscreens. The enhancement was especially high for titanium dioxide where it can reduce the level of titanium dioxide used in the formulation.

# ACKNOWLEDGEMENTS

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| | |
|---|---|
| UV | Ultra violet |
| SPF | Sun protection factor |
| UVA | Ultra violet A |
| rpm | round per minute |
| o/w | oil in water |
| w/o | water in oil |
| UVB | Ultra violet B |
| $TiO_2$ | Titanium dioxide |
| ZnO | Zinc oxide |
| SEM | Scanning electron microscopy |
| UV-Vis | Ultra violet- Visible |
| UVA-PF | Ultra violet A- protection factor |
| w/w | weight per weight |
| UVC | Ultra violet C |
| nm | nanometers |
| UVR | Ultra violet radiations |
| UVA-I | Ultra violet A- I |
| UVA-II | Ultra violet A- II |
| DNA | Deoxyribonucleic acid |
| FDA | Food and drug administration |
| RI | Refractive index |
| $CaCO_3$ | Calcium carbonate |
| MED | Minimal erythema dose |
| PPD | Persistent pigment darkening |
| IPD | Immediate pigment darkening |
| h | hour |
| MPF | Monochromatic protection factor |
| PMMA | Polymethylmethacrylate |
| ISO | International organization for standardization |

| | |
|---|---|
| mm | millimeters |
| cm | centimeters |
| NPCC | Calcium carbonate nanoparticles |
| $Al(OH)_3$ | Aluminum hydroxide |
| ZrO | Zirconia dioxide |
| SEI | Secondary electron image |
| kV | kilovolts |
| PEG | polyethylene glycol |
| USP | United states pharmacopeia |
| NF | National formulary |
| SD | Standard deviation |
| RSD | Relative standard deviation |
| $abs_o$ | Overall UV absorption |
| $abs_s$ | Specific UV absorption |
| $Sc_s$ | specific UV scattering |
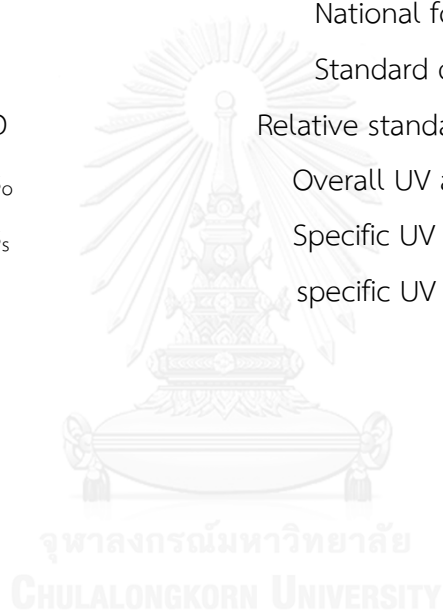
# CHAPTER I
# INTRODUCTION

Ultraviolet ray consists of ultraviolet A (UVA), ultraviolet B (UVB) and ultraviolet C (UVC). UVA, wavelength at 320-400 nanometers (nm), can penetrate to dermis layer and then denature collagen and elastin structure which is the cause of skin aging and tanning in humans. UVB, wavelength at 290-400 nm, is higher energy than UVA. It penetrates into epidermis layer causing sunburn or solar erythema. Additional, exposure high intensity of both UVA and UVB closely link photocarcinogenesis development. While UVC can be absorbed by ozone layer, so it cannot rise on earth (Diffey, 2005; Lin and Lin, 2011; Nelson, 2005; Shaath, 2005a).

Sunscreen is the most effective in preventing humans from the dangers of ultraviolet radiations (UVR). There are commercially two types of sunscreens, physical and chemical sunscreens. Physical sunscreens show some advantages over chemical sunscreens, such as non irritation, safe, photostability and broader spectral protection (Couteau et al., 2008; Smijs and Pavel, 2012). Moreover, it is useful for people with sensitive skin and who are allergic to chemical sunscreens (Manaia et al., 2013). Titanium dioxide and zinc oxide have been used as physical sunscreens for many decades. When light falls on materials, an incident light is either reflected, scattered or absorbed. Depending on the material characteristics. At wavelengths below 360 nm, approximately 90% of UVR is attenuated by absorption (More, 2007). Titanium dioxide is a scatterer in UVA range (wavelengths 320-400 nm) and an absorber in UVB range (wavelengths 290-320 nm). Whereas zinc oxide is a broad spectrum absorber at wavelengths from 200 to 380 nm (Morfesis and Fairhurst, 2005).

The pigment grade of both physical sunscreens present white coloration when applied to the skin because of the reflection of most visible light. Nanotechnology has been used to reduce whiteness on the skin providing cosmetical acceptability as its less visible light reflection (Lin and Lin, 2011; Manaia et al., 2013; Wokovich et al., 2009). Nevertheless, physical sunscreens appear to have lower photoprotection efficiency than chemical sunscreens, especially in UVB range (Shaath, 2005b). Thus, this study aims to select another potential substance to enhance photoprotection efficiency of physical sunscreens. Calcium carbonate is

chosen for its safety, inexpensiveness and environmentally friendly. The pigment of calcium carbonate also creates white appearance when applied so Metallurgy and Materials Science Research Institute, Chulalongkorn University synthesized nanoparticle of calcium carbonate (particle size 30-70 nm) by precipitation reaction to avoid this drawback (Krisana Siralertmukul et al., 2012a, 2012b).

Sato and Ikeya (2004) reported that calcium carbonate by itself do not absorb ultraviolet radiation but when combined with vitamin C, it was found to improve UVB absorption of vitamin C. In addition, combinations of calcium carbonate and chemical sunscreens (ethylhexylmethoxy cinnamate and bis-ethylhexyloxyphenol methoxyphenol triazine) increased the Sun Protection Factor (SPF) depending on calcium carbonate concentrations (Mueller et al., 2008). Kose Cosmetics incorporated calcium carbonate in their sunscreen formulations which consisted of physical and chemical sunscreens, such as titanium dioxide, zinc oxide, benzophenone, benzoic acid and salicylic acid, to induce complete photoprotection throughout UVA and UVB range (Fukuda and Naito, 1985).

One of the objectives of this study is to evaluate the sun protection efficiency of titanium dioxide and zinc oxide when combined with calcium carbonate nanoparticles in various concentrations. There are two commonly methods of *in vitro* photoprotection evaluation, UV-Visible spectrophotometry and sun protection factor analysis. Spectrophotometric analysis operates by measuring the amount of ultraviolet radiation absorbed by a substance in vehicles. This method is simple, rapid and reasonable cost. The other method is sun protection factor analysis, which is the procedure designed for predicting the Sun Protection Factor of cosmetic sunscreen products and raw materials. UV light passes through the product, which is spread on a special plate, which simulates skin humans. A detector on the other side of the plate records the level of absorbance at each wavelength (Shaath, 2005b; Taylor, Lynch and Dlugos, 2013). This investigation will compare the sun protection efficiency of physical sunscreens when combined with calcium carbonate nanoparticles between using UV-Vis spectrophotometer and SPF analyzer instruments.

Emulsion is the most popular vehicle for the inorganic materials. Either water in oil (w/o) and oil in water (o/w) emulsions can be used in physical sunscreens, thereby allowing any type of skin feel to be achieved. Any other desired ingredients, including chemical sunscreens, can be readily incorporated (Lowe, 1997). The another aims of this study is to compare the sun protection factor and UVA protection factor of physical sunscreens combined with calcium carbonate between water in oil and oil in water emulsions to determine the suitable emulsion type of the combination.

**Objective of this study**

1. To evaluate UV protection efficiency of titanium dioxide and zinc oxide when combined with calcium carbonate nanoparticles in various concentrations.

2. To investigate the correlation of the UV protection efficiency of titanium dioxide and zinc oxide when combined with calcium carbonate nanoparticles between using UV-Visible spectrophotometer and SPF analyzer.

3. To compare UV protection efficiency of titanium dioxide combined with calcium carbonate nanoparticles between water in oil and oil in water emulsions.

# CHAPTER II
# LITERATURE REVIEWS

Sunlight is "good" and beneficial to human's health in synthesis of vitamin D. However, the particularly ultraviolet radiation of sunlight (UVR 290-400 nm) is a major factor which is dangerous to human health. For example, UVR cause sunburn, tanning, pigmentation, skin aging, solar keratosis, skin cancer (non-melanoma and melanoma), and immunosuppression. The electromagnetic spectrum of solar radiation is divided from higher energy and shorter wavelength, gamma and x-rays waves, to the lower energy and longer wavelength, visible, infrared, microwaves, and radio waves. The ozone layer of earth can absorb approximately 30-40% of solar radiation in the atmosphere (Lowe and Friedlander, 1997)

Constituents of solar radiation on earth is nearly 50% visible light (wavelength 400-800 nm), 40% infrared light (wavelength 1300-1700 nm), and 10% UVR (wavelength 10-400 nm). There are three types of ultraviolet radiation (UVR) divided by the wavelength. The first is ultraviolet A (UVA), wavelength 320 to 400 nm, it is subdivided into UVA-II or the shorter UVA rays (320-340 nm), and UVA-I or the longer UVA rays (340-400 nm). The second type of UVR presents broad wavelength between 290-320 nm, it is called ultraviolet B (UVB) which has maximum intensity peak approximately at 307 nm. And the last one is the most energetic ray, ultraviolet C (UVC), from 10-100 nm. UVC and the shortest UVB which wavelength shorter than 290 nm are eliminated by ozone layer on earth (Lowe, Shaath and Pathak, 1997; Shaath, 2005b). Therefore, the UV radiation that can reach to the earth's ground is about 90% UVA and 10% UVB. UVA intensity is still constant throughout the day but UVB does not remain at the noontime apex. Human eye can detect only visible range (Lowe and Friedlander, 1997).

UVA rays can cause skin tanning because it can penetrate into the deep layer of human skin, dermis layer, to increase the melanin formation. These weak energy rays have more penetration into skin, and higher incidence of UVA rays can cause skin cancers. Many researchers have found that UVA radiation related with tissular and molecular effects of skin. If UVA penetrates into deep skin, it can introduce the incision in cellular DNA. UVA damage tends to implicate with the oxygen and trace

metals presented and collapse structure of collagen, skin then becomes aging (Shaath, 2005b). Thus, using antioxidant products and singlet oxygen free radical scavengers become more popularity. However, recent studies are detailing the integral role of UV-A exposure in carcinogenesis as well as skin aging.

Exposure to UVB for prolonged time leads to dangerous effects on human skin. The high energetic of UVB radiation can cause erythema, skin burn, skin aging and non-melanoma skin cancers (Mitchnick, Fairhurst and Pinnell, 1999; Schulz et al., 2002; Turkoglu and Yener, 1997; Wang, Stanfield and Osterwalder, 2008).

## 1. Photoprotection against UVA and UVB radiation

By the 1970s, dermatologists were beginning to realize the deleterious effects of UV radiation exposure and were recommending sun protection (Nelson, 2005). Avoidance of sun light is a paramount way to protect harmful light, it can be achieved by using sunscreen products adequately along with wearing UV protective clothing, UV filtering sunglasses and hats.

Protection from UVA and UVB is necessary for people who have normal skin including skin phototypes I and II (fair-skinned), or skin phototypes III, IV, V, and VI (tanned, light brown, or black skin). The protection of skin against UV radiation seems not easy, it is usually incomplete because of excessive sun exposure. All skin phototypes of human can be sufficiently protected less than 3-4 hour against exposure of UVB and UVA radiation (Lowe et al., 1997).

Nowadays, sunscreen products have become an important role for many people, dermatologists and many cosmeceutical companies have recommended to protect the skin from any adverse effects of UV radiation. FDA and many countries regulations have approved several standards for helping the consumers to select suitable sunscreen products by themselves. Because of much information considering the harmful effects of prolonged ultraviolet exposure, the sun protection factor (SPF) value is widely used to indicate the degree of UV protection. (Shaath, 2005b).

## 2. Sunscreens active ingredients

Active ingredients in sunscreen can be mainly classified into chemical sunscreens and physical sunscreens according to the type of protection.

1. Chemical sunscreens : These active ingredients can absorb the harmful UVR and they are divided into either UVA or UVB blocker depending on the type of absorbed radiation. UVA absorbers tend to absorb UVR in the wavelength 320-380 nm, for example benzophenone, meradimate and avobenzone. UVB absorbers can absorb UVR in the wavelength 290-320 nm, for example para-amino benzoates, salicylates, cinnamates and camphor derivatives.

2. Physical sunscreens : These UV filters can absorb, reflect or scatter UV radiation. They are currently used in combination with chemical sunscreens to improve SPF, UVA protection or broader spectrum protection.

Particles size of physical blocking agents, for example titanium dioxide, zinc oxide, iron oxide and kaolin, can absorb, reflect or scatter UV radiation, visible light and even infrared light.

The old physical sunscreens have many disadvantages for being good sunscreen products (Shi et al., 2011; Yabe and Sato, 2002). They were comedogenic and have to be applied very thick layer which caused staining in the clothes. Moreover, they were opaque which making them cosmetically unacceptable. Luckily, older physical sunscreens were potentially necessary for person who have photosensitivity disorders because of their efficacy in broad spectrum coverage, and for children who apply slightly these sunscreen products in limited areas because it is safety for sensitive skin.

Nowadays micronized and nanonized physical sunscreens have been developed available worldwide (Labouta and Schneider, 2013; Newman, Scotland and Ellis, 2009). They do not reflect visible spectrum, but reflect at shorter wavelengths, thus they are invisible and make more cosmetically acceptable sunscreen products (Nelson, 2005; Shaath and Walele, 2005; Shi et al., 2011; Wiechers et al., 2013). Moreover, micronized and nanonized form of titanium dioxide are chemically stable and they do not cause any contact dermatitis and photo allergic (Smijs and Pavel, 2012). A dominant problem for the formulation is

aggregation or agglomeration of the particles. If this phenomenon is happened, the reflection of the spectrum will shift into the longer wavelength (visible light) and it will cause white coloration when apply to the skin like traditional pigment. The other disadvantages of physical sunscreens is the higher cost of production than the available chemical sunscreens nowadays and the difficulty of the formulations (Shaath, 2005b).

### 3. Physical sunscreens

### 3.1 Optical behaviors for physical sunscreens

When the light falls into a particle of materials, particles can reflect, scatter, or absorb the light. There is a simple equation for the interaction of light with a particle (Figure 1). In this equation, $I_t$, $I_0$, $I_r$, $I_s$ and $I_a$ are the intensities of transmitted light, incident light, reflected light, scattered light and absorbed light, respectively. For submicron particles, UV reflection mechanism is very small. Scattering and absorption are the main mechanism of protection (Auger, Martinez and Stout, 2009; Schlossman and Shao, 2005).
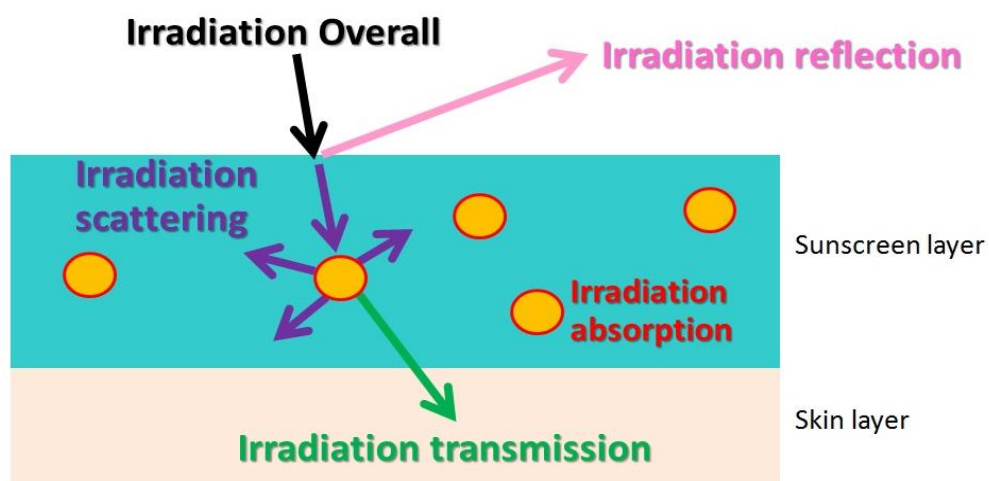


Figure 1 The mechanism of UV protection of particles, applied from Shaath (2005)

$$I_t = I_0 - ( I_r + I_s + I_a)$$

Equation 1 Equation of the interaction of light with a particle

### 3.1.1 Scattering

The scattering effect from very small particle size is predominated by Rayleigh scattering theory. And the scattering effect from the particle size with the close magnitude to the wavelength is predominated by Mie' theory. This effect has an antenna pattern like, a larger particle is sharper and more intense forward lobe (Figure 2). Figure 3 shows the scattering effect by Mie's theory, where N is a number of particles, $I_0$ is an intensity of the incident light, d is a particle's diameter, l is a wavelength of the light, and m is a relative refractive index, which means the ratio between the refractive index of the particle and refractive index of the medium (Schlossman and Shao, 2005; Taylor et al., 2013)

From equation of Mie, the scattering intensity is sixth power of a particle diameter, thus, large particle is much more potential in scattering effect. Avoidance of the whitening effect can be achieved by reducing the scattering of visible light. However, if particle size is reduced too much, a main mechanism of UV attenuation will change from scattering to absorption. The relative refractive index is one of the critical factors for this equation. The vehicles (like oils) in most sunscreen products have refractive index (RI) at 1.33–1.6 (Song et al., 2014). If rutile $TiO_2$ (RI = 2.76) is used, the relative refractive index is approximately 1.8. When ZnO (RI = 1.99) is used, the result of a relative refractive index is about 1.3. According to the Mie's theory, the efficacy of scattering UV light of $TiO_2$ will be about three times of ZnO (Schlossman and Shao, 2005).
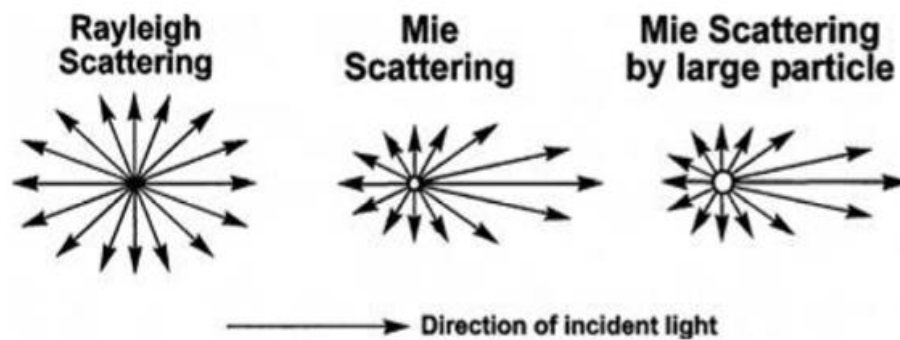
Figure 2 Scattering patterns for Mie and Rayleigh law (Schlossman and Shao, 2005)

$$I_S \propto \frac{Nd^6}{\lambda^4} * \left[\frac{m^2-1}{m^2+2}\right]^2 * I_0$$

Figure 3 Mie scattering's equation

### 3.1.2 Absorption

The wavelength of solar radiation with enough energy to excite electrons in the valence band is 420 nm. Thus, this wavelength can be absorbed by rutile titanium dioxide. However, at longer wavelength, the UV absorption gets weaker and it has a plateau at 360 nm. From this reason, $TiO_2$ is not a potential absorber for UVA radiation, especially for UVA-II absorber, but it is a potential absorber for UVB radiation. UVA protection by $TiO_2$ mainly takes places by scattering mechanism (Schlossman and Shao, 2005; Serpone, Dondi and Albini, 2007).

Zinc oxide has longer band gap wavelength than titanium dioxide, thus it makes these material can absorbs broader spectrum than $TiO_2$. Moreover, due to the difference in electron energy states like band structure, the UV cutoff of ZnO is sharper than for $TiO_2$. The absorption is function of the atom's number that can interact with the incident light in its pathway. When the particle size is reduced, there will be more particles to interact with and it means more UVR can be absorbed. Therefore, if the weight of physical sunscreens is fixed, smaller particle size will provide stronger UV absorption than larger one (Innes et al.).

### 3.2 Physical and chemical properties of titanium dioxide

Titanium dioxide ranks 9th elements in the earth's crust. There are three forms of $TiO_2$, namely rutile, anatase and brookite. Rutile and anatase in the nature are not pure, they contain by variable metals which can pose health hazards to humans. Thus, synthesized commercial $TiO_2$ is always used in all industries. Rutile and anatase forms have the same chemical identity but they have different crystalline structure. Rutile form is a tetragonal crystal lattice while anatase form is a regular octahedral one. Brookite form is an orthorhombic crystalline, but it is not commercially important. Rutile is the most thermal stability of all three form of titanium dioxide. Anatase and brookite will convert into rutile when they are heated at a very high temperature. Because of their crystal difference, rutile and anatase form are different in some of physical and chemical properties which are shown in Table 1. Rutile is widely used for many industries due to the most stability (Auger and McLoughlin, 2014). Titanium dioxide has very good chemical stability (Becheri et al., 208). It is still stable in acids and bases except the high concentration of strong acids. $TiO_2$ is an insoluble material in all organic solvents, thus, it is essentially inert. These properties make $TiO_2$ very safe to use in many applications (Schlossman and Shao, 2005).

Table 1 Physical and chemical properties of $TiO_2$

| Parameters | Rutile | Anatase |
|---|---|---|
| Density (g/cm$^3$) | 4.2 | 3.9 |
| Hardness (Mohs) | 6-7 | 5.5-6 |
| Refractive index | 2.76 | 2.52 |
| Dielectric constant | 114 | 48 |
| Melting point (°C) | 1855 | Convert into rutile |

### 3.3 Physical and chemical properties of zinc oxide

The 24th most common element in abundant is zinc. However, it does not occur freely in the nature. Pure zinc oxide powder is commonly white or yellow-

white color. The structure of zinc oxide is hexagonal crystalline. The oxidizing zinc vapor in burners or the precipitation of zinc salt can produce zinc oxide. Zinc vapor can be generated from the purity of zinc metal, and it can provide the highest purity of zinc oxide which used in cosmetics products. Physical properties of zinc oxide are shown in Table 2. Zinc oxide is slightly soluble in water (Jancikova et al., 2012). This material can convert to zinc hydroxide, it depends on many factors, and the most critical one is about temperature. Zinc oxide does not react with most substances, but it can adsorb carbon dioxide and carbon monoxide. Zinc oxide is amphoteric. It can react with acid to generate zinc salts or react with alkali to generate zincates (Schlossman and Shao, 2005).

Table 2 Physical and chemical properties of ZnO

| Parameters | Value |
|---|---|
| Density (g/cm$^3$) | 5.7 |
| Hardness (Mohs) | 4 |
| Refractive index | 1.99 |
| Dielectric constant | 1.7-2.5 |
| Melting point (°C) | 1975 |

## 3.4 Influence of particle size on UV attenuation of titanium dioxide

Reducing the white coloration of physical sunscreen when apply to the skin can be achieve by using the attenuation grade of $TiO_2$ and ZnO with a primary particle size less than 100 nm. Even though a primary particle size of physical sunscreens is very small, the agglomeration can be occurred, thus, an effective milling is always important process to disperse the particles. When the particles are properly formulated, the primary particle size is a critical factor to get the highest possible transparency. Schlossman and Shao (2005) compared the properties of all dispersion of titanium oxide in the same treatment but the different four primary particle sizes which were milled in the same controlled conditions. The particle sizes are exhibited in Table 3 and the UV-visible transmittance curves are illustrated in Figure 4.

Table 3 Particle size of TiO$_2$ dispersions

| Samples | Primary particle size (nm) | Particle size (nm) |
|---------|---------------------------|--------------------|
| 1 | 15 | 125.3 |
| 2 | 35 | 154.1 |
| 3 | 100 | 251.1 |
| 4 | 180 | 263.4 |
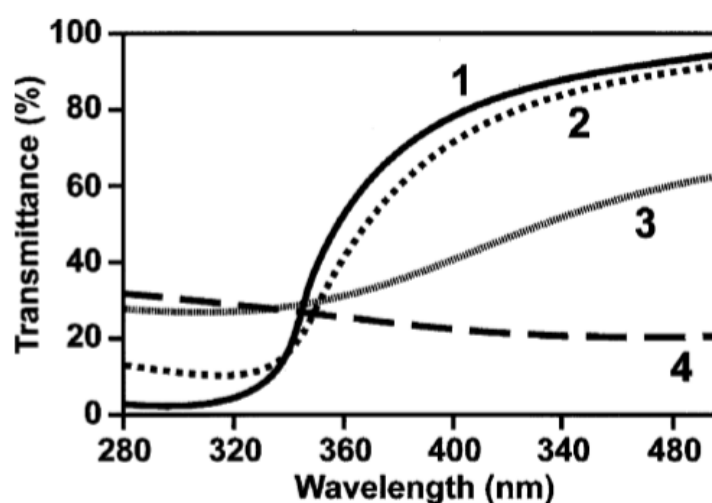


Figure 4 The UV transmittance spectra of TiO$_2$ dispersed in isononyl isononanoate; primary particle sizes: 1=15 nm, 2=35 nm, 3=100 nm, 4=180 nm (concentration = 0.001%TiO$_2$ in CHCl$_3$).

The transmittance curves in Figure 4 shows very clear result that transmittance of visible light, wavelength > 400 nm, is much more when a particle size get smaller. The result could be confirmed by comparing the drawdown of the dispersions on a glass plate. When particle size of TiO$_2$ dispersion is more than 200 nm (samples 3 and 4), the transmittance curves become flatter in both UV and visible range. They cannot be used in personal care products including sunscreen products because of too much whitening effect and too weak UV attenuation (Schlossman and Shao, 2005). Both samples 1 and 2 are proper to be used in sunscreen products as they are high transmittance in visible region. Sample 1 is the smallest size and the transmittance graph below 320 nm is also lowest, indicating it

can provide highest SPF when the concentration used of $TiO_2$ is the same for all samples.

However, sample 2 is lower transmittance curve than sample 1 in almost UVA region at wavelength 335–400 nm. Therefore, it is assumed to give a better PFA (protection factor for UVA) score. The results from Stamatakis et al. (1990) and Sakamoto et al. (1995) explained that the UVR attenuation of $TiO_2$ at wavelength 300 nm (UVB) was increased when a particle size was decreased but at wavelength 350 nm (UVA) was decreased when a particle size was 100 nm or less than. Therefore, it seems to be clear for the UV attenuation of $TiO_2$ that UVB protection is predominated by the absorption effect of $TiO_2$, which increases when a particle size decreases. And UV-A protection is predominated by scattering effect. Therefore, it is necessary to control the particle size of $TiO_2$ in sunscreen products to get the maximum of UV attenuation without causing any whitening effect.

## 3.5  Influence of particle size on UV attenuation of zinc oxide

Schlossman studied the characteristically dispersion of various four primary particle size of ZnO which dispersed and milled in the fair conditions. The particle sizes are shown in Table 4 and the transmittance curves are illustrated in Figure 5. Unlike $TiO_2$, the dispersions of ZnO seem to be transparent although the particle sizes get larger. ZnO can absorb UV radiation more uniformly, and the sharp cutoff starts at 375 nm. When a particle size becomes smaller, it has a little shift to shorter wavelength. It can be explained from the UV transmittance curves (Figure 5) that the smaller primary particle size the less UVR is transmitted in UVB range but most at wavelength around more than 380 nm (UVA-I range), indicating a broader spectrum attenuation of ZnO (Pinnell et al., 2000; Schlossman and Shao, 2005).

Table 4 Particle size of ZnO dispersions

| Samples | Primary particle size (nm) | Particle size (nm) |
|---------|---------------------------|--------------------|
| 1 | 20 | 228.3 |
| 2 | ~60 | 246.0 |
| 3 | ~100 | 263.6 |
| 4 | ~200 | 292.2 |
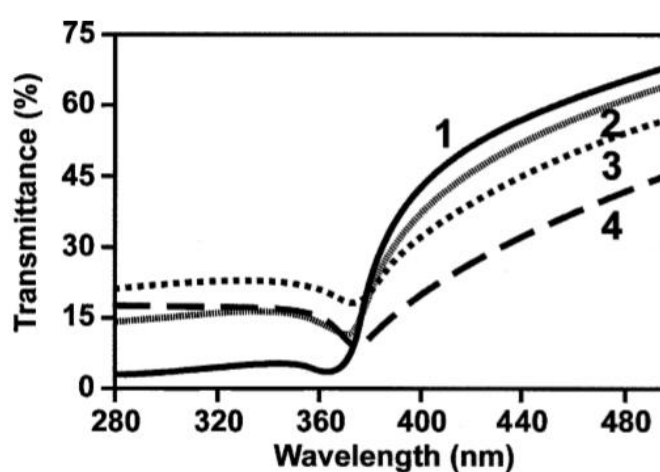


Figure 5 The UV transmittance spectra of ZnO dispersed in isononyl isononanoate; primary particle sizes: 1=20 nm, 2=60 nm, 3=100 nm, 4=200 nm (concentration: 0.005%ZnO in CHCl$_3$).

## 4. Dispersion of physical sunscreens

J. M. Oyarzu ́n (2000) reported the process of pigment dispersion which has the objective to make a stable and homogeneous dispersion of fine particles. The critical points of this process were about a mechanical breakdown, wetting, and stabilization. About the mechanical breakdown, it is so important to have some energy for breaking down the cohesion forces which is defined as the intermolecular forces that can hold solid particles together. The cohesive forces which can cause agglomeration between particles are specifically physical in nature and it does not happen by any chemical bonding. In addition, the surface forces of the particles are strong, hence, it is necessary to use high shear or high speed disperser to achieve the

mechanical breakdown, and the agglomeration of particles may be reduced. The common dispersion formulation is contained by the particles' surface treatment, dispersant, and medium. The surface treatment is important to pre-wet the particles in medium, while the dispersant can be used to help the particle wet and stabilize the particles in medium.

Currently, the number of demand about using dispersions instead of the powder form of physical sunscreens is increasing. It can be easier to achieve the desired particle size or desired UVR protection by using the dispersions since the particles' level in a medium of the formulation seems to be too low to cause agglomeration or aggregation of the particles. Additionally, when large particles are milled, the whitening effect, which is the disadvantage of physical sunscreens, will be reduced. However, it seems difficult to control the fine particle size of the pigments in the finished products. Therefore, it is necessary to use powerful and proper mixing method for wetting these pigments due to their hardness, density, including its high specific gravity. The pigment concentration and the viscosity of premix dispersion play important roles in choosing the suitable choice of mill.

The pigment grade of both physical sunscreens present white coloration when applied to the skin because of the reflection of most visible light. Nanotechnology has been used to reduce whiteness on the skin providing cosmetic acceptability as its less visible light reflection (Manaia et al., 2013). Nevertheless, physical sunscreens appear to have lower photoprotection efficiency than chemical sunscreens, especially in UVB range.

For this reason, this study aims to select another potential substance to enhance photoprotection efficiency of physical sunscreens. It was reported that titanium dioxide and zinc oxide at 20% concentration combined with 1%iron oxide by spectrophotometric analysis can reduce the transmittance of UVA and visible light at maximum of approximately 20%. Besides that zinc oxide combined with iron oxide provided a synergistic effect, the combination can effectively reduce the transmittance in UVA and visible region to as low as 1.5%. The results were confirmed by *in vivo* evaluation method using a guinea pig as a model (Shaath, 2005b). Other interesting material is calcium carbonate because of its safety,

inexpensiveness and environmentally friendly. There are very few studies on the calcium carbonate safety. The Calcium Carbonate Association (Association, 2005) in Europe studied the acute dermal and eye irritation tests from OECD Guidelines for Testing of Chemicals. It was found that calcium carbonate is safe and caused no irritation to the skin and eye of rabbits. The pigment of calcium carbonate also creates whiteness when applied, thus Metallurgy and Materials Science Research Institute, Chulalongkorn University had synthesized nanoparticles of calcium carbonate (particle size 30-70 nm) by precipitation reaction to avoid this disadvantage. These nanoparticles were found to be safe in mitochondria cell by MTT assay with $IC_{50} > 1$ mg/ml (Krisana Siralertmukul et al., 2012a, 2012b). However, more study of safety in human skin is needed.

## 5. Calcium carbonate in ultraviolet photoprotection

There was reported that calcium carbonate by itself is not good absorber in ultraviolet radiation. However, when calcium carbonate was combined with vitamin C by solution mixing to incorporate 0.5 and 1% vitamin c derivative ($ASc^{2-}$) into crystalline structure of calcium carbonate, it was found that this combination can absorb UVB radiation which has maximum peak at 292 nm (Sato and Ikeya, 2004). Nevertheless, UVB attenuated ability of the combination may occur by $ASc^{2-}$ itself because $ASc^{2-}$ can absorb at 297.5 nm (UVB region) but it is not stable, hence matrix of calcium carbonate can make $ASc^{2-}$ more stable. Mueller at al. (2008) studied the combination of calcium carbonate and chemical sunscreens, such as ethylhexylmethoxy cinnamate and bis-ethylhexyloxyphenol methoxyphenol triazine. They varied calcium carbonate concentrations at 2.5, 5, 10 and 15% and then incorporated into a commercial available w/o lotion. The result showed calcium carbonate can enhance SPF in all concentration levels when comparing with lotion without $CaCO_3$. Additionally, Kose Cosmetics used calcium carbonate in their sunscreen formulations which consisted of physical and chemical sunscreens, such as titanium dioxide, zinc oxide, benzophenone, benzoic acid and salicylic acid, to induce complete photoprotection throughout UVA and UVB range (Fukuda and Naito, 1985). There are no reports which combine calcium carbonate nanoparticles with

physical sunscreens. Therefore, calcium carbonate nanoparticle is an interesting material to use as enhancer photoprotection efficiency of physical sunscreens.

### 6.  Physical sunscreens formulation

Formulation of sunscreen products have been developing for various forms, for example emulsions, gels, sprays, sticks or oil. The most popular form is emulsion because it can be flexible in term of SPF values, product viscosity, placement of the active ingredients, cosmetical aesthetics and can be incorporated other ingredients such as moisturizers, humectants or emollients (Anderson, Hewitt and Spruce, 1997; Klein, 1997). When the active ingredients are applied to the skin, they must be resistant to any chemical, photodegradation and to remove by skin oil, water or perspiration. It must be odorless and non-staining to skin or the clothing. Most consumers like the formulation which should be rub easily or leave the skin with no stickiness. Moreover, it should be transparent when apply on the skin.

The three requirements to achieve the desirable efficiency of physical sunscreens formulation by Hewitt (1999) are :

1. It is considered to choose the suitable particles size and particle size distribution of materials. As described above, the particle sizes more than 100 nm may cause whitening effect on skin. Hence, the information of materials should be reported by the supplier with data of UV-visible curve.

2. It is important to make the homogeneous dispersion in emulsion. The factors which can affect the homogeneity are the coating of the particles (hydrophobic or hydrophilic), type of emulsion (w/o or o/w emulsion) and other ingredients in formulation.

3. Formulators should ensure an even distribution of the active ingredients on the skin after application.

### 7.  Ultraviolet Protection Evaluation

The Sun protection factor (SPF) value on commercial sunscreen products illustrates the amount of erythema ultraviolet protection (Osterwalder and Herzog,

2009). Numerical SPF means when sunscreen products are applied on the skin, it has more protective times than without sunscreen application. SPF value can be calculated by dividing minimal erythema dose (MED) on protected skin by MED dose on unprotected skin (Equation 2). MED is a measurement of the amount of energy per unit area ($J \cdot cm^{-2}$) which can cause minimal erythema.

$$SPF = \frac{\text{Minimal erythema dose on protected skin}}{\text{Minimal erythema dose on unprotected skin}}$$

(Equation 2)

Although *in vivo* SPF determination is more credible, it has many disadvantages. This method is expensive for both money and time for determination. Additionally, there are ethical considerations because a volunteer skin has to be damaged by high UVR energy. However, SPF determination is an indicator for only erythema caused by UVB and UVA-II, SPF value does not include the effects of another important ray, that is, UVA-I which can induce damages to cellular DNA.

*In vivo* UVA evaluation requires high intensity of UVA, hence it will have drawbacks on ethics. There are three methods to determine UVA protection, namely PPD (Persistent Pigment Darkening), IPD (Immediate Pigment Darkening), and UVA-PF (UVA Protection Factor). PPD and IPD are the methods for persistent and immediate skin pigment changes, respectively. The IPD value is the smallest dose which can produce skin darkening, it is observed after UVR exposure immediately. While PPD value is the end-point of pigmentation changes, it is maximum at 2–4 h after UVR exposure (Lowe et al., 1997; Shaath, 2005b). UVA-PF is the ratio of minimal UVA dose which can induce pigmentation of protected and unprotected skin (Equation 3).

$$UVA\text{-}PF = \frac{\text{Minimal persistent pigmentation dose protected skin (MPPDDp)}}{\text{Minimal persistent pigmentation dose unprotected skin (MPPDDu)}}$$

(Equation 3)

Recently, *in vitro* SPF determination have been developed to be faster, more convenient, inexpensive, and the results was reported to be reliable due to the correlation with *in vivo* evaluation.

There are two common methods for *in vitro* UV determination. The first is the method which measures the UV absorption or UV transmission by applying sunscreen products onto films in quartz plates or biomembranes and analyzed by SPF analyzer. The second method measures the UV absorption or UV transmission of sunscreen active ingredients through diluted solutions based on UV-Visible spectrophotometric analysis.

### 7.1  SPF analyzer



Ultra-violet (UVB) and near ultra-violet (UVA) radiation is provided by a 125 W CW Xenon arc lamp.

A horizontal sample area permits testing of liquids, creams, emulsions and sprays.

An integrating sphere placed just below the sample collects light scattered by the product and its supporting substrate, increasing measurement accuracy.
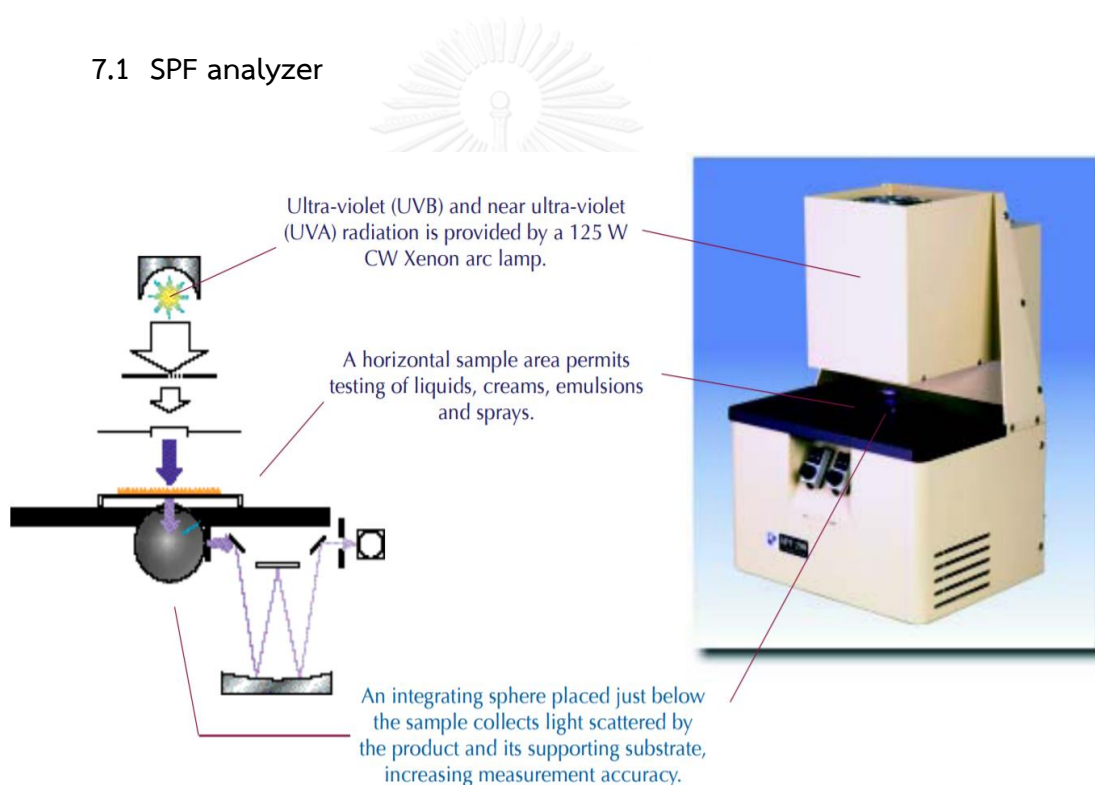
Figure 6 Diagram of SPF-290S analyzer system (Optometrics, 2009)

SPF-290S analyzer is a recording UV spectrophotometer which is designed for SPF determination. This analysis correlates well with human *in vivo* evaluation (ISO, 2011). Various forms of sunscreens and cosmetic products can be used such as lotions, sticks, cosmetics creams, liquids and sprays. Additionally, it can be used for reducing the need and cost of *in vivo* evaluation in human subjects. Nevertheless,

the cost of this analysis seems expensive for the first step in the development of a new active ingredient.

The system automatically scans the coverage both UVA and UVB range, from 290 to 400 nm, the intervals are 1, 2 or 5 nm. The monochromatic protection factor (MPF) is determined for each of the selected wavelengths and is used to calculate the SPF value by programming into the software. There are an integrating sphere below the sample collecting light scattered by the product and its supporting substrate which can increase measurement accuracy (Optometrics, 2009). According to ISO 24443, COLIPA 2011 and FDA final rule 2011, polymethylmethacrylate (PMMA) plate is now recommended as a substrate used for SPF analyzer. They recommend HELIOPLATE HD 6 (50mm×50mm) about 6 microns roughness because PMMA plate is fairly transparent to UV and simulates the porosity and texture of human skin more than the old substrates, such as, 3M Transpore™ Tape, Vitro Skin™ and Polyvinyl Chloride Film (Saran Wrap), which all give variable results (Padera, 2014). The SPF results of Optometrics SPF-290S using PMMA plate show that *in-vitro* evaluation give similar results to the *in-vivo* evaluation for variable forms of sunscreen products (Optometrics, 2009).

### 7.2 UV-Visible spectrophotometric analysis

Although this method cannot provide exact data, it is currently used to reveal the trends of new active ingredients during development (Shaath and Flores, 2005). There are many studies and suppliers providing UV absorption spectra or %UV transmittance of new sunscreens active ingredients (Osakasasaki, 2015) and physical sunscreens (Goh, Xu and McCormick, 2014; Innospec, 2010; KOBO, 2015a, 2015b; Manaia et al., 2013; More, 2007; Morfesis and Fairhurst, 2005; Schlossman and Shao, 2005) in solution or dispersion forms. Due to the time-consuming and high cost of *in vivo* and *in vitro* SPF determination using SPF analyzer, UV-Visible spectrophotometric analysis is used in this study for preliminary selecting suitable concentrations of physical sunscreens combined with calcium carbonate. Then SPF analyzer will be used to correlate and evaluate those suitable combinations to confirm the results of UV-Visible spectrophotometer.

UV-Visible spectrophotometry can investigate the quantity of molecules absorbing the UV or visible radiation in both solution and dispersion forms of physical sunscreen active ingredients. Most physical sunscreen's suppliers always provide UV-Visible absorption spectra of their materials which has a close relationship to the active ingredient's properties in the final products (Hewitt, 2005). This machine can measure the ratio of two beam intensities of UV and visible light (Figure 7). This method is rapid, simple, moderately specific and it can be used with a small quantities of the samples (Behera et al., 2012; Lowe et al., 1997; Shaath, 2005b). In addition, Beer-Lambert law is always obeyed in this analysis.

Beer's law: Absorbance of substances is proportional to the concentration of substances.

Lambert's law: Absorbance of samples is proportional to a homogeneous thickness of medium.

Beer-Lambert law: when the light beam is passed through a cuvette containing an absorbing substance solution, the decreasing rate of light intensity is proportional to the thickness as well as the concentration of the solution.

The mathematic is $A = a \times b \times c$ Where, A=absorbance, a=absorptivity or the extinction coefficient, b=path length or length of sample cell (cm.) and c=concentration. Both a and b are constant values, hence, A is directly proportional to the concentration (c).

The UV and visible radiation wavelength, which is directed through the sample and reference, is recorded as the transmittance light and then it is calculated into overall UV absorption ($abs_o$) where specific UV absorption ($abs_s$) and specific UV scattering ($Sc_s$) are all included (Taylor et al., 2013; Thakur, 2011), as shown in the following equation.

Overall UV absorption = specific UV absorption + specific UV scattering

$(abs_o)$ $\qquad$ $(abs_s)$ $\qquad$ $(Sc_s)$

(Equation 4)

**The instrumentation of UV spectroscopy**
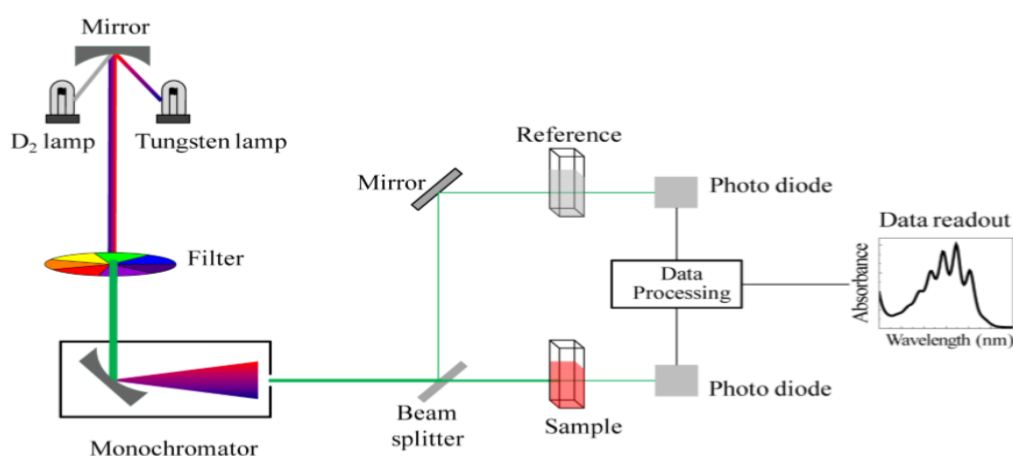


Figure 7 Schematic for a UV-Visible spectrophotometer (Sobarwiki, 2014)

Light Source - Tungsten and Hydrogen-Deuterium lamps are the most used light source because of the coverage of whole UV range. Tungsten filament lamps fall the wavelength at 375 nm, while Hydrogen-Deuterium lamps emit below 375 nm.

Monochromator – it composes of the prisms and slits. The UV and visible radiation which are emitted from the light source is dispersed by rotating prisms. Prism will separate the wavelengths and slit then selects them. The light beam which is selected by the slit is called monochromatic and divided into two beams by another prism (double beam spectrophotometers).

Sample and reference cells - One beam is passed through the reference cuvette cell and other is passed through the sample cuvette cell. These cells are made of either silica or quartz because they do not absorb UV radiation.

Detector - Generally two photocells serve the purpose of detector in UV spectrophotometry. One of the photocells receives the beam from sample cell and the second receives the beam from the reference. Normally, the intensity of the radiation from the reference cell is stronger than the beam of sample cell.

<u>Amplifier</u> - The main purpose of amplifier is to magnify the signals many times.

<u>Recording devices</u> - All the data were recorded and then produces the spectrum by computer.

From Figures 6 and 7, the transmitted light detectors between SPF analyzer and UV-visible spectrophotometer are different. SPF analyzer has an integrating sphere which can collect the transmitted and scattered light from many directions increasing more accuracy in measuring absorption (Optometrics, 2009). Whereas the detectors of UV-visible spectrophotometer is not integrating sphere, it can collect only straight line of transmitted light. Therefore, in this study, the UV protection measurement from these two instruments will be measured and compared to investigate the correlation of both results.
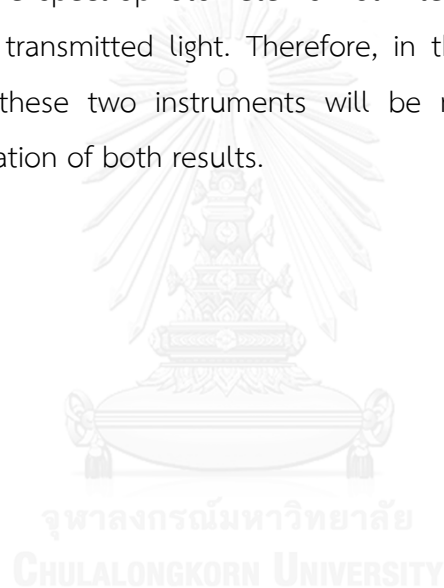
# CHAPTER III
# METHODOLOGY

## 1. Materials

The following materials were obtained from commercial sources.

- Calcium carbonate nanoparticles (NPCC), average particle size 30-70 nm (Innova Nano Tech Company Limited, Thailand)

- Titanium dioxide (TTO-D2) coated with $Al(OH)_3$ $ZrO_2$ Stearic acid, average particle size 40-70 nm (Lot.No.0114, Ishihara Sangyo Kaisha, Ltd, Japan)

- Zinc oxide (ZnO-610Si(4)) coated with 4% Hydrogen dimethicone, average particle size 15-55 nm (Lot.No.K86747, Sumitomo Osaka Cement, Japan)

- Dimethicone (Lot.No.0008122852, Ter Chemicals, Germany)

- C12-15 Alkyl benzoate (Saboderm AB sample size, P.C.Intertrade Co., Ltd., Italy)

- Mineral oil (Lot.No.2015031330, Kukdong oil & Chemical Co., Ltd., Seoul, Korea)

- White beeswax (Lot.No.022376, S. Tong Chemicals Co., Ltd., Thailand)

- Propyl paraben (Lot.No.GBG0004232, Clariant Production UK Ltd., United Kingdom)

- Methyl paraben (Lot.No.BH1711, Ueno Fine Chemicals Industry, Ltd., Osaka, Japan)

- PEG-30 dipolyhydroxystearate (Arlacel™ P135, Croda Thailand Co. Ltd., United Kingdom)

- Sorbitan stearate (and) sucrose cocoate (Arlacel™ 2121, Croda Thailand Co. Ltd., United Kingdom)

- Magnesium sulfate (Lot.No.570260, Wendt-Chemie Vertriebsges, mbH & Co. KG, United State)

- Propylene glycol (Lot.No.C815E69TD1, Dow Chemical Thailand Ltd., United State)

- 95% Ethyl alcohol pharmacuetical, food and industrial grade (Lot.No.5C120716, Liquor Distillery Organization, Thailand)

- Deinoized water

## 2. Equipments

- Analytical balance 4 digits (Model AG285, Mettler Toledo, Zürich, Switzerland)

- Analytical balance 3 digits (Model PG403-S, Mettler Toledo, Zürich, Switzerland)

- Field Emission Scanning Electron Microscope (Model JSM-7610F, Japan)

- Polarizing optical microscope (Model Eclipse E200, Nikon, Tokyo, Japan)

- Digital camera (Model DS126271, Canon, Tokyo, Japan).

- High Speed Disperser (IKA®-WERKE EUROSTAR digital, Germany)

- Homogenizer (Model D-7801 type x1020, Ystral GmbH, Dottingen, Germany)

- UV-Visible Spectrophotometer (Model uv-1800, Shimadzu, Japan)

- SPF 290s-analyzer (Model Optometrics LLC, Massachusetts, United States)

## 3. Methods

## 1. Morphology characterization of physical sunscreens and calcium carbonate

In this study, morphology of titanium dioxide, zinc oxide and calcium carbonate was observed using Field Emission Scanning Electron Microscope and Energy Dispersive X-Ray Spectrometer. All samples were submitted to Scientific and Technological Research Equipment Centre, Chulalongkorn University for evaluation. Each sample was scattered onto metal stub. The resolution to characterize the sample using SEM is to assay with 5.00 kV and use Secondary Electron Image (SEI) probe.

## 2. Determination for proper sample preparation condition

### 2.1 Preparation of physical sunscreen dispersions

As samples, 10%w/w titanium dioxide, 10%w/w zinc oxide, 5%w/w calcium carbonate, 10%w/w titanium dioxide combined with 5%w/w calcium carbonate or 10%w/w zinc oxide combined with 5%w/w calcium carbonate were dispersed in dimethicone 350. In preliminary study, four vehicles, namely ethanol, cyclomethicone, capric triglyceride and dimethicone were used to disperse all samples for investigating the suitable vehicles. It was found that dimethicone can disperse all samples homogeneously and didn't occur precipitation for approximately 5-6 hours, as seen in Figure 8. High speed disperser was used at varying speeds and durations to obtain homogeneous particulate dispersion. Stirring speeds were varied of 1000, 1500 and 2000 rpm with varying durations of 5, 10 and 15 minutes (Table 5). Concentrations of physical sunscreens were modified from Lin and Lin (2011) and Amnuaikit and Boonme (2013).

Table 5 Speeds and durations used for the dispersion of physical sunscreens

| Condition | Speed (rpm) | Duration (minutes) |
|-----------|-------------|--------------------|
| 1 | 1000 | 5 |
| 2 | 1000 | 10 |
| 3 | 1000 | 15 |
| 4 | 1500 | 5 |
| 5 | 1500 | 10 |
| 6 | 1500 | 15 |
| 7 | 2000 | 5 |
| 8 | 2000 | 10 |
| 9 | 2000 | 15 |
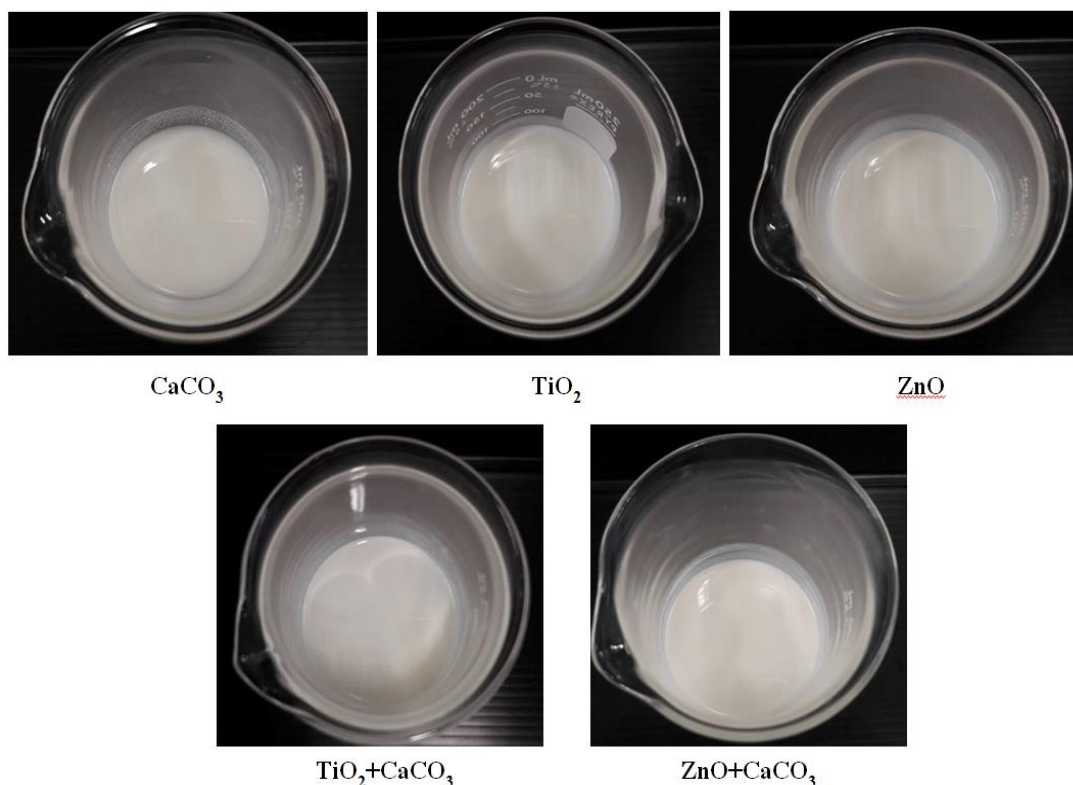
Figure 8 All sample pictures when dispersed in dimethicone

## 2.2 Ultraviolet absorption measurement

Overall UV absorbance ($abs_o$) will be measured throughout this study. Samples were diluted with dimethicone 350 to get optimal absorbance range, all sample dispersions should be diluted to a level at maximum absorbance is less than 2 (Schlossman and Shao, 2005). Thirty milligrams of sample was weighed accurately, then, 50 grams of dimethicone oil was poured into it. Samples were stirred by stirring rod until it became homogeneous. The pictures of all samples can be seen in Figures 9. Ultraviolet absorbance was measured using UV-Visible spectrophotometer connected to UV probe software to measure absorption properties of physical sunscreens at wavelengths ranging from 290 to 400 nm, with step size of 5 nm (Shaath and Flores, 2005). Samples were poured into cuvette cell of 1x1 centimeter. Dimethicone was used as a blank. Each sample was divided into 3 divisions. Then, each division was measured to ensure homogeneity.
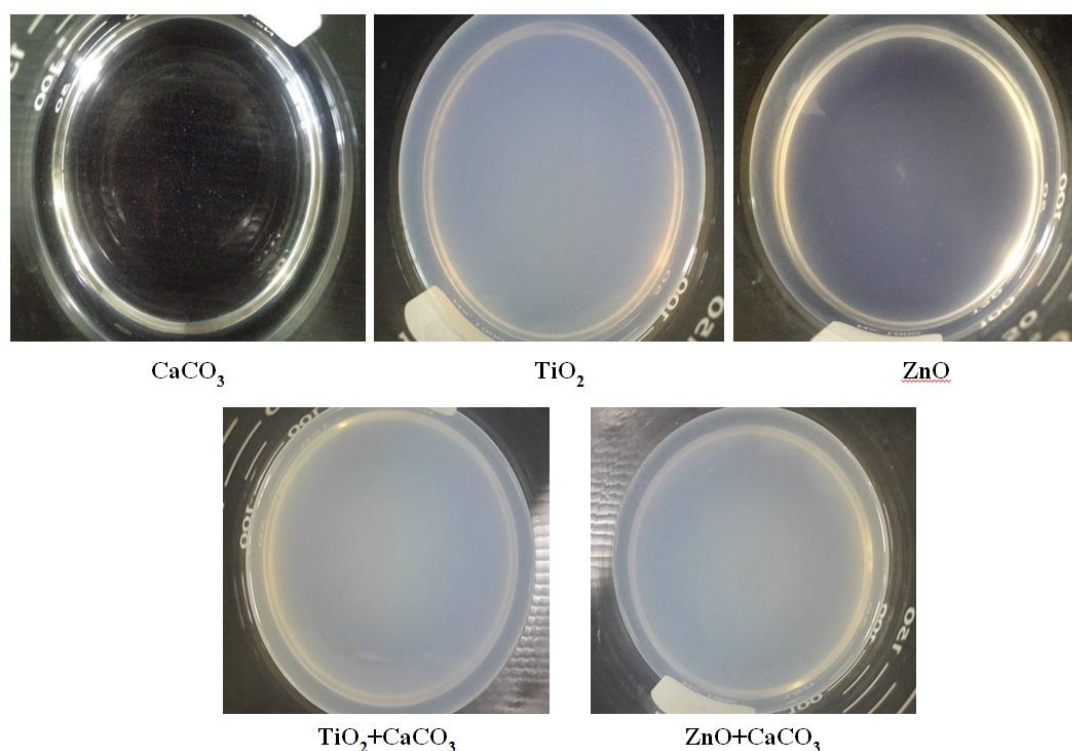
Figure 9 All sample pictures when diluted by dimethicone

## 3. Sun protection efficiency determination of physical sunscreens combined with calcium carbonate in various concentrations

### 3.1 Physical sunscreen dispersions preparation

Either titanium dioxide or zinc oxide was combined with calcium carbonate at various concentrations. As control groups, individual titanium dioxide, zinc oxide and calcium carbonate were used alone. Concentrations of titanium dioxide and zinc oxide were 2.5%, 5% and 10%w/w (concentrations were applied from Lin and Lin (2011) and Amnuaikit and Boonme (2013)) and concentrations of calcium carbonate were 2.5%, 5%, 7.5% and 10%w/w, as seen in Table 6. Dimethicone oil was used as vehicle. Samples were dispersed into vehicle by optimal stirring condition which was previously investigated in section 2.

Table 6 Various concentrations of physical sunscreens

| Calcium carbonate concentrations (%w/w) | Either titanium dioxide or zinc oxide concentrations (%w/w) | | |
|---|---|---|---|
| | 2.5 | 5 | 10 |
| 2.5 | 2.5 + 2.5 | 5 + 2.5 | 10 + 2.5 |
| 5 | 2.5 + 5 | 5 + 5 | 10 + 5 |
| 7.5 | 2.5 + 7.5 | 5 + 7.5 | 10 + 7.5 |
| 10 | 2.5 + 10 | 5 + 10 | 10 + 10 |

### 3.2 Ultraviolet absorption measurement

Overall ultraviolet absorbance ($abs_o$) will be measured by UV-Visible spectrophotometer, wavelength from 290 to 400 nm, step size of 5 nm (Shaath and Flores, 2005). Thirty milligrams of each sample was placed in the beaker then approximately 50 grams of dimethicone oil was added, Stir until homogeneous. The samples were poured into cuvette cell slowly. Dimethicone was used as control to define the baseline. Each sample was measured in triplicates.

### 3.3 Determination of the sun protection factor

Optimum concentrations of the combinations of physical sunscreens and calcium carbonate in section 3.2 were selected to measure the sun protection factor (SPF) by SPF 290s analyzer. One calcium carbonate concentration which could enhance to get highest ultraviolet absorption when combined with various concentrations of titanium dioxide or zinc oxide was chosen as shown in table 7. Various concentrations of titanium dioxide, zinc oxide and calcium carbonate alone were also carried out as control.

All samples were prepared as same in section 3.1 and then they were assigned to Chulalongkorn University Drug and Health Products Innovation Promotion Center (CU.D.HIP) for analyzing the photoprotection efficiency. SPF 290s analyzer was needed to warm more than 15 minutes before measurement. Neat polymethylmethacrylate (PMMA) plate was used as a blank substrate by applying

glycerine approximately 0.004 grams into PMMA plate and then it was left to dry for 15 minutes. For sample measurements, syringe was used to suck each sample up about 0.5 ml. After that the sample was pointed into PMMA plate approximately 0.032-0.033 grams (it should have more than 50 points), sample was applied homogeneously about 1.3 mg/cm$^2$ thickness with finger glove and it was left to dry for 15 minutes. Subsequently, PMMA plate was placed on SPF analyzer holder to scan. This experiment was trialed according to COLIPA *in vitro* UV method, each sample was analyzed with 3 PMMA plates and every plates were scanned 9 times (Optometrics, 2009).

Table 7 Concentrations selected of physical sunscreens combined with calcium carbonate

| Calcium carbonate concentration (%w/w) | Titanium dioxide or zinc oxide concentrations (%w/w) |
|:---:|:---:|
| X | 2.5 |
| X | 5 |
| X | 10 |

*X is calcium carbonate concentration which could enhance to highest ultraviolet absorption when combined with various concentrations of physical sunscreens.

### 3.4 Correlation Analysis between UV-Visible Spectrophotometer and SPF analyzer

Excel 2010 was used to create the correlation graph between UV absorbance values using UV-visible spectrophotometer instrument and SPF/UVA-PF values obtained by SPF analyzer instrument. Coefficient of determination ($R^2$) was calculated to determine the correlation.

## 4.  Physical sunscreens formulations

### 4.1  Preparation of water in oil (w/o) and oil in water (o/w) emulsions

Either concentration combination of titanium dioxide or zinc oxide with calcium carbonate was selected. The selected one should enhance the sun protection factor when compared to individual physical sunscreen and can be incorporated to form emulsion.

Preparation method of simple and stable water in oil (w/o) emulsion in this experiment was quoted from Woodruff (2001) who formulated the physical sunscreen emulsion containing 5% titanium dioxide. In this study, the w/o formula consists of C12-15 alkyl benzoate, mineral oil, beeswax, magnesium sulfate, propylene glycol, methyl paraben, propyl paraben, deionized water and PEG-30 dipolyhydroxystearate (arlacel P135). Arlacel P135 is nonionic w/o emulsifier which can be used to make stable and high water content w/o emulsions. It is unconcerned by all different oils and viscosities.

Meanwhile, oil in water (o/w) emulsion was prepared by changing type, percentage of emulsifier and ordering preparation method from w/o formulation. Nonionic o/w emulsifier used is sorbitan stearate (and) sucrose cocoate available as *arlacel™* 2121 which is excellent spreading properties. It can improve water resistance of formulations and is compatible with both chemical and physical sunscreens. All ingredients are shown in table 8.

Emulsifiers used in this experiment were determined by Hydrophile-lipophile balance (HLB) system as shown below.

Calculate require HLB for formulations

Oil phase

1.  Alkyl benzoate (HLB=13)   = 10%  ;  $\dfrac{10}{30.5}$   = 0.328 x 13 = 4.264

2.  Mineral oil   (HLB=10)   = 17.5% ;  $\dfrac{17.5}{30.5}$   = 0.574 x 10 = 5.740

3.  Beeswax   (HLB=12)   = 3%  ;  $\dfrac{3}{30.5}$   = 0.098 x 12 = 1.176

Total percentages   = 30.5%

Require HLB   = 4.264 + 5.740 + 1.176 = 11.180

Table 8 Water in oil (w/o) and oil in water (o/w) formulas

| Ingredients | w/o (%w/w) | o/w (%w/w) |
|---|---|---|
| <u>Oil phase</u> | 33.16 | 30.56 |
| titanium dioxide or zinc oxide | qs. | qs. |
| calcium carbonate | qs. | qs. |
| C12-15 alkyl benzoate | 10.00 | 10.00 |
| mineral oil | 17.50 | 17.50 |
| beeswax | 3.00 | 3.00 |
| propyl paraben | 0.06 | 0.06 |
| PEG-30 dipolyhydroxystearate (arlacel™ P135) | 2.60 | - |
| <u>Water phase</u> | 66.84 | 69.44 |
| sorbitan stearate (and) sucrose cocoate (arlacel™ 2121) | - | 5.00 |
| magnesium sulfate | 0.70 | 0.70 |
| propylene glycol | 5.00 | 5.00 |
| methyl paraben | 0.10 | 0.10 |
| deionized water | 61.04 | 58.64 |
| Total | 100.00 | 100.00 |

HLB of arlacel 135 is 5.5. Percentage used of this emulsifier can be calculated by $\dfrac{11.18}{5.5}$ = 2.033. However, Woodruff used 2.6% arlacel 135 in w/o emulsion and confirmed the stability. Thus, w/o emulsifier level in this study is quoted from Woodruff (2001). Whereas HLB of arlacel 2121 is 6. Percentage used = $\dfrac{11.18}{6}$ = 1.87. Nevertheless, in preliminary study, o/w formulation of 1.87% arlacel 2121 can't be formed an emulsion. There was some water separated. Therefore, percentage of arlacel 2121 was increased as 5% to form o/w emulsion.

Figure 10 shows w/o emulsion preparation, pre-dispersion of physical screen was prepared before emulsification. Combination of titanium dioxide or zinc oxide with calcium carbonate were incorporated into C12-15 alkyl benzoate and mineral oil, followed by dispersing all particles in both oils by High speed disperser of 800 rpm for 10 minutes. After that residual of oil phase (beeswax, propyl paraben and PEG-30 dipolyhydroxystearate) were added into the dispersion and heated by water bath to 70 Celsius degree (°C).

Meanwhile, water phase, including magnesium sulfate, propylene glycol, methyl paraben and deionized water, were heated to 75℃. Heated-water phase was added slowly to heated-oil phase using homogenizer instrument with high sheer of 10,000 rpm for 5 minutes. Stirring speed was decreased to 1,000 rpm and continued stirring for 10 minutes, then emulsion was formed and cooled to room temperature.
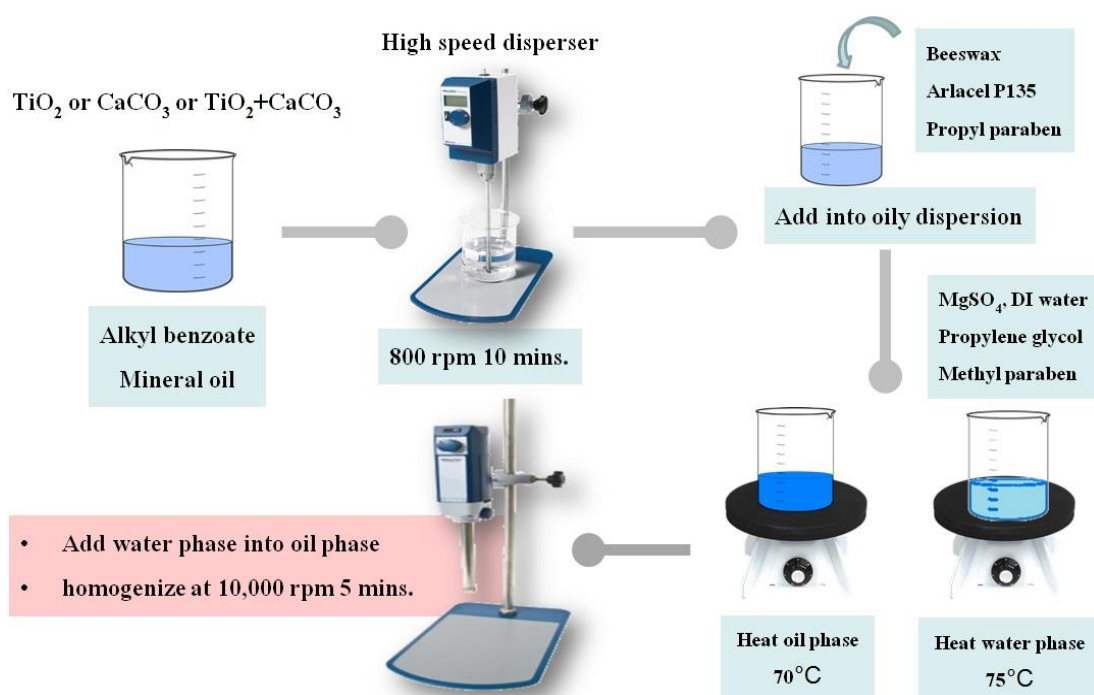


Figure 10 w/o emulsion preparation

Figure 11 illustrates o/w emulsion preparation, the method of pre-dispersion was same with w/o emulsion. Beeswax and propyl paraben were added and heated the oil phase to 70℃. For water phase, deionized water was heated above 80℃ and then mixed with sorbitan stearate (and) sucrose cocoate (arlacel™ 2121). Subsequently, propylene glycol and methyl paraben were added into water phase at 75℃. Heated-oil phase was added slowly to heated-water phase using homogenizer instrument. The next step was done as same way with w/o preparation. In addition, w/o and o/w blank emulsions were prepared as cream base. All sample pictures were shown in Figure 12.
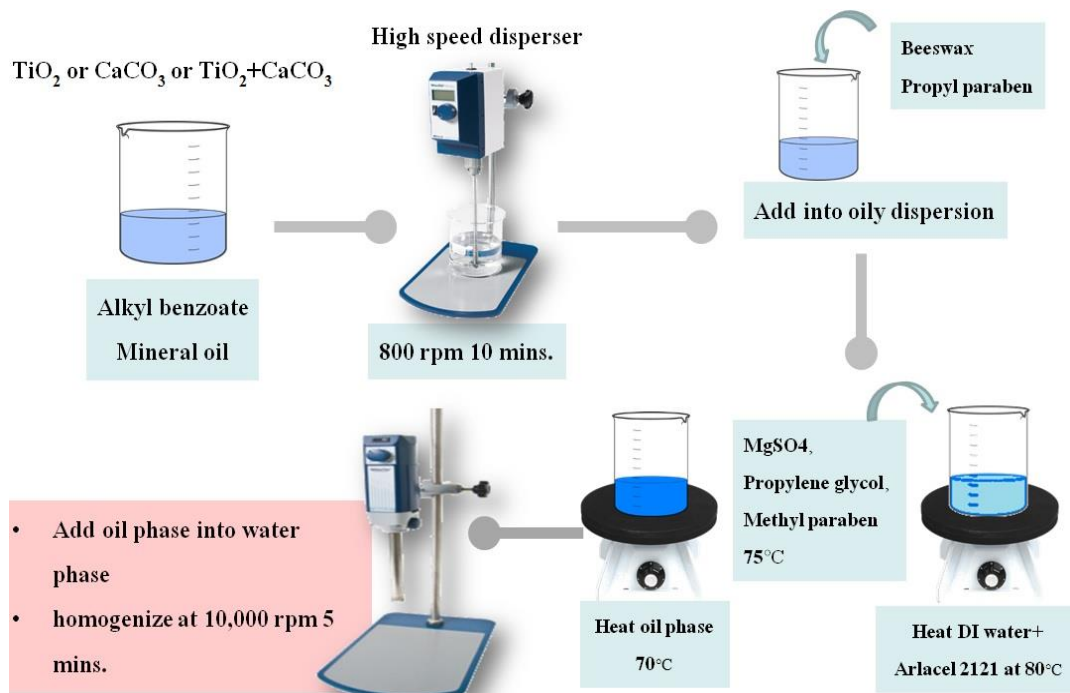
.Figure 11 o/w emulaion preparation
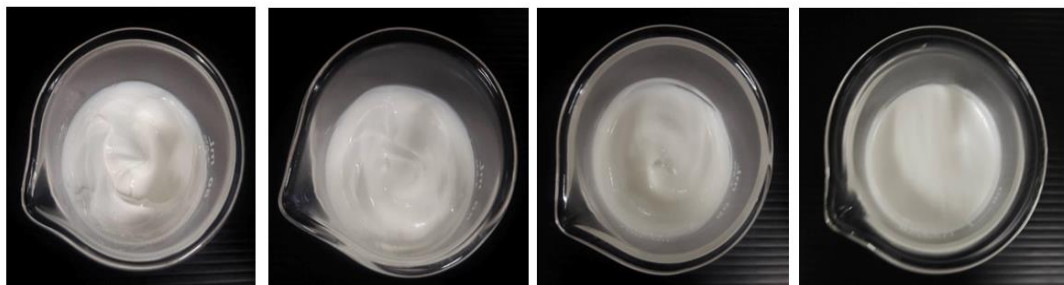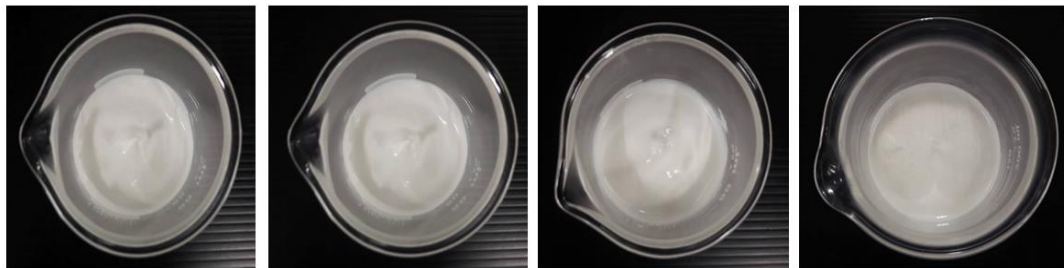


Figure 12 w/o and o/w formulations

Emulsion types were determined by observing incidents when each emulsion was dropped in pure water or mineral oil, respectively. W/O emulsion drops will disperse when they are dropped in a volume of oil but continue as drops in a volume of water. In contrast, o/w emulsion drops will disperse in water and remain as drops in oil (Henríquez, 2009).

### 4.2  Determination the Sun Protection Factor of Emulsions

Nine samples, namely w/o and o/w physical sunscreen combined with calcium carbonate emulsions, w/o and o/w individual physical sunscreen emulsions, w/o and o/w individual calcium carbonate emulsions, w/o and o/w cream base and standard sunscreen formulation, were sent to Chulalongkorn University Drug and Health Products Innovation Promotion Center (CU.D.HIP) to analyze SPF/UVA-PF values using SPF 290s analyzer. The method is same as in section 3.3. Briefly, Neat PMMA plate was used as blank substrate. Sample was pointed into PMMA plate, and then it was applied homogeneously in PMMA about 1.3 ml/cm$^2$ thickness. (Optometrics, 2009).

## 5.  Validation for determination of UV-Visible Spectrophotometer

Test procedures for assessment the quality methods of the experiment should be measured to ensure acceptability criteria of the United States of pharmacopeia. The analytical methods of United States Pharmacopeia 35–National Formulary 30 (USP 35–NF 30) were evaluated for accuracy, precision, specificity and linearity.

### 5.1  Linearity

Six points for the calibration curve were carried out in a concentration range, the combined concentration percentages of physical sunscreens and calcium carbonate were 2+2, 3+3, 4+4, 5+5, 6+6 and 7+7%w/w, the ratio was 1:1. The

calibration curve was created by plotting mean absorbance peak value (A) against concentration (C). Linearity was calculated using a mathematic to the regression line such as least mean square ($R^2$). The acceptance criteria of $R^2$ should be more than 0.9950 (USP 35–NF 30).

### 5.2 Accuracy

Accuracy of the analytical method was ascertained in seven replications (n=7). Standard quantity equivalent, 80, 100 and 120 % of target concentration, was added in the sample. The concentrations of titanium dioxide and zinc oxide combined with calcium carbonate are 4+4, 5+5 and 6+6%w/w, respectively. The ratio is 1:1, the target concentration is 5+5 which means 100%. Average recovery values for the combination of physical sunscreens and calcium carbonate were calculated as mean and standard deviation (mean±SD). The acceptance criteria range should be between 95% and 102%.

### 5.3 Precision

Precision of the method was determined by carrying out the analysis as within run precision and between run precision. Standard deviation (SD) and % relative standard deviation (%RSD) were calculated for the repeatability analysis.

**Within run precision**

Intra-day repeatability method was analyzed in six determinations (n=6) for each three concentrations of the combinations of physical sunscreens and calcium carbonate dispersion by preparing in the same day. Percentage of relative standard deviation (%RSD), which should be less than 2%, was calculated (USP 35–NF 30).

\

**Between run precision**

Inter-day repeatability method was analyzed in three determinations (n=3) for each three concentrations of the combinations of physical sunscreens and calcium carbonate dispersion by preparing on 3 days. Percentage of relative standard deviation (%RSD), which should be less than 2%, was calculated.

### 5.4 Specificity

Specificity analytical method is to ensure that the experimental procedure is unaffected by other components. The signal measurement should come from only interesting substance and the other components must not interfere. This assay was to compare between the absorbance spectra between dispersion of physical sunscreens combined with calcium carbonate and dimeticone oil which was used as a blank.

## CHAPTER IV
## RESULTS AND DISCUSSION

### 1. Morphology characterization of physical sunscreens and calcium carbonate

Morphology of calcium carbonate, titanium dioxide and zinc oxide powder were observed under Field Emission Scanning Electron Microscope. Figure 13 shows the morphology of calcium carbonate at magnifications of 5,000x and 30,000x. It exhibits that calcium carbonate is spherical shape with particle size of approximately by 100 nm. In addition, some particles are found to agglomerate.

Photomicrographs of titanium dioxide indicate that the shape of titanium dioxide is needle shape and length of its long axis is approximately 100-200 nm (Figure 14). Individual particles of zinc oxide, however, cannot be seen because of the aggregation (Figure 15). The shape of individual zinc oxide is predicted to be spherical and the particle size is approximately 100-200 nm.

(13a)                           (13b)
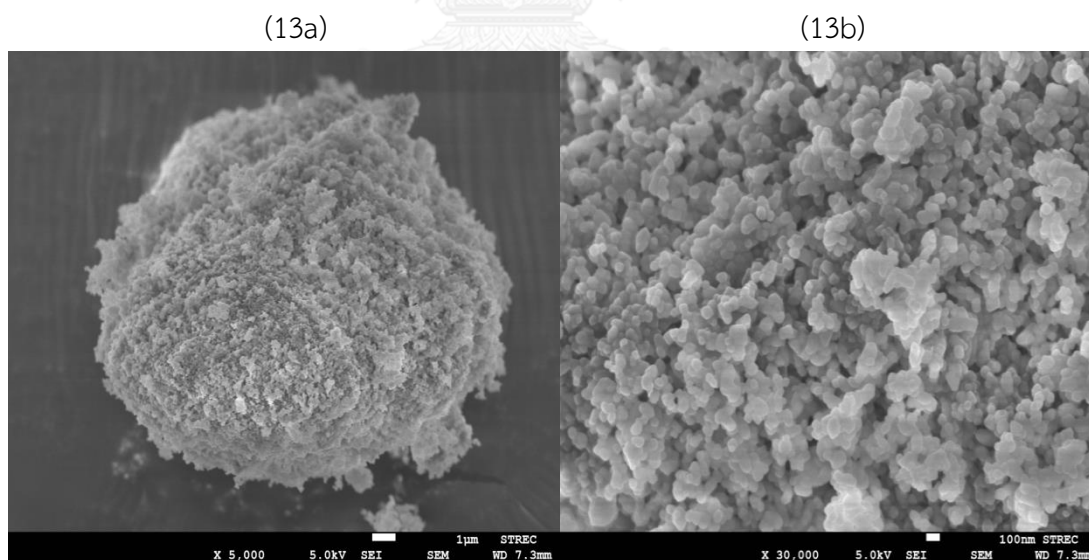


Figure 13 Scanning electron microscopy (SEM) of calcium carbonate nanoparticles
(13a) 5,000x and (13b) 30,000x

(14a)                                    (14b)
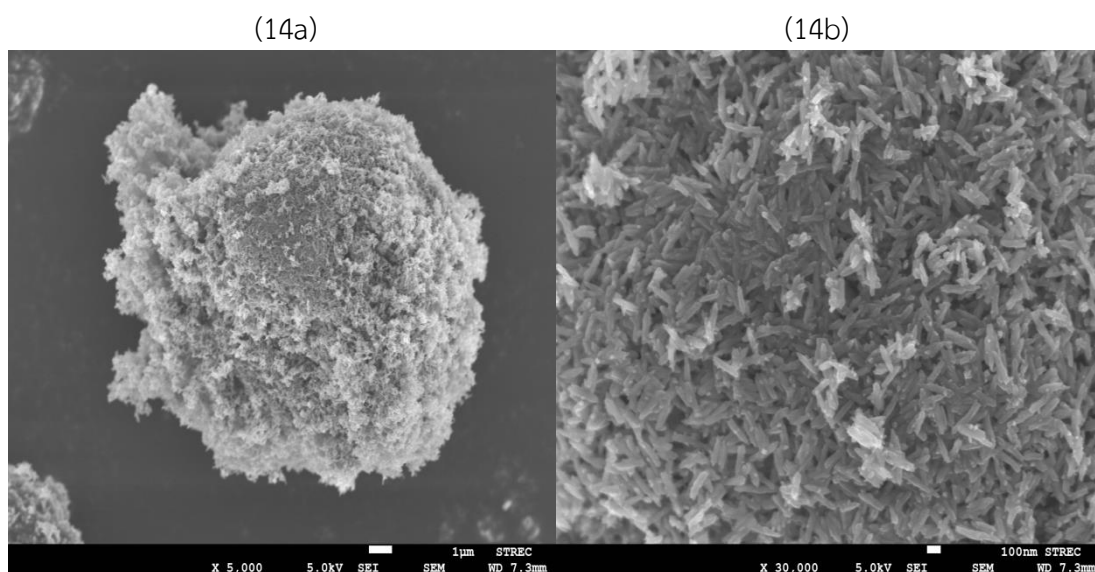


Figure 14 Scanning electron microscopy (SEM) of titanium dioxide nanoparticles
(14a) 5,000x and (14b) 30,000x

(15a)                                    (15b)
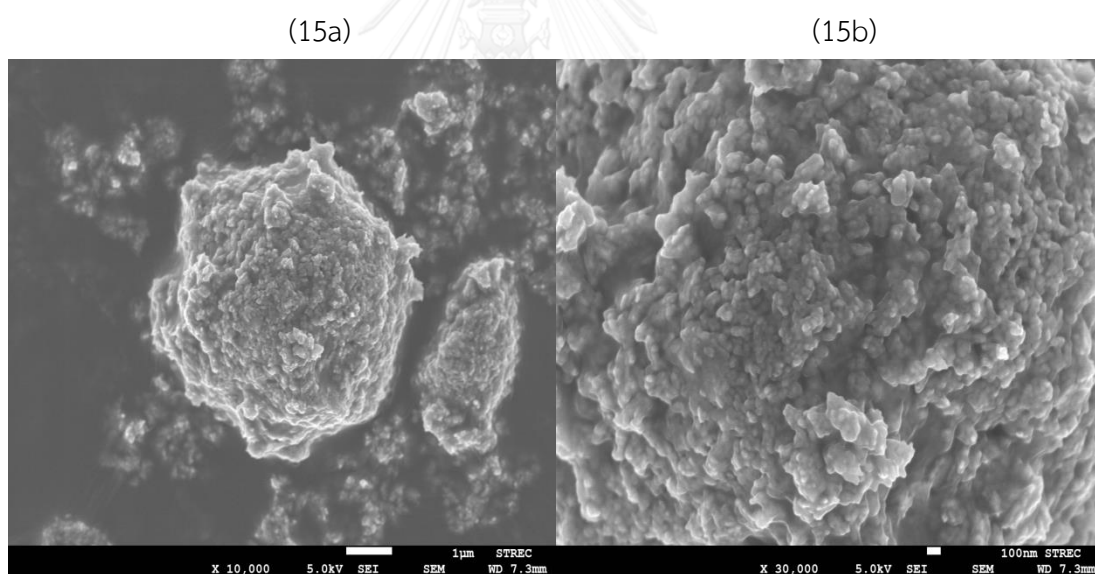


Figure 15 Scanning electron microscopy (SEM) of zinc oxide nanoparticles
(15a) 10,000x and (15b) 30,000x

## 2. Determination for proper sample preparation conditions

### 2.1 Preparation of physical sunscreen dispersions

Combination of 10%w/w titanium dioxide and 5%w/w calcium carbonate, 10%w/w zinc oxide and 5%w/w calcium carbonate and each individual component were dispersed in dimethicone. Individual samples were used as control group. Ultraviolet absorption was measured by UV-Visible spectrophotometer. Various speed and duration of high speed disperser were investigated for obtaining the optimal conditions uniformly dispersing samples and achieve excellent ultraviolet absorption.

### 2.2 Ultraviolet absorbance measurement

Overall UV absorbance ($abs_o$) was measured throughout this study. Figures 16, 17 and 19 indicate that calcium carbonate shows very low absorption at 320 nm while absorption of titanium dioxide and zinc oxide occur at 325 and 375 nm, respectively. According to Schlossman and Shao (2005), titanium dioxide with particle size of 250 nm showed the absorption peak at 318 nm which correlates well with this study. Also, the absorption peak of zinc oxide in this experiment was the same as the results obtained by Schlossman and Shao (2005) at wavelength of 375 nm where particle size of zinc oxide was 260 nm. The absorption peak of physical sunscreens depended partly on particle size in the carrier. Bigger particle size exhibits longer absorption wavelengths. Therefore, it can be speculated that the particle size of titanium dioxide is slightly larger than 250 nm and zinc oxide was approximately 260 nm.

According to speed and durations used for dispersing physical sunscreens (Table 9), some dispersing conditions showed similar peak absorbance values, as can be seen in Figures 16-21, and relative orders are summarized in Table 9.
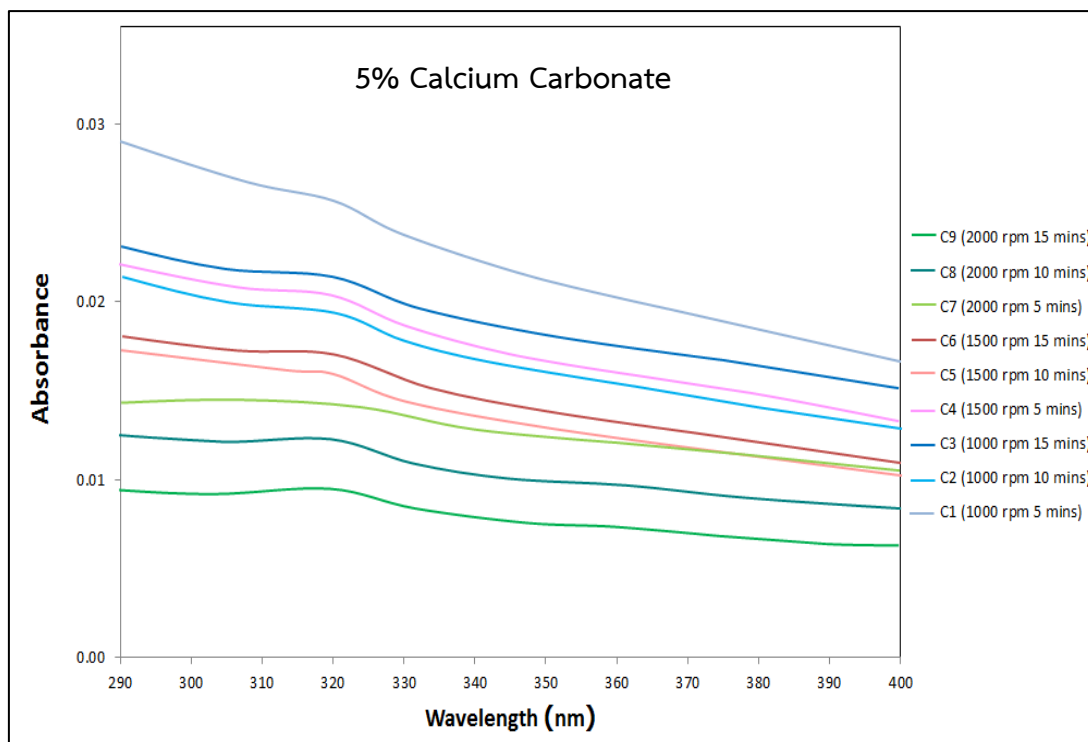
Figure 16 Absorption spectra of CaCO$_3$ using various dispersing conditions
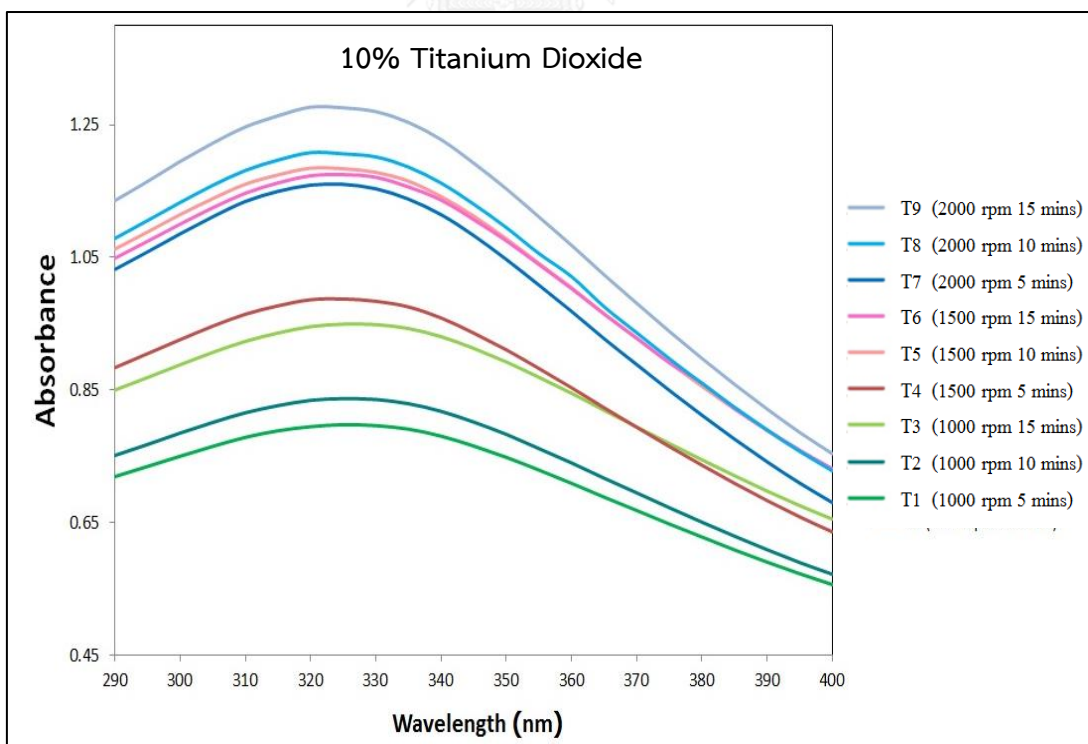


Figure 17 Absorption spectra of TiO$_2$ using various dispersing conditions
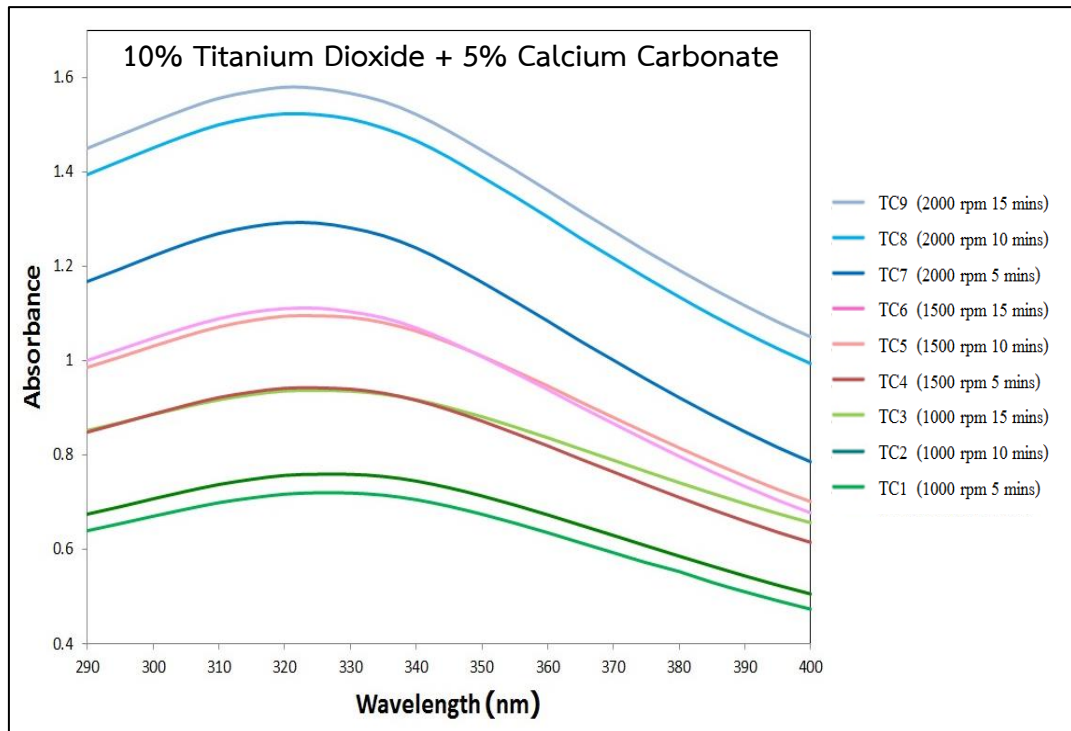
Figure 18 Absorption spectra of $TiO_2$ combined with $CaCO_3$ using various dispersing conditions
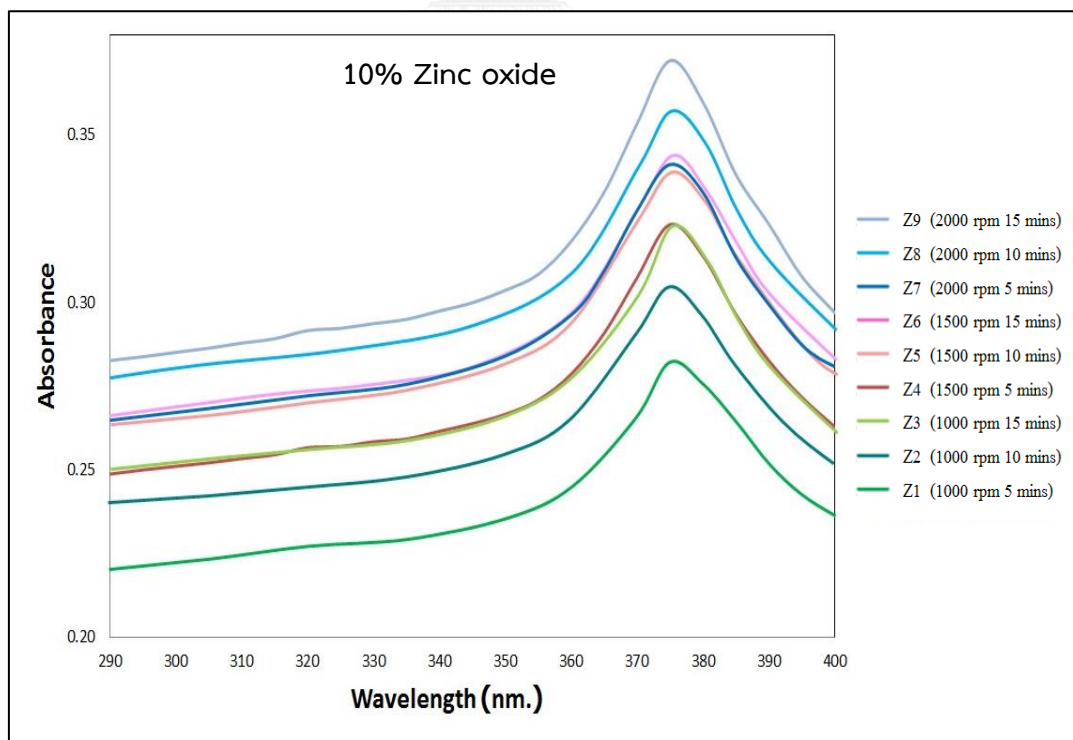


Figure 19 Absorption spectra of ZnO using various dispersing conditions
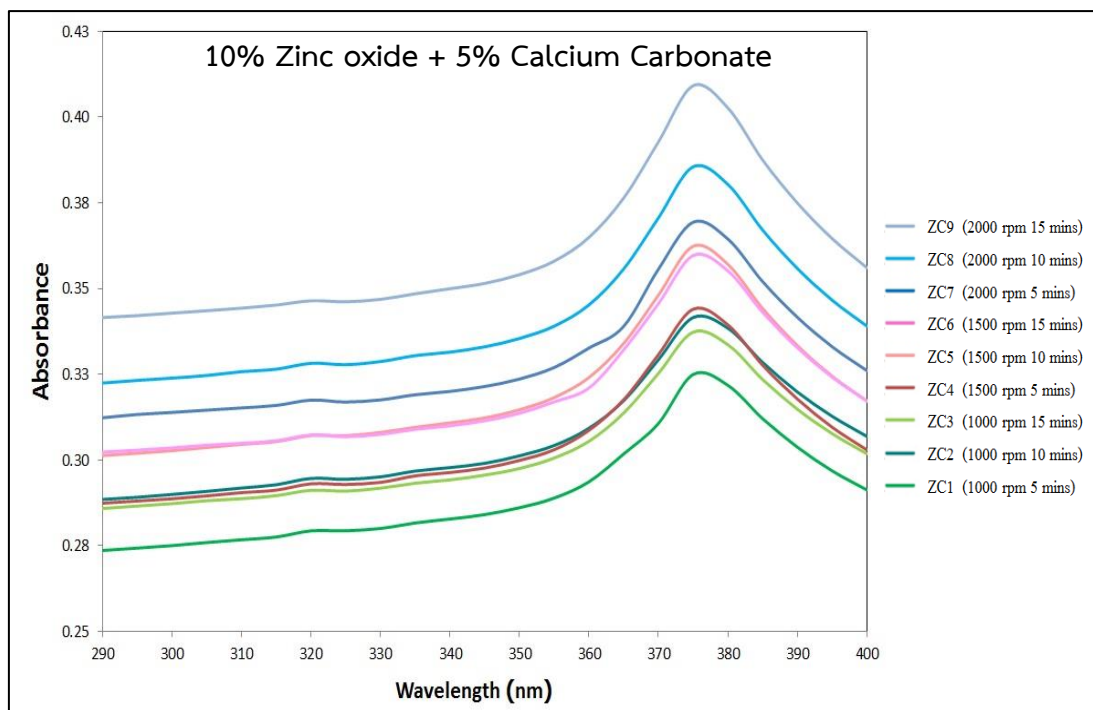
Figure 20 Absorption spectra of ZnO combined with CaCO$_3$ using various dispersing conditions

Figure 21 shows UV absorbance peak values under various dispersing conditions. Figure 21a is the graph for titanium dioxide, calcium carbonate and their combination, and 21b is for zinc oxide, calcium carbonate and their combination. These graphs illustrate that both speed and duration affected ultraviolet absorption of individual physical sunscreen or in combination with calcium carbonate. When speed and duration increased, most absorbance spectra increased, as shown in Table 9. It can be seen that, higher shear dispersion can reduce the time used similar to the report by Markee and coworkers (2008). In Figure 21b, it was found that absorbance values of conditions 1-4 for titanium dioxide alone increased when speed and time increased, but absorbance values of conditions 5-9 were constant. It is noteworthy that conditions 5-9 can disperse titanium dioxide particles well and homogeneously.

For titanium dioxide in combination with calcium carbonate, it was found that the absorbance values for conditions 1-7 increased while conditions 8-9 are constant. It means that conditions 8-9 can disperse particles well. Both of conditions 8 and 9 showed high UV absorption but condition 8 is shorter time used. Therefore, condition 8 was chosen as an optimal condition for dispersing particles in dimethicone oil. This

was due to its high absorbance and no particle structure changes because the absorption peaks of all conditions are same.

In Figure 21b, it was found that the absorbance values for both of zinc oxide alone and zinc oxide combined with calcium carbonate is increased slightly from conditions 1-9 and conditions 8-9 is higher than 1-7. Thus, condition 8 was chosen for optimal condition as same reason with titanium dioxide combined with calcium carbonate. All absorption values are shown in Table 10.

In summary, proper dispersion method could increase the degree of UV attenuation (Innospec, 2010). In sun care products, effective distribution of pigments and active ingredients are highly necessary to ensure sufficient ultraviolet protection. Cost saving can be made by reducing each physical sunscreen concentrations if proper dispersion is used.

Table 9 Order of absorption values for each physical sunscreen under various dispersing conditions

| Samples | conditions |
|---------|------------|
| $TiO_2$ | 9 > 8, 7, 6, 5 > 4, 3 > 2, 1 |
| $TiO_2 + CaCO_3$ | 9, 8 > 7 > 6, 5 > 4, 3 > 2, 1 |
| ZnO | 9 > 8 > 7, 6, 5 > 4, 3 > 2 > 1 |
| $ZnO + CaCO_3$ | 9 > 8 > 7 > 6, 5 > 4, 3, 2 > 1 |
| $CaCO_3$ | 9 > 8, 6 > 7 > 5, 4 > 3 > 2 > 1 |

(21a)
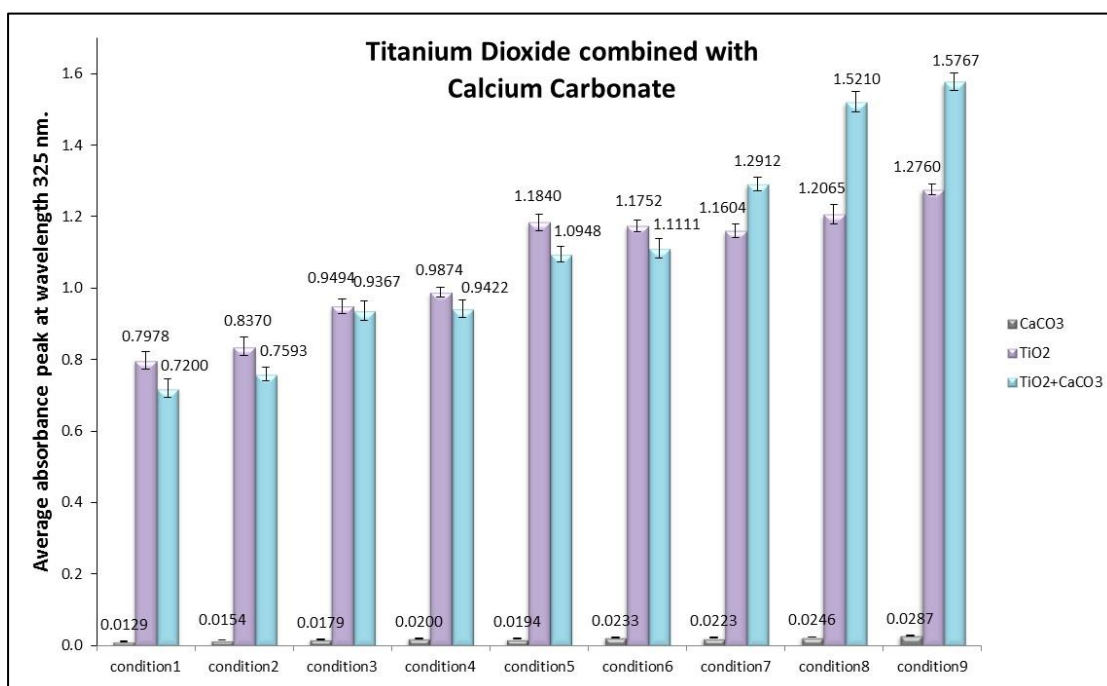


(21b)
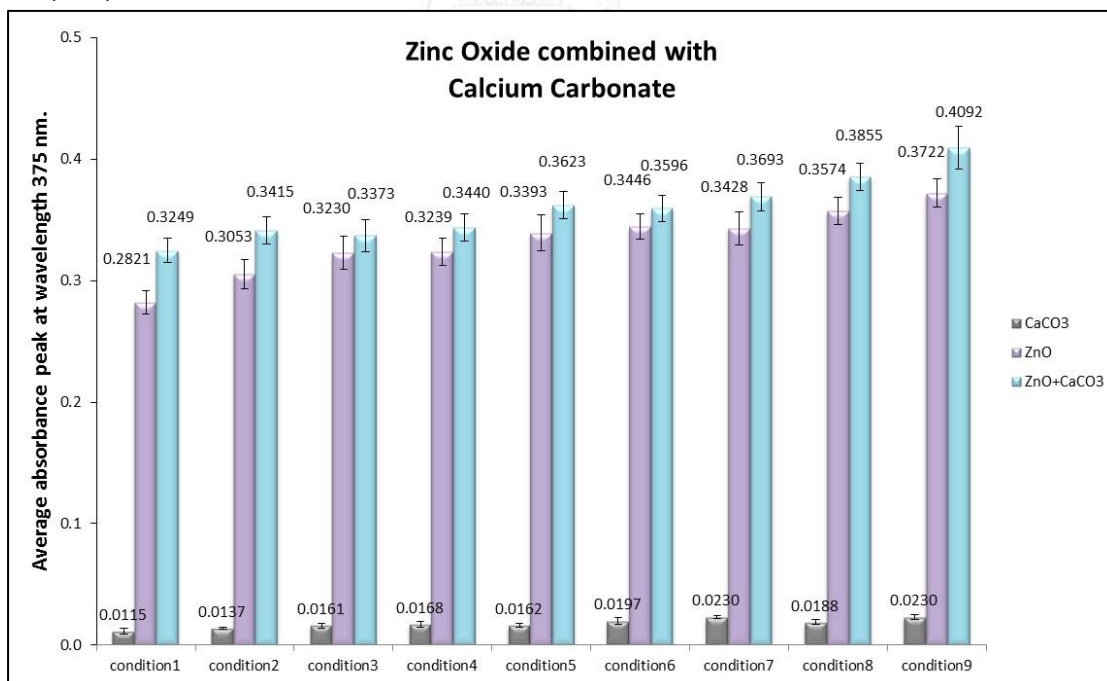


Figure 21 Absorbance values obtained by utilizing various dispersing conditions of (21a) $CaCO_3$, $TiO_2$ and their combinations and (21b) $CaCO_3$, ZnO and their combinations.

Table 10 UV absorption values of physical sunscreens combined with calcium carbonate

| | Conditions | | Average peak absorbance at 325 nm (mean±SD) | | | Average peak absorbance at 375 nm (mean±SD) | | |
|---|---|---|---|---|---|---|---|---|
| | | | $TiO_2$ | $TiO_2+CaCO_3$ | $CaCO_3$ | ZnO | ZnO+CaCO3 | $CaCO_3$ |
| 1 | 1000 rpm | 5 mins | 0.7978±0.0003 | 0.7200±0.0011 | 0.0129±0.0004 | 0.2821±0.0010 | 0.3249±0.0061 | 0.0115±0.0005 |
| 2 | | 10 mins | 0.8370±0.0016 | 0.7593±0.0039 | 0.0154±0.0003 | 0.3053±0.0008 | 0.3415±0.0021 | 0.0137±0.0005 |
| 3 | | 15 mins | 0.9494±0.0017 | 0.9367±0.0057 | 0.0179±0.0003 | 0.3230±0.0033 | 0.3373±0.0025 | 0.0161±0.0002 |
| 4 | 1500 rpm | 5 mins | 0.9874±0.0013 | 0.9422±0.0029 | 0.0200±0.0002 | 0.3239±0.0018 | 0.3440±0.0040 | 0.0168±0.0002 |
| 5 | | 10 mins | 1.1840±0.0012 | 1.0948±0.0023 | 0.0194±0.0002 | 0.3396±0.0092 | 0.3623±0.0018 | 0.0162±0.0002 |
| 6 | | 15 mins | 1.1752±0.0008 | 1.1111±0.0020 | 0.0233±0.0006 | 0.3446±0.0017 | 0.3596±0.0041 | 0.0197±0.0005 |
| 7 | 2000 rpm | 5 mins | 1.1604±0.0011 | 1.2912±0.0038 | 0.0226±0.0008 | 0.3428±0.0026 | 0.3693±0.0026 | 0.0190±0.0014 |
| 8 | | 10 mins | 1.2065±0.0025 | 1.5210±0.0010 | 0.0246±0.0001 | 0.3574±0.0011 | 0.3855±0.0026 | 0.0230±0.0002 |
| 9 | | 15 mins | 1.2760±0.0024 | 1.5767±0.0018 | 0.0287±0.0004 | 0.3722±0.0008 | 0.4092±0.0040 | 0.0230±0.0004 |

**3. Sun protection efficiency determination of physical sunscreens combined with calcium carbonate in various concentrations**

3.1 Physical sunscreen dispersion preparation

Titanium dioxide and zinc oxide were combined with calcium carbonate in various concentrations by physical mixing. Titanium dioxide, zinc oxide and calcium carbonate alone were also used as control group. All samples were dispersed into dimethicone by High speed disperser at 2,000 rpm for 10 minutes which is the optimal stirring condition that was investigated in previous experimental section 2.

3.2 Ultraviolet absorption measurement

Overall UV absorbance ($abs_o$) was measured. UV absorbance of calcium carbonate in Figure 17 shows that when the concentrations increased, the absorption also increased. However, the intensity of absorption in both UVA and UVB regions were very low compare to that of titanium dioxide and zinc oxide. Every concentrations of calcium carbonate showed maximum peak in the UVB range at 320 nm. Absorbance values of calcium carbonate were very low where 2.5, 5, 7.5 and 10%w/w resulted in 0.0136, 0.0260, 0.0356 and 0.0427, respectively (Figure 17). Correlation coefficient ($R^2$) between concentration of calcium carbonate and average absorbance was 0.9853 (Figure 20). From these results, it can be concluded that calcium carbonate alone is not a good UV absorber similar to conclude made by Sato and Ikeya (2004).

Titanium dioxide had UV absorbance peak at 325 nm and also showed higher ultraviolet absorption when the concentration increased. Samples with concentrations of 2.5, 5 and 10%w/w had UV absorption intensities at 0.3037, 0.5898 and 1.2448, respectively (Figure 18). Titanium dioxide concentrations and average absorbance also showed strong correlation with $R^2$ of 0.9989 (Figure 21).

When titanium dioxide at concentrations of 2.5, 5 and 10%w/w was combined with calcium carbonate at 2.5, 5 and 10%w/w by physical mixing method. every combinations also showed concentration-dependence UV absorption (Figure

22). Correlation coefficient ($R^2$) on absorbance of every titanium dioxide concentrations when combined with 2.5, 5, 7.5 and 10%w/w calcium carbonate were 0.9960, 0.9992, 1.000 and 0.9998, respectively.

The ultraviolet absorption of titanium dioxide alone was compared with UV absorption of the combination of titanium dioxide and calcium carbonate. Figure 19 indicates that calcium carbonate could increase UV absorption of titanium dioxide for both UVA (320-400 nm) and UVB (290-320 nm) range. Interestingly, calcium carbonate could increase the intensity of UVB absorption of titanium dioxide more than UVA. The maximum absorption peak of titanium dioxide was shifted from 325 nm to shorter wavelength of calcium carbonate around 320 and 315 nm with increasing calcium carbonate concentrations.

Table 11 illustrates the % absorption enhancement of the combination when compare with titanium dioxide alone. Enhancing percentages at each titanium dioxide concentration increased when calcium carbonate concentrations increased. Calcium carbonate concentration which could provide highest UV absorption when combined with each titanium dioxide concentration is 10%w/w. In addition, Table 3 shows that the combined mixture of titanium dioxide and calcium carbonate also gave enhancing percentages when compared with the sum of absorbance values of each individual titanium dioxide and calcium carbonate at wavelength 325 nm.

Absorption enhancing percentages seem to decrease as titanium dioxide concentrations increased when combined with 7.5 and 10%w/w of calcium carbonate as seen in Table 3. However, at low concentrations of calcium carbonate (2.5 and 5%w/w) no decreased in photoprotection enhancement of $TiO_2$ were observed.

Thus, it seemed consistent that titanium dioxide when combined with calcium carbonate, with proper concentration and mixing method, could provide satisfactory synergistic photoprotection efficiency.
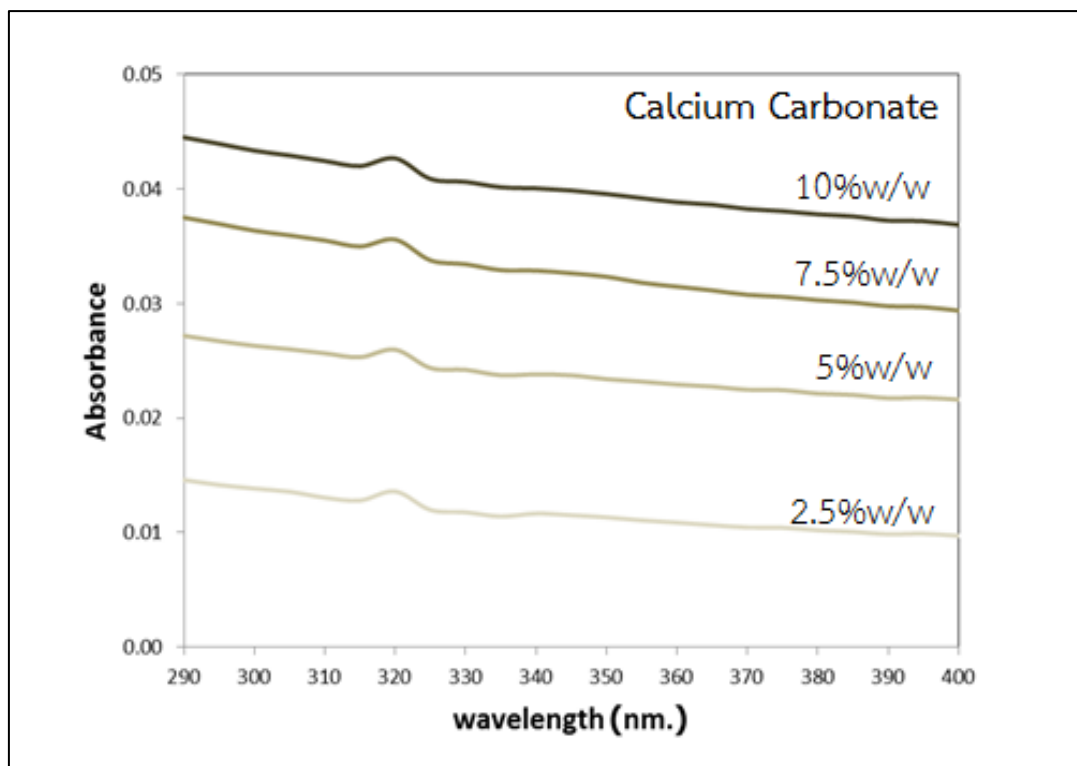
Figure 22 Absorbance spectra of $CaCO_3$ at various concentrations (n=3)



Figure 23 Absorbance spectra of $TiO_2$ at various concentrations (n=3)

Figure 24 Absorbance spectra of TiO$_2$ combined with CaCO$_3$ at various concentrations
(n=3)



Figure 25 Plot between CaCO$_3$ concentrations and average UV absorption values
(n=3)

Figure 26 Plots between concentrations of $TiO_2$ combined with various $CaCO_3$ concentrations and average UV absorption values (n=3)



Figure 27 Average UV absorbance of $TiO_2$ and when combined with $CaCO_3$ in various concentrations (n=3)
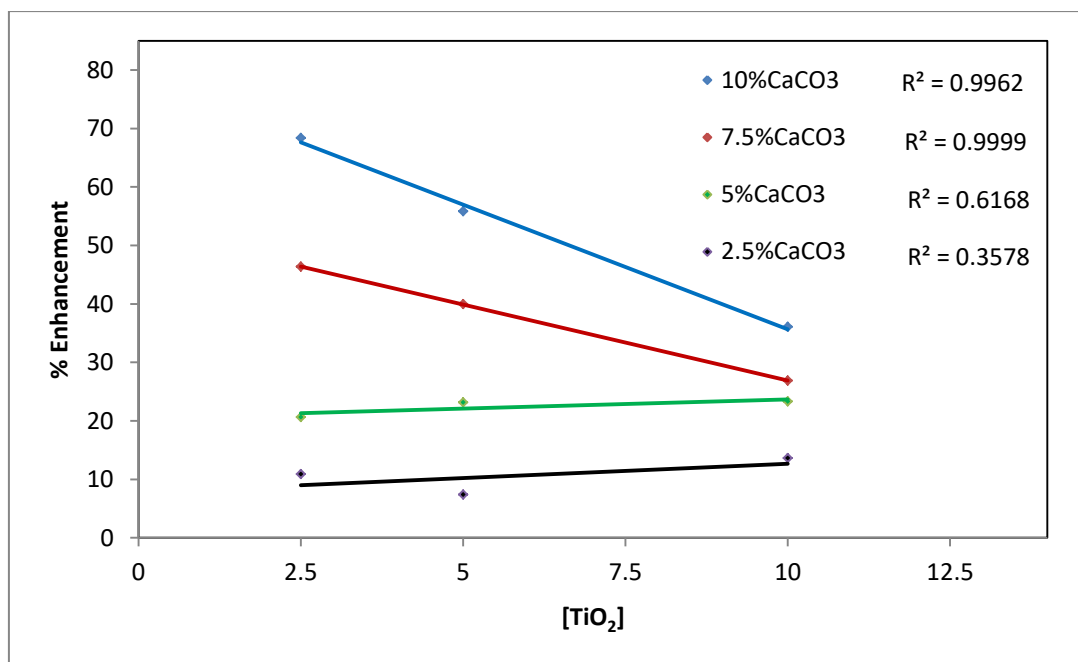
Figure 28 Plots between concentrations of $TiO_2$ when combined with various $CaCO_3$ concentrations and percent enhancement

Figure 31 illustrates strong correlation between zinc oxide concentrations and average UV absorbance ($R^2$ = 0.9997). Concentrations used for zinc oxide were 2.5, 5 and 10%w/w, as shown in Figure 29 where absorption values at 375 nm were 0.0918, 0.1897 and 0.3732, respectively (Figure 32). These results show intense absorption in UVA region.

The ultraviolet absorbance spectra of individual zinc oxide were compared with the combination of zinc oxide and calcium carbonate. The results showed that calcium carbonate can increase both UVA and UVB absorption of zinc oxide with equal intensity along both UVA and UVB regions (Figure 30). The maximum peak of this combined mixture was still at wavelength 375 nm which was the same as zinc oxide alone.

When zinc oxide at concentrations of 2.5, 5 and 10%w/w was combined with calcium carbonate at 2.5, 5 and 10%w/w by physical mixing method. Every combinations also showed concentration-dependence UV absorption (Figure 31).

Table 11 Percent enhancement of combination between $TiO_2$ and $CaCO_3$ when compare with pure $TiO_2$ and when compare with additive absorption value of $TiO_2$ and values of $CaCO_3$ (n=3)

| Samples | | Absorption values at 325 nm | % Enhancement of combination compare to $TiO_2$ alone | Additive absorption values of $TiO_2$ +$CaCO_3$ | % Enhancement of combination compare to additive values |
|---|---|---|---|---|---|
| % $TiO_2$ | % $CaCO_3$ | | | | |
| - | 2.5 | 0.0120 | - | - | - |
| | 5 | 0.0244 | - | - | - |
| | 7.5 | 0.0338 | - | - | - |
| | 10 | 0.0409 | - | - | - |
| 2.5 | - | 0.3037 | - | - | - |
| | 2.5 | 0.3368 | 10.90% | 0.3157 | 6.68% |
| | 5 | 0.3663 | 20.61% | 0.3281 | 11.64% |
| | 7.5 | 0.4444 | 46.33% | 0.3375 | 31.67% |
| | 10 | 0.5113 | 68.36% | 0.3446 | 48.37% |
| 5 | - | 0.5898 | - | - | - |
| | 2.5 | 0.6335 | 7.41% | 0.6018 | 5.27% |
| | 5 | 0.7264 | 23.16% | 0.6142 | 18.27% |
| | 7.5 | 0.8156 | 39.98% | 0.6236 | 30.79% |
| | 10 | 0.9190 | 55.82% | 0.6307 | 45.71% |
| 10 | - | 1.2448 | - | - | - |
| | 2.5 | 1.4142 | 13.61% | 1.2568 | 12.52% |
| | 5 | 1.5349 | 23.30% | 1.2692 | 20.93% |
| | 7.5 | 1.5795 | 26.89% | 1.2786 | 23.53% |
| | 10 | 1.6935 | 36.05% | 1.2857 | 31.71% |

Correlation coefficient ($R^2$) on absorbance of every zinc oxide concentrations when combined with 2.5, 5, 7.5 and 10%w/w calcium carbonate were 0.9990, 0.9989, 0.9998 and 1.000, respectively.

The exponential graph between enhancing percentages from individual zinc oxide and concentrations, which consist of 2.5, 5 and 10%w/w zinc oxide and 2.5, 5, 7.5 and 10%w/w calcium carbonate, were linearity with $R^2$ of 0.9555, 0.9944, 0.9298 and 0.9334, respectively (Figure 33).

When combined, higher calcium carbonate concentrations caused increased intensities of UV absorption (Figure 30 and 32). At every zinc oxide concentrations, 2.5, 5 and 10%w/w, resulted in higher enhancing percentages when increasing calcium carbonate concentrations. The best concentration of calcium carbonate which could provide the highest UV absorption in every zinc oxide concentrations was also found to be 10%w/w which was the same as when titanium dioxide was combined with calcium carbonate.

Percent enhancement for 10%w/w calcium carbonate combined with 2.5, 5 and 10%w/w zinc oxide when compared with individual zinc oxide were 59.91%, 23.62% and 11.42%, respectively (Table 12). When the absorption values of combination were compared to the addition of zinc oxide absorption values with calcium carbonate absorption values, percent enhancement were respectively 13.09%, 2.98% and 1.09%. They had low enhancing percentages, as seen in Table 12. Thus zinc oxide combined with calcium carbonate provided only addition effect, and did not show any synergy for ultraviolet absorption.
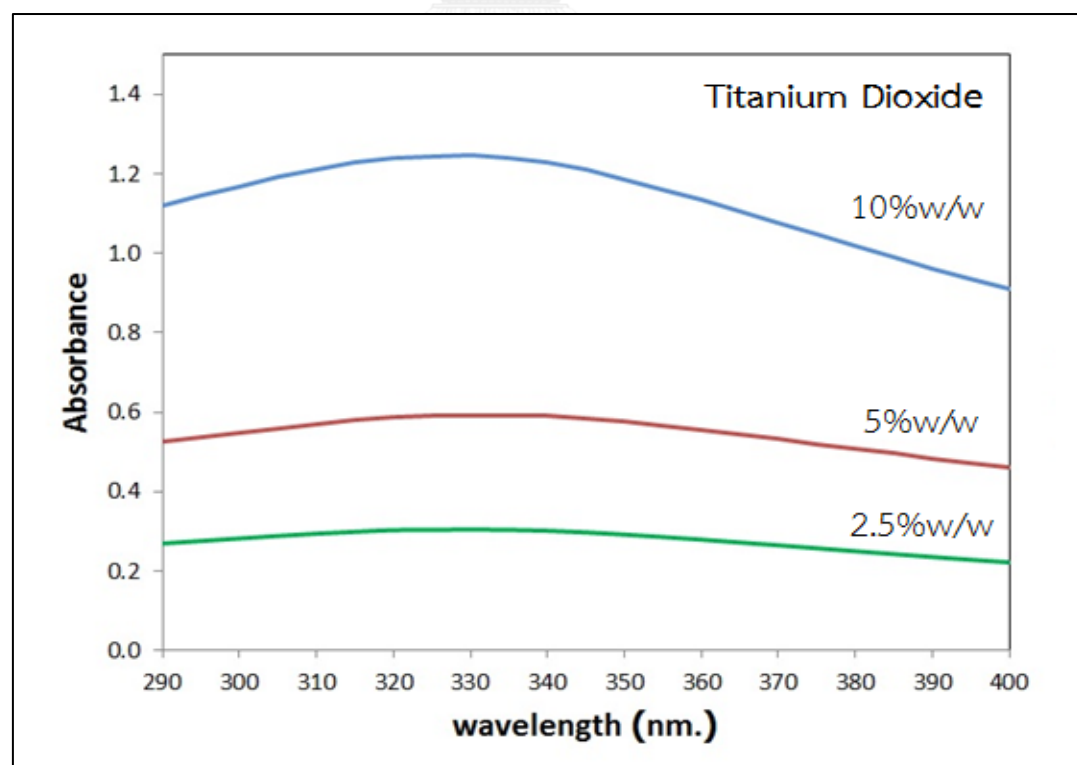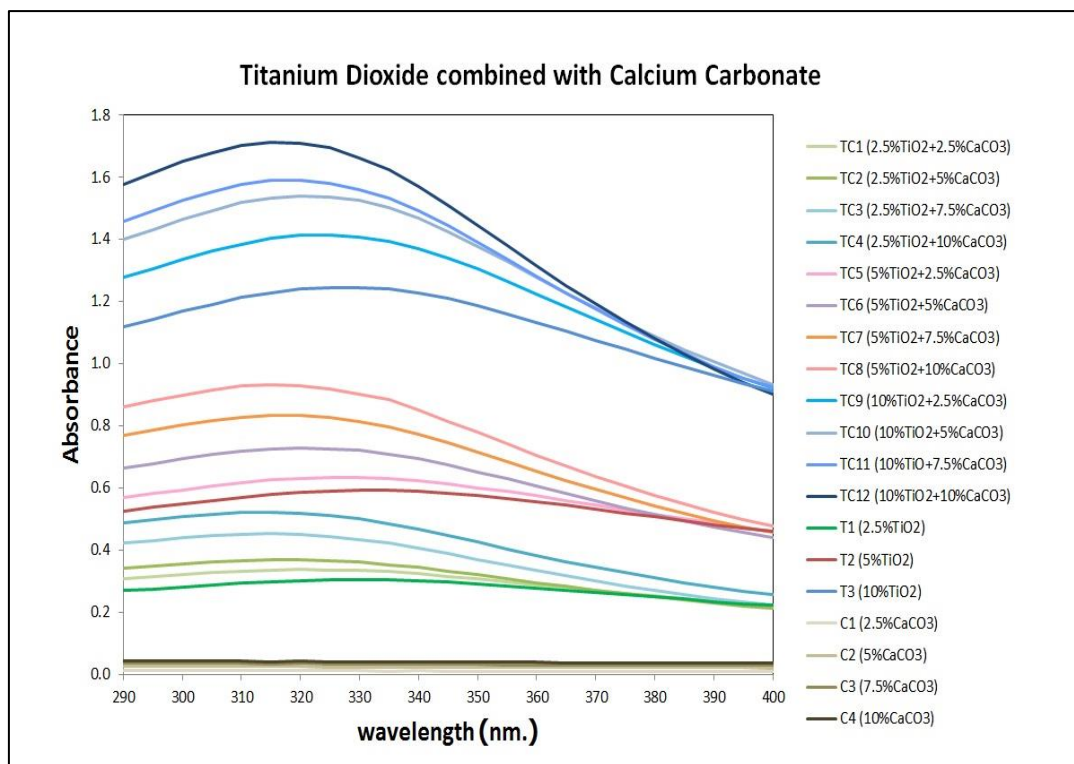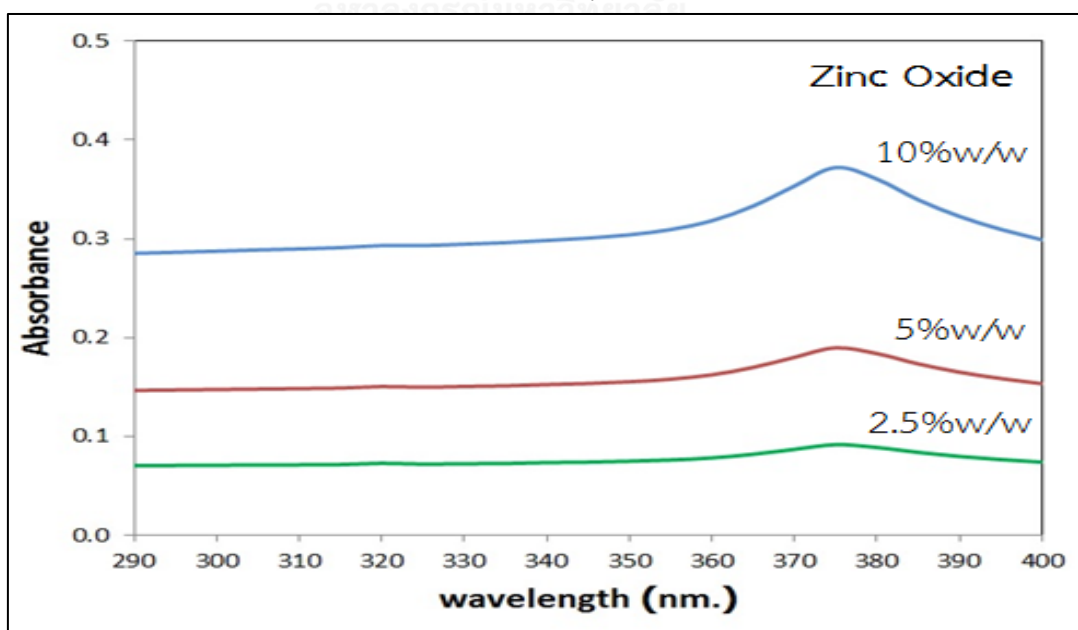


Figure 29 Absorbance spectra of various ZnO concentrations (n=3)
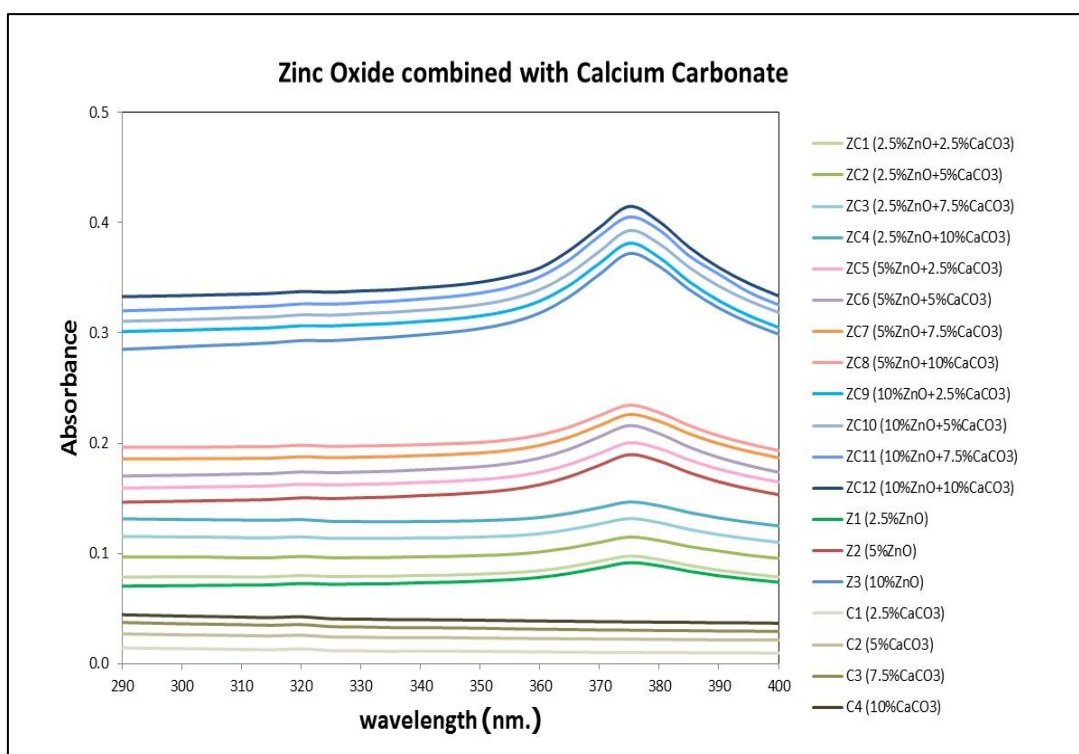
Figure 30 Absorbance spectra of ZnO combined with CaCO$_3$ at
various concentrations (n=3)
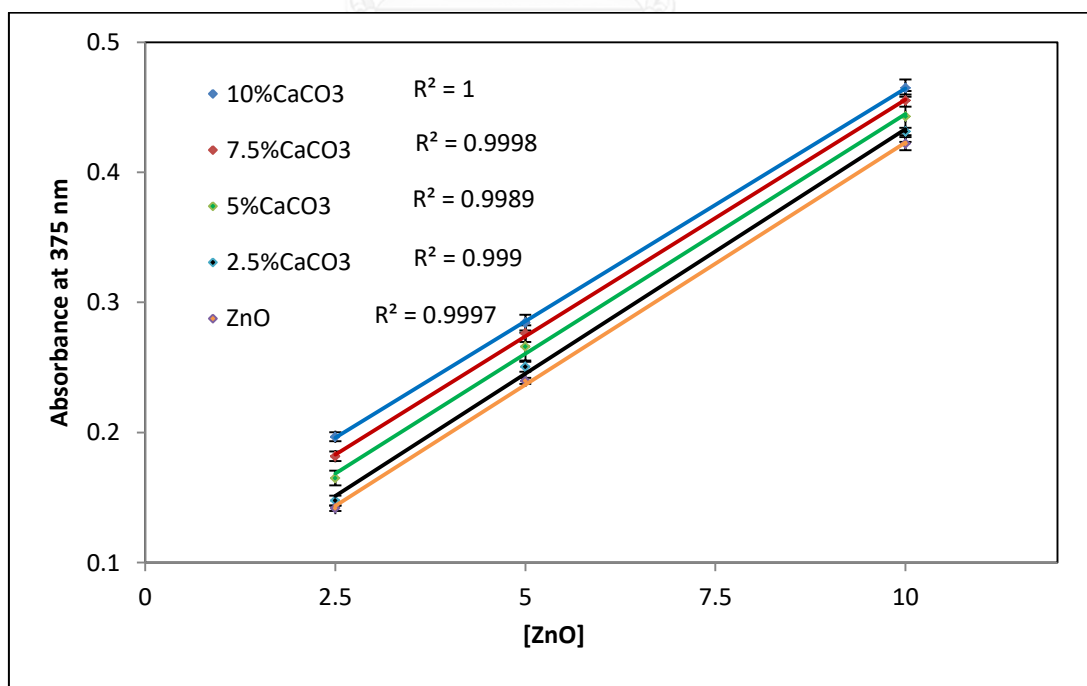


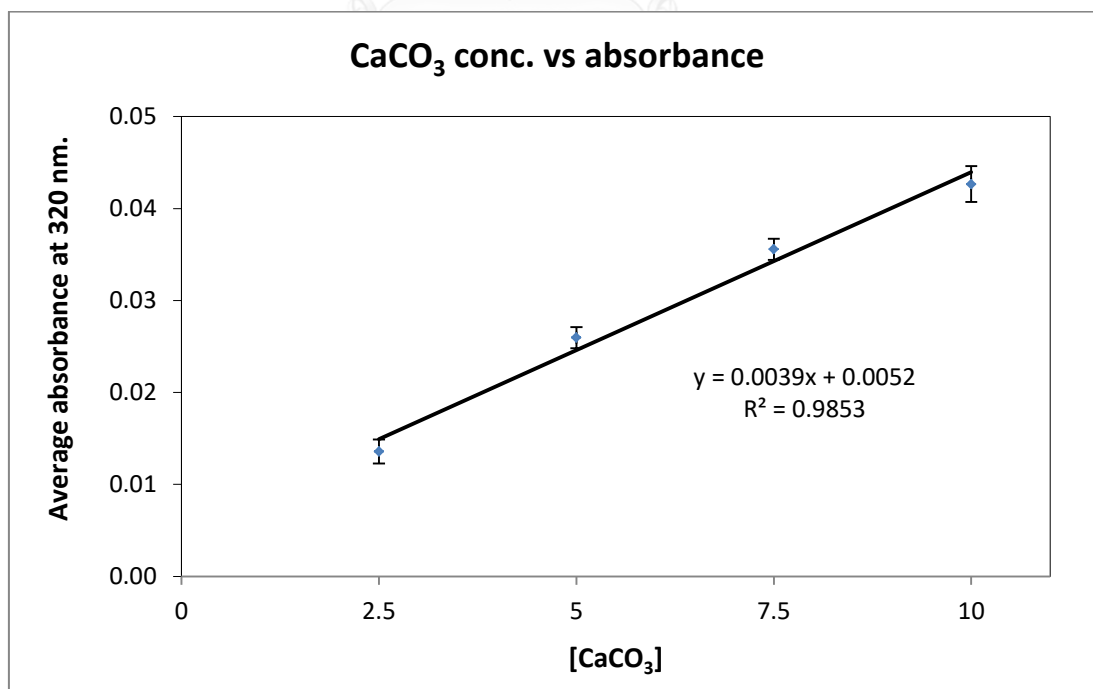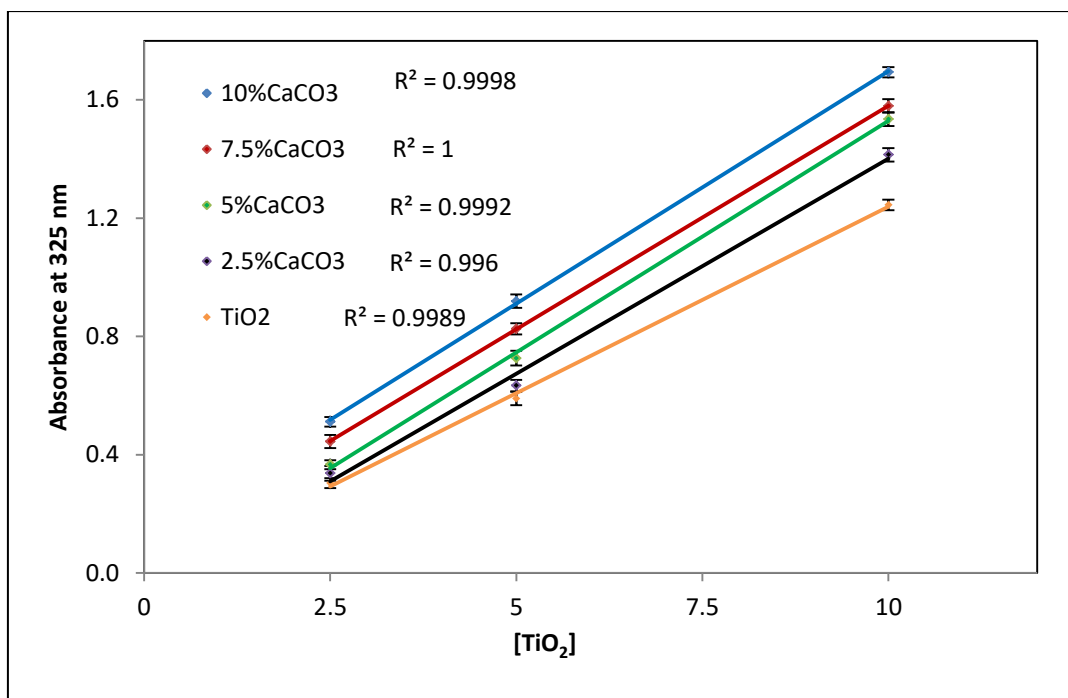Figure 31 Plots between concentrations of ZnO combined with various CaCO$_3$
concentrations and average UV absorption values (n=3)
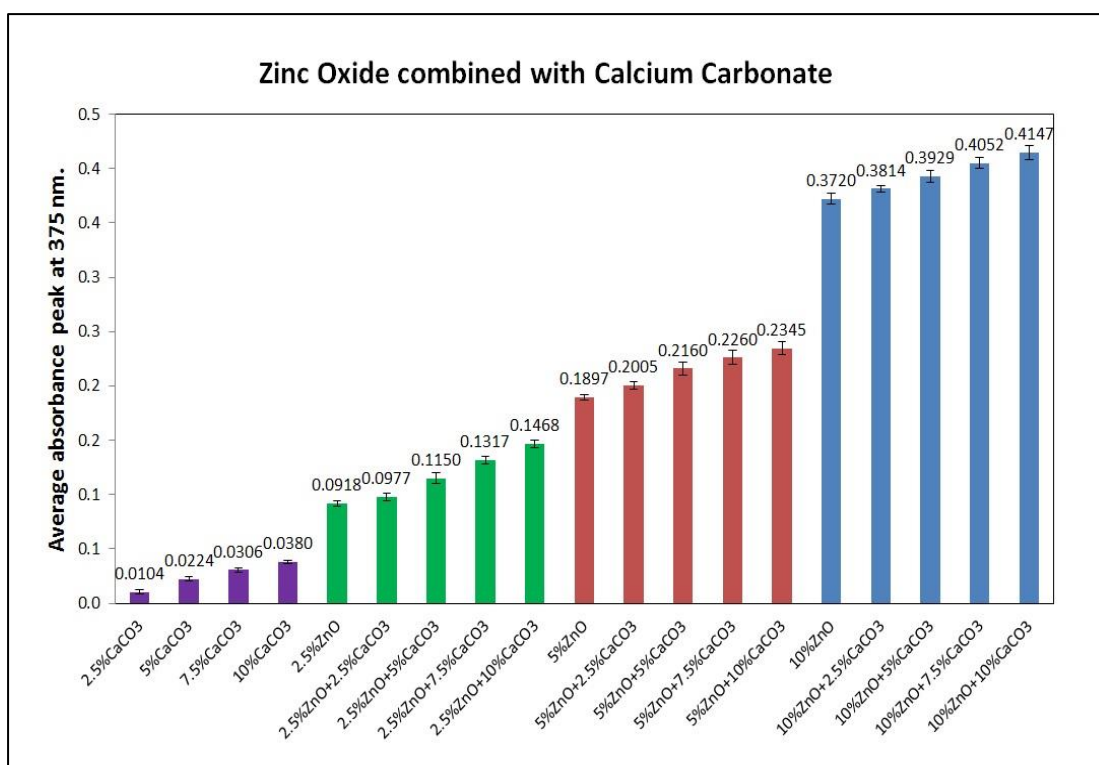
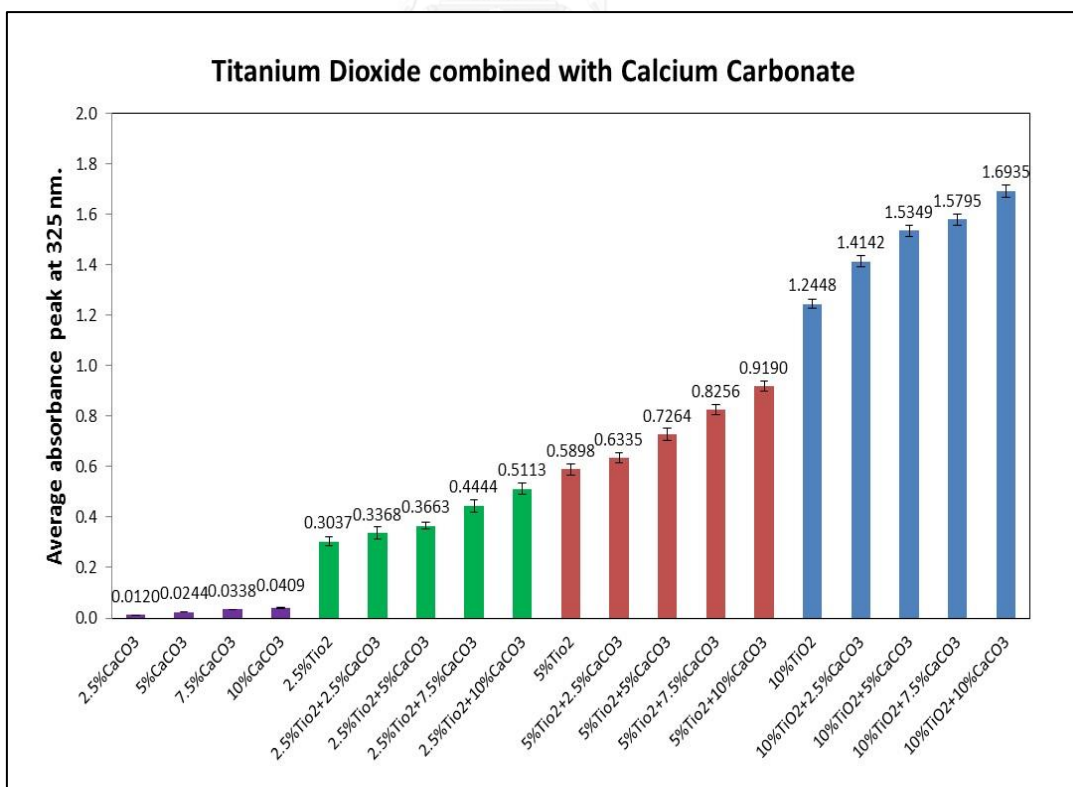Figure 32 Average UV absorbance of ZnO and when combined with CaCO$_3$ at various concentrations (n=3)
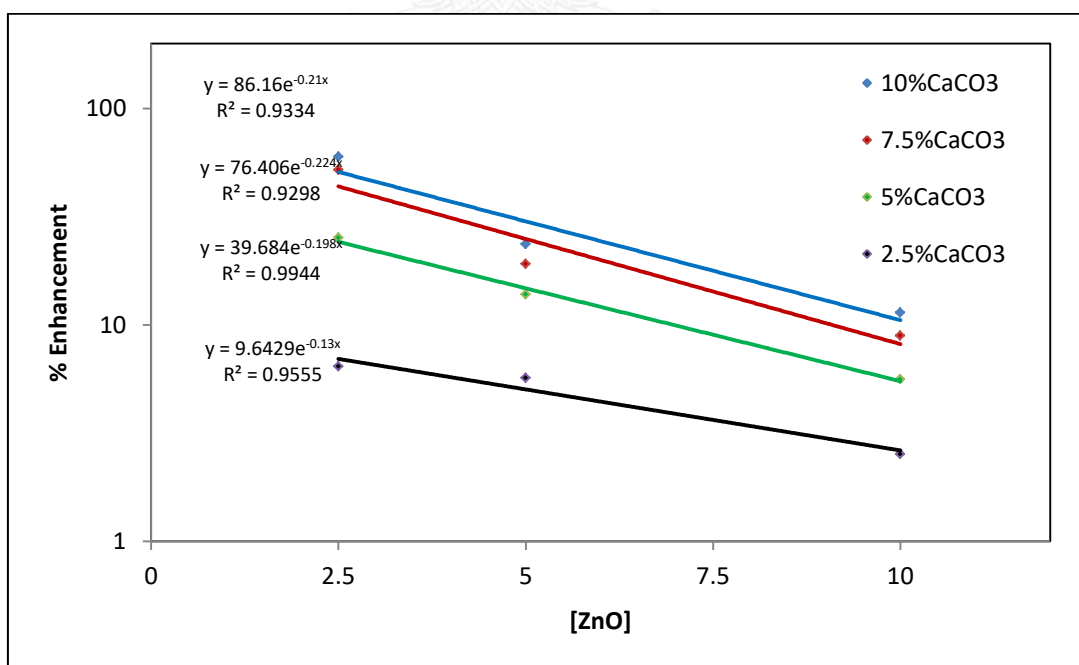


Figure 33 Plot between concentrations of ZnO when combined with CaCO$_3$ and percent enhancement

Table 12 Percent enhancement of combination between ZnO and $CaCO_3$ when compare with pure ZnO and when compare with additive absorption value of ZnO and values of $CaCO_3$ (n=3)

| Samples | | Absorption values at 375 nm | % Enhancement of combination compare to ZnO | Additive absorption values of ZnO +$CaCO_3$ | % Enhancement of combination compare to additive values |
|---|---|---|---|---|---|
| % ZnO | % $CaCO_3$ | | | | |
| - | 2.5 | 0.0104 | - | - | - |
| | 5.0 | 0.0224 | - | - | - |
| | 7.5 | 0.0306 | - | - | - |
| | 10.0 | 0.0380 | - | - | - |
| 2.5% | - | 0.0918 | - | - | - |
| | 2.5 | 0.0977 | 6.43% | 0.1022 | -11.90% |
| | 5.0 | 0.1150 | 25.27% | 0.1142 | 0.70% |
| | 7.5 | 0.1317 | 52.16% | 0.1224 | 7.59% |
| | 10.0 | 0.1468 | 59.91% | 0.1298 | 13.09% |
| 5% | - | 0.1897 | - | - | - |
| | 2.5 | 0.2005 | 5.69% | 0.2001 | 0.19% |
| | 5.0 | 0.2160 | 13.86% | 0.2121 | 1.83% |
| | 7.5 | 0.2260 | 19.14% | 0.2203 | 2.58% |
| | 10.0 | 0.2345 | 23.62% | 0.2277 | 2.98% |
| 10% | - | 0.3720 | - | - | - |
| | 2.5 | 0.3814 | 2.53% | 0.3824 | -0.26% |
| | 5.0 | 0.3929 | 5.62% | 0.3944 | -0.38% |
| | 7.5 | 0.4052 | 8.92% | 0.4026 | 0.64% |
| | 10.0 | 0.4145 | 11.42% | 0.4100 | 1.09% |

With regards to our experiment (Sunazuka, 2016), the abs$_s$ characteristics were evaluated and compared between two calcium carbonate powder samples, sized nanometer and micrometer, using UV/Visible spectrophotometer fitted with an integrating sphere to detect solid powder samples. The results showed that the smaller calcium carbonate powder of 30-70 nm had better abs$_s$ than larger particles

(Figure 31). Others researchers have stated that when the particle size was reduced, more surface area of particles are available to interact and absorb ultraviolet light (Schlossman and Shao, 2005; Taylor et al., 2013). However, when particles were dispersed in dimethicone to measure the $abs_o$ using regular UV-Visible spectrophotometer, the result was the opposite. It means that the smaller calcium carbonate in dispersion had lower $abs_o$ than larger ones. This can be explained by the fact that this regular UV-visible spectrophotometer detects the light which can pass through samples, which is called transmittance light, and then it can be calculated into $abs_o$ values which includes both $abs_s$ and $Sc_s$ in the value. Thus, the overall absorption values obtained from this instrument includes both $abs_s$ and $sc_s$ (Shaath, 2005b; Taylor et al., 2013).

From Mie scattering theory, larger particles are more efficient in scattering UV attenuation than smaller ones. Therefore, the higher $abs_o$ of large calcium carbonate particles in dispersion may be initiated from this strong scattering when compare to the smaller particles. However, when titanium dioxide was combined with small and large calcium carbonate by physical mixing, the $abs_o$ of titanium dioxide with smaller particles had better $abs_o$ than titanium dioxide combined with larger ones. In addition, $abs_o$ resulted that combination of titanium dioxide and large calcium carbonate was not different from titanium dioxide alone. This results denied the possibility that $Sc_s$ by calcium carbonate enhanced the $abs_o$ of titanium dioxide, because large calcium carbonate had weak effect on absorption improvement. Instead, it can be speculated that the $abs_s$ of calcium carbonate nanoparticles is the main contributing factor for the $abs_o$ enhancement of titanium dioxide mixture.

In the UV absorption spectra of calcium carbonate (Figure 17), absorption peak was shown to be at 320 nm and was the same location with that found in the dispersed sample in dimethicone (Figure 31). Moreover, the $abs_o$ peak position of titanium dioxide was shifted gradually from 325 nm (characteristic of $TiO_2$ peak position) to 320 nm (characteristic of $CaCO_3$ peak position), as increasing amount of calcium carbonate nanoparticle was added (Figure 19). These data support the speculation that the specific $abs_s$ of calcium carbonate nanoparticles is a main factor for UV attenuated absorption improvement of titanium dioxide when combined with

calcium carbonate nanoparticles. This is the advantage of nanoparticles that they show very different physicochemical properties than what is expected from regular larger particles. These nanoparticles have dramatically high surface area to absorb $abs_s$ more energy of UV light instead of simply scattering like most physical sunscreens.

From the speculation above, some hypotheses about the absorption enhancement can be made. The hypothesis is that the $abs_o$ enhancement of $TiO_2$ combined with $CaCO_3$ in dispersion derived from $abs_s$ of calcium carbonate itself, not its scattering. It means that calcium carbonate nanoparticles have the ability to absorb UV light with weak scattering intensity, but it only can absorb limited amount of UV light when dispersions were measured for $abs_o$. By the presence of UV scattering of titanium dioxide, calcium carbonate nanoparticles can absorb higher amount of UV light by increasing path length from scattering effect of $TiO_2$, the scheme is shown in Figure 30.

The $sc_s$ of titanium dioxide inhibits the incident light from passing the sunscreens film with straight line, and the light will scattered more to calcium carbonate nanoparticle to absorb it. As a result, $abs_s$ of calcium carbonate nanoparticles are improved with the same reason concluded by Hewitt (2005) when titanium dioxide was combined with chemical sunscreens (Figure 33). Moreover, incorporation of calcium carbonate nanoparticles can improve overall skin coverage result in enhanced UV photoprotection for physical sunscreens. Figure 34 shows idealized distribution of sunscreens film on skin which incident light cannot penetrate to human skin. However, in reality, sunscreen films tend to pool in the wrinkles of the skin causing uneven distribution of sunscreen on skin, the thin or broken film then occur (Figure 35). As a result, more UV light can penetrate though the skin (Hewitt, 2005; Lowe et al., 1997). From these reasons, it is reasonable to improve photoprotection efficiency of titanium dioxide by utilizing calcium carbonate nanoparticles.

On the other hand, the combination of zinc oxide and calcium carbonate resulted in only additive effect. From Figure 3, Mie scattering's equation, shows that there are many factors affecting $Sc_s$ of physical sunscreens, including refractive index

of materials. Particles with high refractive index have high $Sc_s$. The refractive index of titanium dioxide is 2.7 but zinc oxide is 1.9, therefore titanium dioxide has higher $Sc_s$ than zinc oxide. The possible mechanism can be explained as same as when titanium dioxide was combined with calcium carbonate. Due to the lower $Sc_s$ of zinc oxide, the incident light can be attenuated by $abs_s$ of zinc oxide and calcium carbonate separately. There are rarely any interaction by scattering of the UV light to other particles. Hence, the $abs_o$ resulted from this combination showed only additive effect.
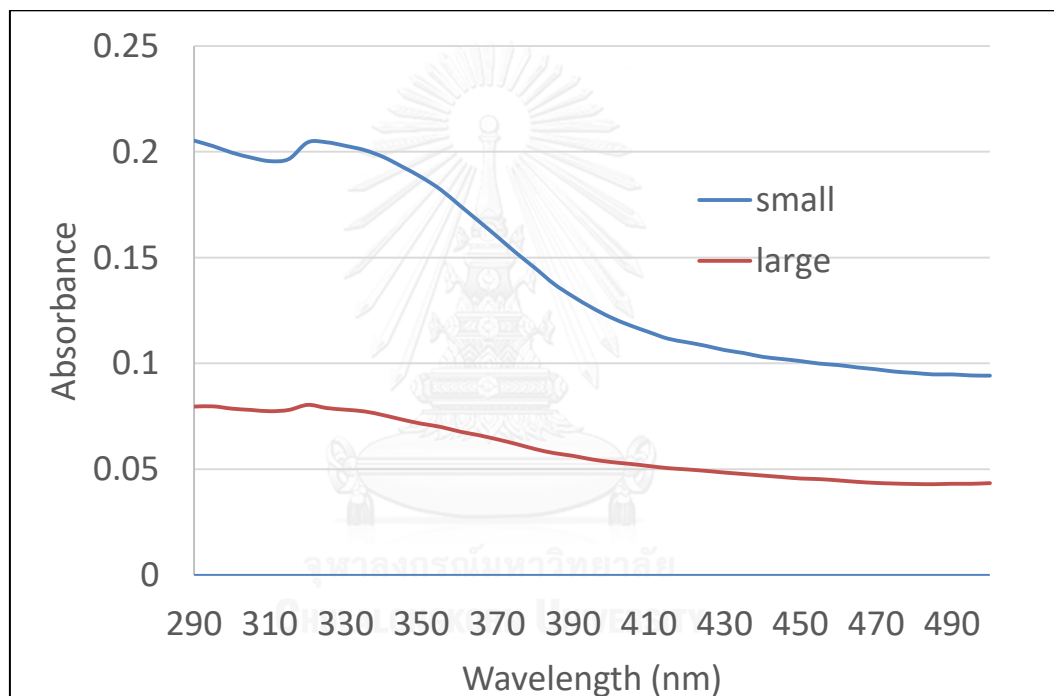


Figure 34 UV absorption measurement of solid compacts for small and large particle size $CaCO_3$ powder by UV/Visible spectrophotometer fitted with an integrating sphere. (Sunazuka, 2016, unplublished data)
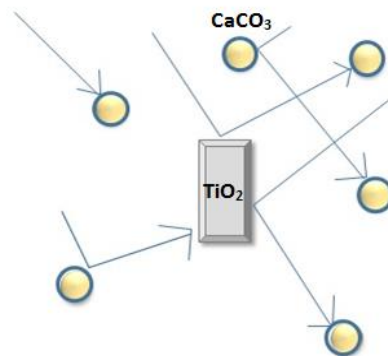
Figure 35 The hypothesis of overall absorption mechanism for synergistic effect of
$TiO_2$ when combined with $CaCO_3$ nanoparticles.
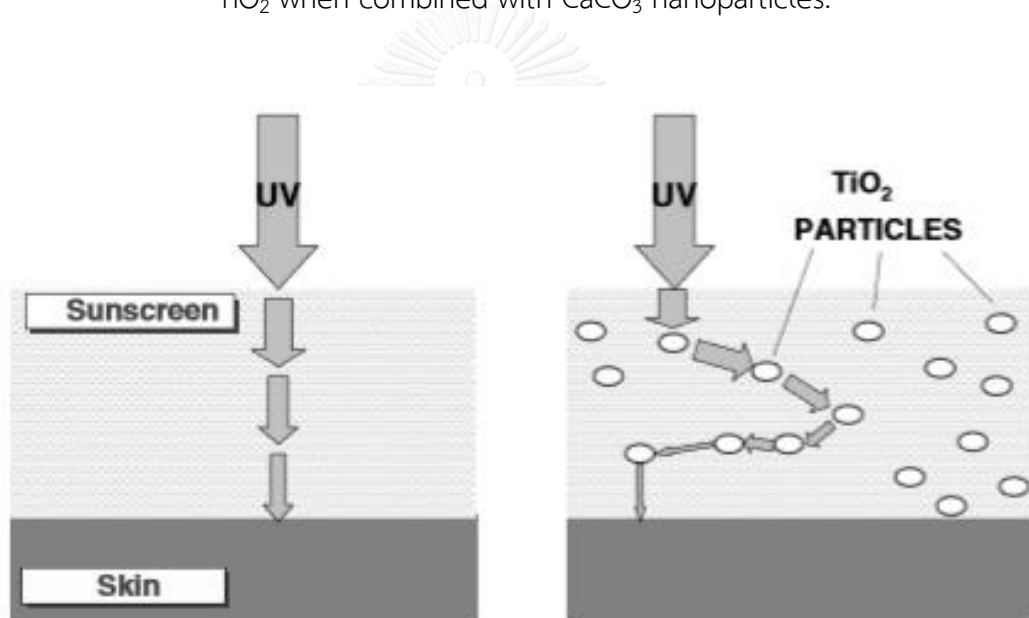


Figure 36 Increasing the optical path length due to scattering effect
by $TiO_2$ particles (Hewitt, 2005)



Figure 37 Idealized distribution of sunscreen on the skin (Hewitt, 2005)
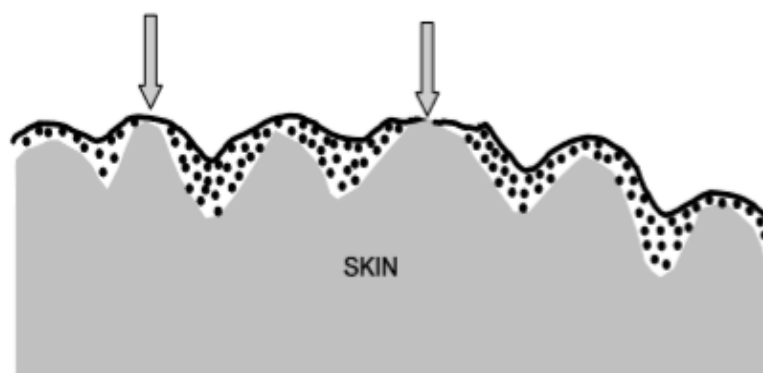
Figure 38 Uneven distribution of sunscreen on the skin (Hewitt, 2005)

### 3.3 Determination the Sun Protection Factor of dispersion

The concentration of calcium carbonate which gave the highest absorption values when combined with both physical sunscreens was 10%w/w. Therefore, calcium carbonate was held constant of 10%w/w and three concentrations of titanium dioxide or zinc oxide were varied, at 2.5, 5 and 10%w/w (Table 13) to evaluate the sun protection factor (SPF) and UVA protection factor (UVA-PF) values by SPF 290s analyzer and correlate these results to the values obtained by UV-absorption in previous section. Individual titanium dioxide, zinc oxide and calcium carbonate were used as control.

Table 13 Concentrations of physical sunscreens in combination
with calcium carbonate

| Calcium carbonate concentration (%w/w) | Titanium dioxide or zinc oxide concentrations (%w/w) |
|---|---|
| 10 | 2.5 |
| 10 | 5 |
| 10 | 10 |

Table 14 shows SPF and UVA-PF values of titanium dioxide and calcium carbonate combination. Calcium carbonate 10%w/w combined with titanium dioxide

2.5, 5 and 10%w/w showed SPF of 6.17 ± 0.41, 18.54 ± 1.11 and 59.46 ± 1.22, respectively and they can be calculated to the enhancing percentages from individual titanium dioxide of 40.23%, 50.12% and 26.81%, respectively. They provided UVA-PF of 3.59 ± 0.13, 9.10 ± 0.48 and 29.09 ± 1.15 which yielded percent enhancement from $TiO_2$ alone of 21.28%, 34.42% and 23.05%, respectively.

Figure 39 exhibits that combinations of 2.5, 5 and 10%w/w titanium dioxide with 10%w/w calcium carbonate showed higher SPF and UVA-PF than titanium dioxide alone with p-value<0.05 (student t-test). Although 10%w/w calcium carbonate has more concentration and particles than 2.5 and 5%w/w titanium dioxide, or same concentration with 10%w/w titanium dioxide, SPF and UVA-PF of 10% calcium carbonate is lower than 2.5, 5 and 10% titanium dioxide with statistical significance (p<0.05). From this reason, it was clear that the photoprotection enhancement of calcium carbonate on physical sunscreens doesn't from the solid content but it depends on characteristic of this material.

Table 14 The Sun Protection Factor (SPF) and UVA-Protection Factor (UVA-PF) of various concentrations of $TiO_2$ in combination with $CaCO_3$ in dimethicone oil (n=3)

| Conc. (%w/w) | | Mean SPF ± SD | % Enhancement from $TiO_2$ alone | Mean UVA-PF ± SD | % Enhancement from $TiO_2$ alone |
|---|---|---|---|---|---|
| $TiO_2$ | $CaCO_3$ | | | | |
| - | 10* | 1.12 ± 0.03 | - | 1.11 ± 0.02 | - |
| 2.5* | - | 4.40 ± 0.08 | - | 2.96 ± 0.03 | - |
| 2.5 | 10 | 6.17 ± 0.41 | 40.23% | 3.59 ± 0.13 | 21.28% |
| 5* | - | 12.35 ± 0.62 | - | 6.77 ± 0.32 | - |
| 5 | 10 | 18.54 ± 1.11 | 50.12% | 9.10 ± 0.48 | 34.42% |
| 10* | - | 46.87 ± 1.95 | - | 23.64 ± 1.15 | - |
| 10 | 10 | 59.46 ± 1.22 | 26.81% | 29.09 ± 1.15 | 23.05% |
| -** | - | 0.94 ± 0.00 | - | 0.95 ± 0.01 | - |

*Individual $TiO_2$ and $CaCO_3$ were used as control.

**Dimethicone oil was used as negative control.

The sun protection factor (SPF) and UVA protection factor (UVA-PF) of combination of zinc oxide and calcium carbonate are presented in Table 15. Calcium carbonate 10%w/w combined with zinc oxide 2.5, 5 and 10%w/w showed SPF of $1.64 \pm 0.19$, $1.93 \pm 0.03$ and $4.09 \pm 0.04$ and yielded percent enhancement from individual zinc oxide of 23.31%, 10.29% and 45.04%, respectively. UVA protection factor (UVA-PF) of the combinations provided $1.42 \pm 0.07$, $1.80 \pm 0.02$ and $3.56 \pm 0.03$ and the enhancing percentages were 10.08%, 9.09% and 37.45%, respectively.

Figure 40 depicts the combinations of 5 and 10%w/w zinc oxide and 10%w/w calcium carbonate showed higher SPF and UVA-PF than titanium dioxide alone with p-value<0.05 (student t-test). However, SPF and UVA-PF of 2.5%w/w zinc oxide combined with 10% w/w calcium carbonate are not different from zinc oxide alone (p-value>0.05)
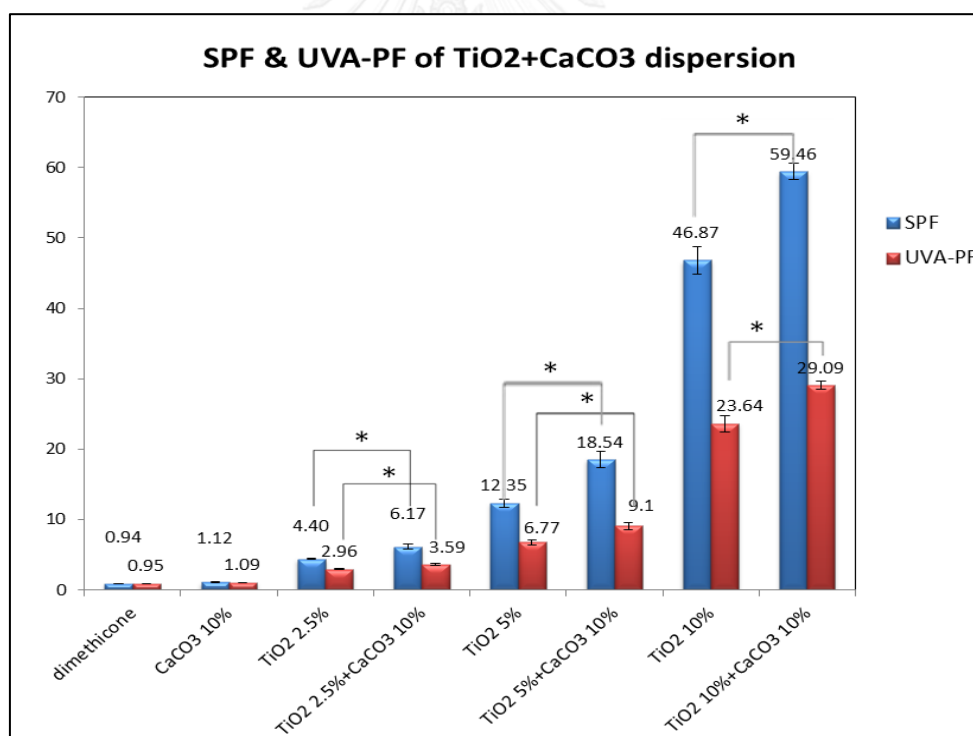


Figure 39 SPF and UVA-PF of $TiO_2$ in combination with $CaCO_3$ in dispersion

Table 15 The Sun Protection Factor (SPF) and UVA-Protection Factor (UVA-PF) of various concentrations of ZnO in combination with $CaCO_3$ in dimethicone oil (n=3)

| Conc. (%w/w) | | Mean SPF ± SD | % Enhancement from ZnO alone | Mean UVA-PF ± SD | % Enhancement from ZnO alone |
|---|---|---|---|---|---|
| ZnO | $CaCO_3$ | | | | |
| - | 10* | 1.12 ± 0.03 | - | 1.09 ± 0.02 | - |
| 2.5* | - | 1.33 ± 0.01 | - | 1.29 ± 0.01 | - |
| 2.5 | 10 | 1.64 ± 0.19 | 23.31% | 1.42 ± 0.07 | 10.08% |
| 5* | - | 1.75 ± 0.02 | - | 1.65 ± 0.01 | - |
| 5 | 10 | 1.93 ± 0.03 | 10.29% | 1.80 ± 0.02 | 9.09% |
| 10* | - | 2.82 ± 0.11 | - | 2.59 ± 0.10 | - |
| 10 | 10 | 4.09 ± 0.04 | 45.04% | 3.56 ± 0.03 | 37.45% |
| -** | - | 0.94 ± 0.00 | - | 0.95 ± 0.01 | - |

*Individual ZnO and $CaCO_3$ were used as control.

**Dimethicone oil was used as negative control.
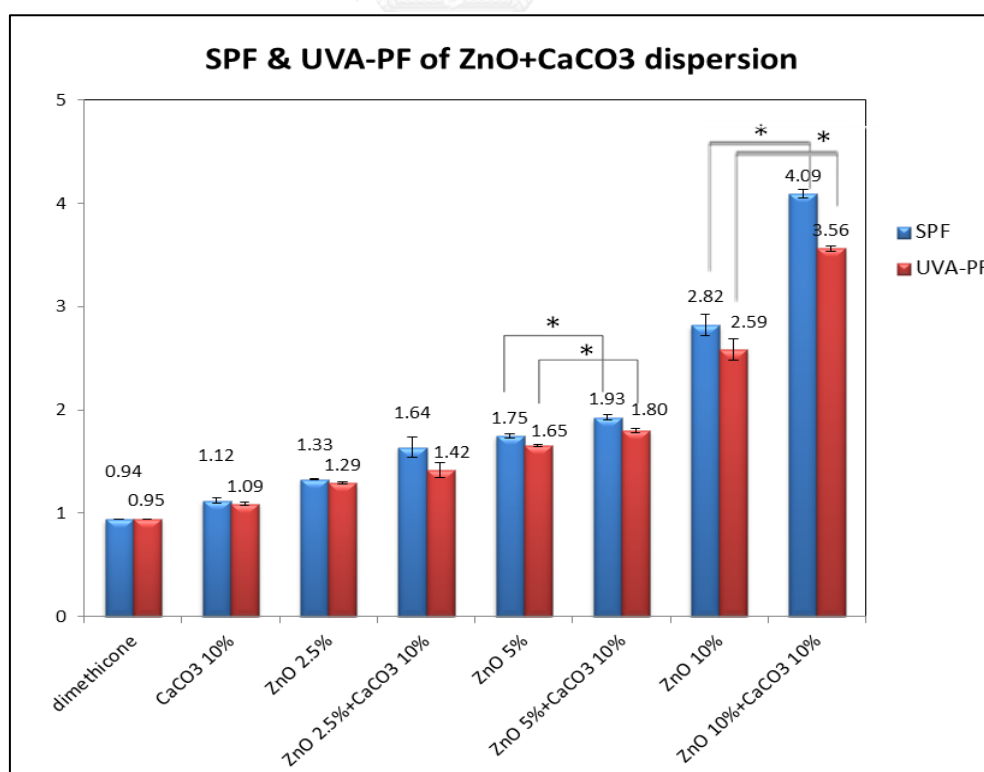


Figure 40 SPF and UVA-PF of ZnO in combination with $CaCO_3$ in dispersion

Figure 41 presents linearity graph between titanium dioxide concentration and SPF values. Titanium dioxide dispersed in dimethicone oil at 2.5, 5 and 10%w/w provided SPF values dependent on titanium dioxide concentration with $R^2$ = 0.9757. Likewise, the combination of 2.5, 5 and 10%w/w titanium dioxide and 10%w/w calcium carbonate also resulted in linearity correlation ($R^2$ = 0.9880). It shows that this combination also have SPF-concentration dependence (Figure 41).

Zinc oxide alone, at concentrations of 2.5, 5 and 10%w/w, and zinc oxide with the same concentrations combined with 10%w/w calcium carbonate resulted in good concentration-dependence on UVA-PF in dimethicone oil with $R^2$ = 0.9964 and 0.9729 (Figure 42), respectively.
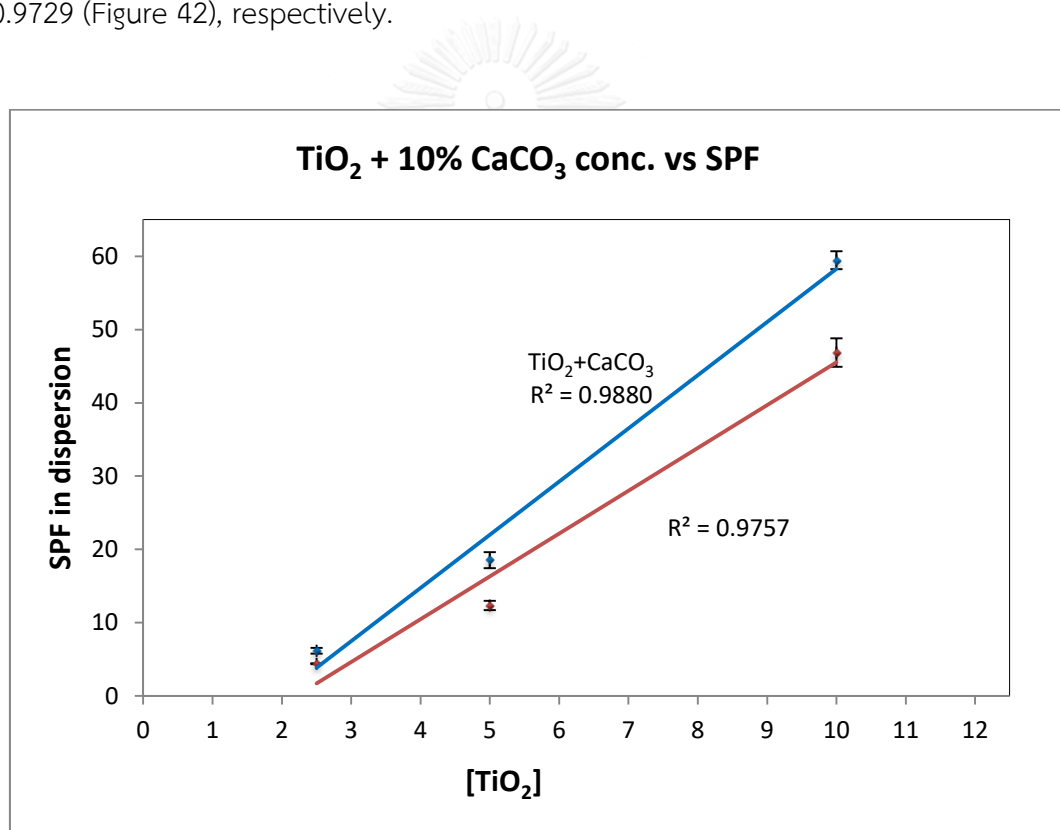


Figure 41 Plot between various $TiO_2$ concentrations and when combined with 10%w/w $CaCO_3$ and SPF values
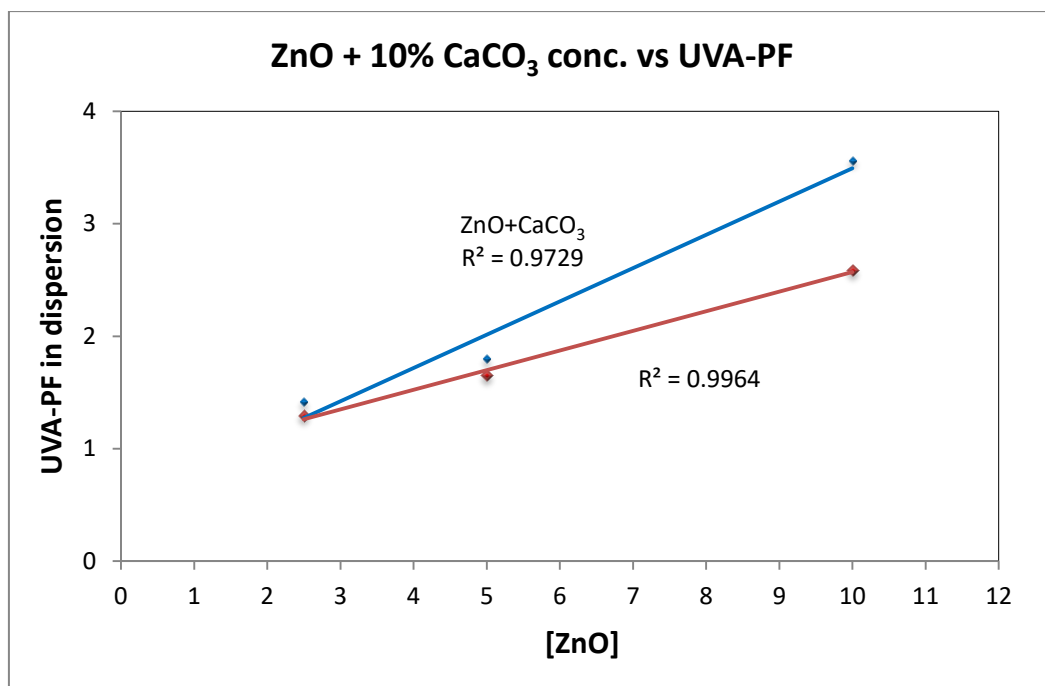
Figure 42 Plot between various ZnO concentrations and when combined with 10%w/w $CaCO_3$ and UVA-PF

## 3.4 Correlation between UV absorbance and Sun Protection Factor/UVA Protection Factor values

The graphs between UV absorbance peak values and sun protection factor or UVA protection factor were created to analyze the correlation between results obtained by two methods using correlation coefficient ($R^2$).

Figure 43 illustrates the correlation between SPF and average absorbance of various $TiO_2$ concentrations at the maximum peak of 325 nm. The correlation was found to be linearity with $R^2$ = 0.9847. Moreover, SPF values and absorbance peak of various $TiO_2$ concentrations in combination with 10%w/w $CaCO_3$ also gave linearity correlation with $R^2$ = 0.9850, as seen in Figure 43.

Various concentrations of individual ZnO and ZnO combined with 10%w/w $CaCO_3$ provided UVA-PF and UV absorption correlation values with $R^2$ = 0.9939 and 0.9750, respectively (Figure 44).

In conclusion, all samples showed linearity correlations between the sun protection factor values or UVA protection factor and UV absorbance values. Thus, it

is significant to note that the results on the absorbance values using *in vitro* UV-Visible spectrophotometry can be used as an alternative to measure the photoprotection efficiency of physical sunscreens in dispersion. It is good correlation to the values obtained by *in vitro* SPF analyzer.

## 4. Physical sunscreens formulations

### 4.1 Preparation of water in oil (w/o) and oil in water (o/w) emulsions

Due to the higher enhancing percentages of calcium carbonate when combined with titanium dioxide than zinc oxide, titanium dioxide was chosen to be incorporated into both water in oil (w/o) and oil in water (o/w) emulsions to compare SPF and UVA-PF values between both emulsion types. Also, to compare SPF and UVA-PF values of the combination and titanium dioxide alone in each emulsion type to investigate suitable emulsion for the combination. The suitable emulsion means emulsion which can provide high enhancing percentages of calcium
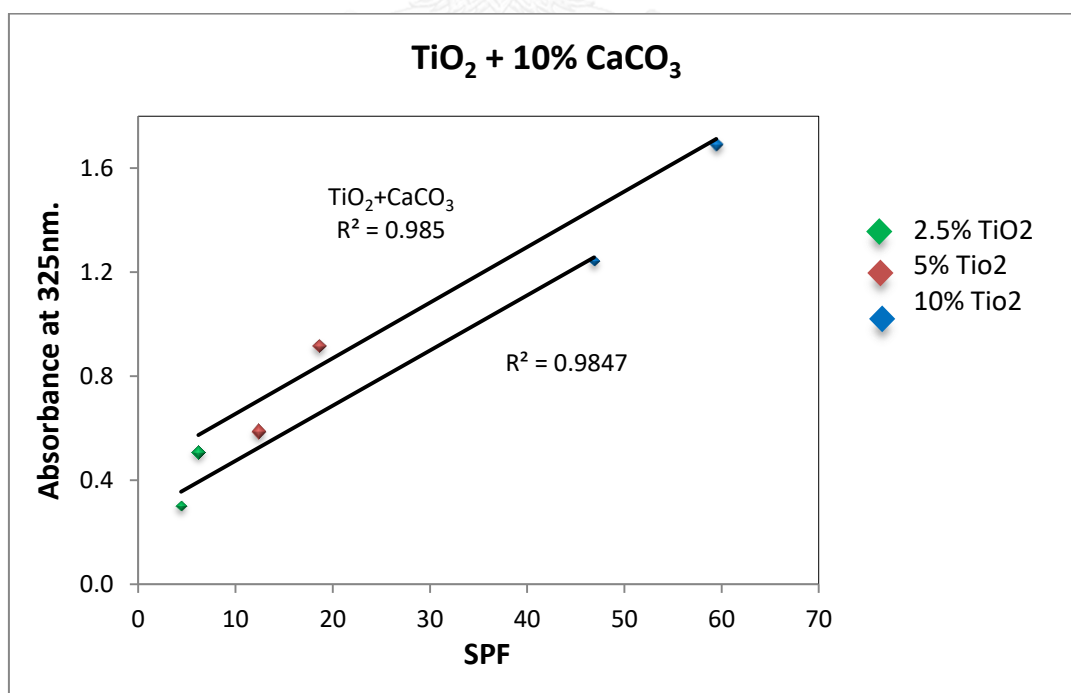


Figure 43 Correlation between SPF and average UV absorbance of various $TiO_2$ concentrations and when combined with 10%w/w $CaCO_3$
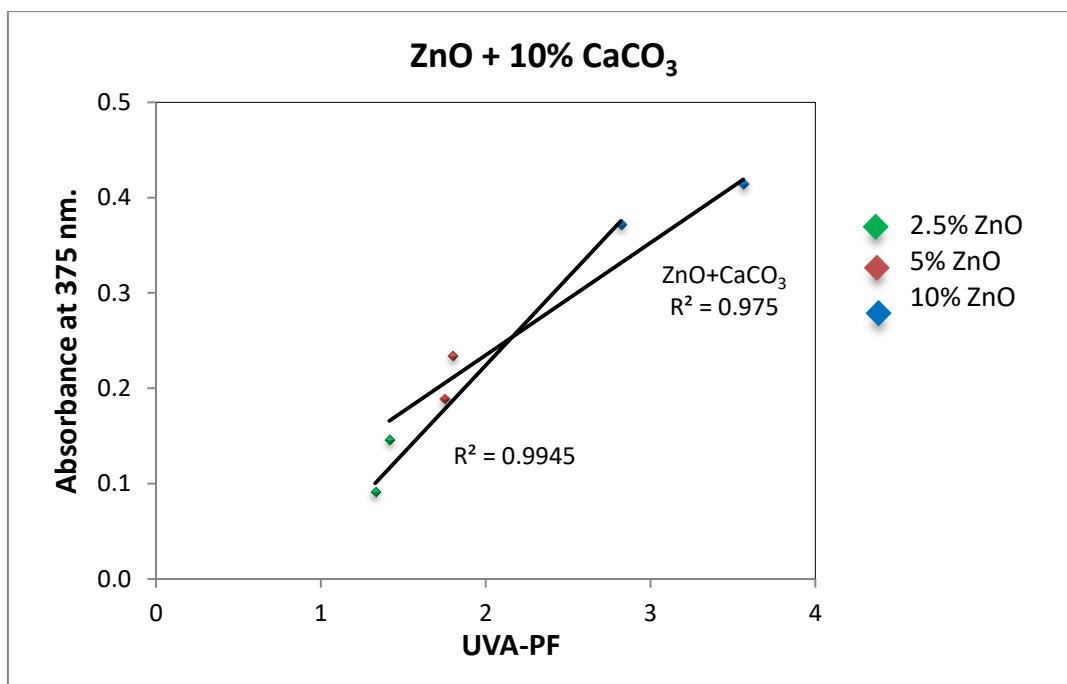
Figure 44 Correlation between UVA-PF and average UV absorbance of various ZnO concentrations and when combined with 10%w/w $CaCO_3$

carbonate on titanium dioxide similar to the combination which was incorporated into silicone oil. Negative controls were w/o and o/w blank emulsions.

The concentration used in this study was 5%w/w for titanium dioxide combined with 5%w/w calcium carbonate. In experiment 3.2, it was clear that 10%w/w calcium carbonate combined with every concentrations of titanium dioxide showed highest absorption values. However, to use calcium carbonate as a sunscreen booster in a formulation, the concentration used should be less than 10% because it is not the main ingredients in the formula. Fairhurst and Mitchnick (1997) advised reasonable level for enhancers of 2-5%. Truffault et al. (2012) used 6% cerium oxide to improve sun protection factor and UVA protection factor of titanium dioxide in water in oil emulsion. Boutard and cowprkers (2013) used 3% cerium oxide combined with chemical sunscreens to enhance the Sun protection factor compared to 3% zinc oxide.

Titanium dioxide 10%w/w can give high SPF when combined with calcium carbonate 5%w/w. However, in preliminary study, 10%$TiO_2$ combined with 5% calcium carbonate has too much particles to incorporate in this formulations which

have only 33.16% and 30.56% for w/o and o/w emulsions, respectively. Therefore, concentration of $TiO_2$ used was decreased to 5%w/w to incorporate in formulation.

Determination of the emulsion types can be seen in Figure 45, all water in oil (w/o) emulsions were released as droplets in both pure water and mineral oil. W/O droplets were dispersed when released in bulk oil but remained as droplets in water. All oil in water (o/w) emulsions also were dropped in both water and same oil. The results gave opposite way, all o/w emulsion drops could disperse in water and remain as drops in oil. From this test, it was confirmed that all emulsion types which were prepared in this study were true.



**o/w**                          **w/o**
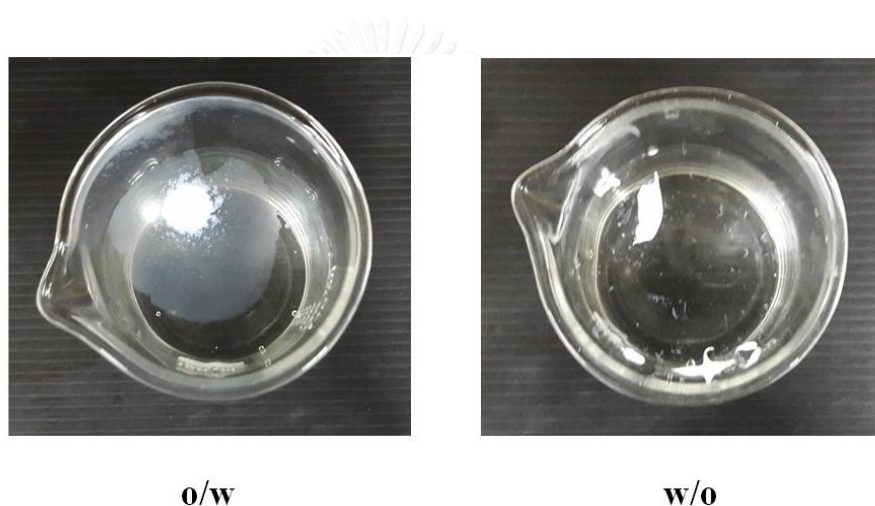
Figure 45 Emulsion types determination when o/w and w/o were dropped into water

4.2  Determination the Sun Protection Factor and UVA protection factor of emulsions

As shown in Table 16, the sun protection factor of w/o titanium dioxide combined with calcium carbonate emulsion showed improvement from individual titanium dioxide with enhancing percentage at 20.16%. Moreover, the UVA-PF value was improved at 10.40% as the enhancing percentage. SPF enhancing percentage of w/o emulsion got along with the enhancing percentage of 5%w/w titanium dioxide combined with 5%w/w calcium carbonate (23.16%) using UV-visible spectrophotometer. From this reason, the result could ensure that the $abs_o$ values

from UV-visible spectrophotometer can be measured to reveal the UV protection trend of physical sunscreens.

In contrast, the same concentration of titanium dioxide combined with calcium carbonate in o/w emulsion provided very low enhancing percentage of SPF and UVA-PF of only 2.90% and 1.80% from individual titanium dioxide, respectively. Therefore, o/w emulsion is not suitable base for this combination.

Table 16 The Sun Protection Factor (SPF) of titanium dioxide combined with calcium carbonate in various concentrations compared between w/o and o/w emulsions (n=3)

| | Concentration (%w/w) | | Mean SPF ± SD | % Enhancement from $TiO_2$ alone | Mean UVA-PF ± SD | % Enhancement from $TiO_2$ alone |
|---|---|---|---|---|---|---|
| | $TiO_2$ | $CaCO_3$ | | | | |
| w/o | *- | - | 1.02 ± 0.01 | - | 0.99 ± 0.01 | - |
| | - | 5 | 1.08 ± 0.04 | - | 1.03 ± 0.03 | - |
| | 5 | - | 14.09 ± 1.10 | - | 6.83 ± 0.40 | - |
| | 5 | 5 | 16.93 ± 0.73 | 20.16% | 7.54 ± 0.33 | 10.40% |
| o/w | *- | - | 1.03 ± 0.01 | - | 1.00 ± 0.01 | - |
| | - | 5 | 1.10 ± 0.01 | - | 1.04 ± 0.01 | - |
| | 5 | - | 11.37 ± 1.02 | - | 5.55 ± 0.50 | - |
| | 5 | 5 | 11.70 ± 1.37 | 2.90% | 5.65 ± 0.50 | 1.80% |

*w/o and o/w blank emulsions were used as negative control.

Figure 46 illustrates the comparison of SPF and UVA-PF values with statistics (student t-test). The resulted showed that w/o emulsion of the combination gave higher SPF than titanium dioxide alone (p-value<0.05), but UVA-PF is not different (p>0.05). While SPF and UVA-PF values of o/w emulsion are not different between the combination and titanium dioxide alone.

When SPF and UVA-PF values of the combination were compared between w/o and o/w. It is shown that w/o emulsion provided higher SPF and UVA-PF than o/w emulsion with statistical significance (p<0.05). It is noteworthy that w/o emulsion

is more suitable for titanium dioxide combined with calcium carbonate. It is also clear that this combination can enhance photoprotection especially for SPF.
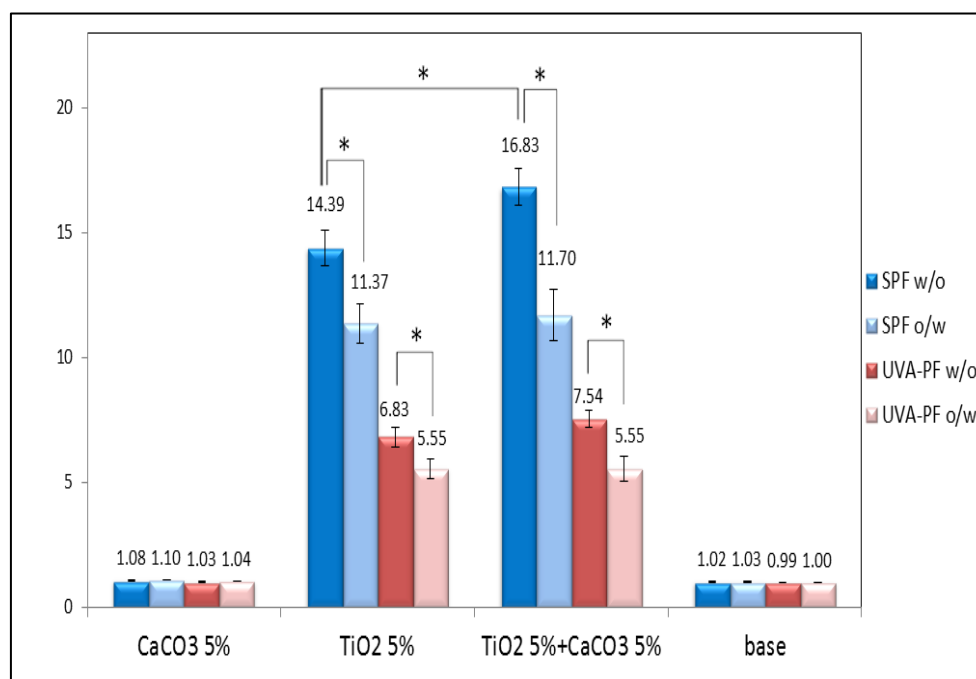


Figure 46 SPF and UVA-PF of $TiO_2$ combined with $CaCO_3$ in w/o and o/w emulsions

Oil in water (o/w) emulsion containing titanium dioxide in oil phase has more limited capacity of active ingredients due to the high concentration level of UV filter in the internal phase can lead to crowding effect, hence aggregation is occurred and then can cause reducing photoprotection efficacy of the product (Anderson et al., 1997; Shaath, 2005b). In this study, both of titanium dioxide and calcium carbonate particles (5%w/w each) were incorporated into oil phase, it may cause aggregation in the oil phase or migrate to water phase because of the crowded particles.

On the contrary, water in oil (w/o) emulsion containing titanium dioxide and calcium carbonate in oil phase was more efficient system. Most samples in this experiment, 5%w/w titanium dioxide and 5+5%w/w titanium dioxide combined with calcium carbonate, exhibited higher SPF and UVA-PF when they were incorporated in w/o emulsion with statistically significance ($p<0.05$, student t-test). The exception was only 5%w/w calcium carbonate, SPF and PFA values of this sample were not different between w/o and o/w emulsions ($p>0.05$, student t-test). Calcium

carbonate at 5%w/w have less particles than   titanium dioxide in the same concentrations, thus it is possible to occur less aggregation than titanium dioxide alone leading to have the same SPF and PFA as w/o emulsion.

Distribution of physical sunscreens is a considerable factor influencing photoprotection efficiency against UV ray. When physical sunscreens are applied on the skin, it is important to remain a continuous film on skin after dry-down to get potential efficacy. If the external phase of emulsion is oil, there is a continuous oil film with particles of physical sunscreens evenly distributed within the film. So it can give a satisfactory distribution and cause effective ability to protect sunlight. If the external phase is water, it may show discontinuous oil film because of the water evaporation, the particles of physical sunscreens simply aggregate in small area, leaving most of skin surface with no coverage, which give a lower efficacy (Anderson et al., 1997; Hewitt, 2005). This tendency does not happen in w/o emulsion because the carrier (oil) does not evaporate and the particles remain to disperse in carrier, thus the higher SPF and PFA of w/o emulsion could be recorded in this experiment.

In conclusion, the particles of both titanium dioxide and calcium carbonate should be placed in the oil phase of the emulsion for potential efficacy of this combination. However, there are several factors which influence the efficacy of sunscreen emulsion. The oil phase of emulsion does not consist of only oil ingredients but it also has emulsifiers, humectants and other active ingredients which can dilute or disperse in oils. All ingredients in oil phase can affect the distribution of a film (Klein, 1997; Woodruff, 2001) and can cause a forming of liquid crystalline structure (Dahms, 1992).

# CHAPTER V
# CONCLUSIONS

In the present study, calcium carbonate was combined with both titanium dioxide and zinc oxide to improve UV protection efficiency. Speed and duration using High speed disperser were varied to investigate suitable condition for preparing dispersion. The results show that speed at 2000 rpm and duration of 10 minutes is an optimal condition for dispersing particles in dimethicone oil due to the high absorbance values within shorter time used.

Concentrations of physical sunscreens (2.5, 5 and 10%) and calcium carbonate (2.5, 5 7.5 and 10%) were varied to study the enhancing effect of the combination using UV-Visible spectrophotometer. It was found that every calcium carbonate concentrations can improve UV absorption for every physical sunscreen concentrations and 10% calcium carbonate is the best. Therefore, 2.5, 5 and 10% physical sunscreens combined with 10% calcium carbonate were chosen to evaluate the correlation between sun protection factor (SPF) or UVA protection factor (UVA-PF) using SPF analyzer with the results from UV-spectroscopic analysis. The values obtained by SPF analyzer are found to correlate well with absorbance values by UV-Vis spectrophotometer. The effect was found to be due to an enhance in specific absorbance of calcium carbonate in the nanometer size range which did not occur with larger particles.

The combination of titanium dioxide and calcium carbonate provided synergistic effect on UV absorption values while zinc oxide combined with calcium carbonate showed only additional effect. For this reason, titanium dioxide combined with calcium carbonate was chosen to be incorporated into the formulation of w/o and o/w emulsions. The results show that 5% titanium dioxide combine with 5% calcium carbonate in w/o emulsion gave better photoprotection efficiency in both of SPF and UVA-PF values ($p < 0.05$) than o/w formulation. Nevertheless, the combination in w/o emulsion provided higher SPF than titanium dioxide alone ($p < 0.05$) but UVA-PF is not different ($p > 0.05$)

In conclusion, calcium carbonate is an interesting nanomaterial for enhancing UV protection of titanium dioxide. It can be added into physical sunscreen products to reduce the active ingredient concentration and decrease the amount of physical sunscreens which must be imported from abroad. Moreover, it can promote innovation in Thai cosmetic industry by using material which is produced in Thailand. However, the mechanism of enhancement still needs further confirmation study.

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# APPENDIX

Analysis of UV-visible spectrophotometer method validation

**Validation for determination of UV-Visible Spectrophotometer**

In this experiment, the assessments of quality methods were evaluated to linearity, accuracy, precision and specificity according to United States Pharmacopeia 35–National Formulary 30 (USP 35–NF 30).

Table 17 Analytical method validation parameter of UV-visible spectrophotometer for titanium dioxide and zinc oxide combined with calcium carbonate

| Parameters | Result values | | Limitation |
|---|---|---|---|
| | $TiO_2+CaCO_3$ | $ZnO+CaCO_3$ | |
| 1. Linearity (Correlation coefficient ) | 0.9992 | 0.9992 | ≥0.9990 |
| 2. Accuracy | | | |
| 2.1 % Recovery | 99.09 | 98.84 | 80-120 |
| 2.2 % RSD | 1.47 | 1.89 | <2.00 |
| 3. Precision | | | |
| 3.1 Within run precision (%RSD) | 1.67 | 1.72 | <2.00 |
| 3.2 Between run precision (%RSD) | 1.29 | 1.17 | <2.00 |
| 4. Specificity | No interfere peak | No interfere peak | No interfere peak |

**1. Linearity**

Linearity of the combination of titanium dioxide and zinc oxide combined with calcium carbonate were validated with concentration at 2+2, 3+3, 4+4, 5+5, 6+6 and 7+7%w/w, total concentrations are 4, 6, 8, 10, 12 and 14%w/w, the ratio was 1:1. The correlation coefficient ($R^2$) of titanium dioxide and zinc oxide combined with calcium carbonate between absorption values at wavelength 325 and 375 nm, the

total concentrations were 0.9992 and 0.9978, respectively (Figure 47). The data was shown in Table 18.
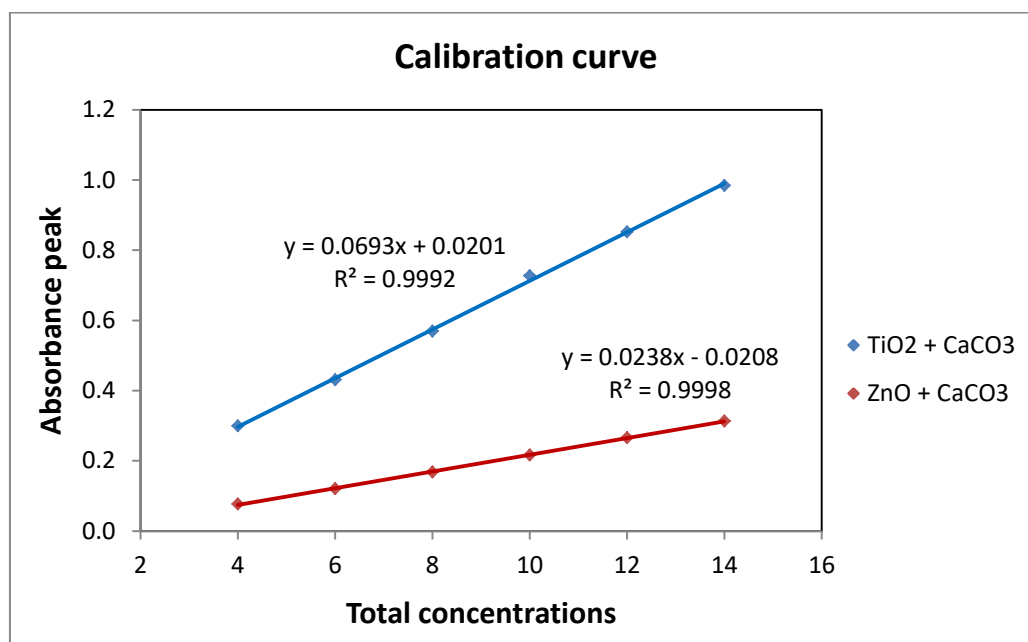


Figure 47 The calibration curve of titanium dioxide combined with calcium carbonate and zinc oxide combined with calcium carbonate

Table 18 Linearity validation for titanium dioxide and zinc oxide combined with calcium carbonate

| $TiO_2$ or ZnO (%w/w) | $CaCO_3$ (%w/w) | Total concentrations | $TiO_2 + CaCO_3$ | | $ZnO + CaCO_3$ | |
|---|---|---|---|---|---|---|
| | | | mean | SD | mean | SD |
| 2 | 2 | 4 | 0.2987 | 0.0226 | 0.0763 | 0.0037 |
| 3 | 3 | 6 | 0.4305 | 0.0195 | 0.1206 | 0.0035 |
| 4 | 4 | 8 | 0.5688 | 0.0243 | 0.1647 | 0.0038 |
| 5 | 5 | 10 | 0.7264 | 0.0198 | 0.2160 | 0.0058 |
| 6 | 6 | 12 | 0.8522 | 0.0208 | 0.2656 | 0.0064 |
| 7 | 7 | 14 | 0.9837 | 0.0225 | 0.3226 | 0.0060 |

## 2. Accuracy

The accuracy assessment was validated with standard samples at 80, 100 and 120% for amount concentrations of titanium dioxide or zinc oxide combined with calcium carbonate of 4+4, 5+5 and 6+6%w/w, respectively. For titanium dioxide combined with calcium carbonate, recovery percentage (%recovery) of the standard samples at 80, 100 and 120% were 98.98, 99.09 and 101.53, respectively. The percentage of relative standard deviation (%RSD) for the standard samples at 80, 100 and 120% were 1.92, 1.47 and 1.79, respectively. For zinc oxide combined with calcium carbonate, percent recovery were 99.23, 98.84 and 106.68, and percent RSD were 1.83, 1.89 and 1.87 for standard samples at 80, 100 and 120%, respectively. All data were in the range of acceptance criteria which were depicted in Table 19 and 20.

Table 19 Accuracy validation of analytical method of titanium dioxide combined with calcium carbonate

| TiO$_2$+CaCO$_3$ Conc.(%w/w) | Absorption | Mean | SD | % RSD | % recovery |
|---|---|---|---|---|---|
| 4+4 | 0.5756 | 0.5630 | 0.0108 | 1.92 | 98.98 |
| | 0.5678 | | | | |
| | 0.5532 | | | | |
| | 0.5717 | | | | |
| | 0.5682 | | | | |
| | 0.5456 | | | | |
| | 0.5587 | | | | |
| 5+5 | 0.7300 | 0.7198 | 0.0106 | 1.47 | 99.09 |
| | 0.7261 | | | | |
| | 0.699 | | | | |
| | 0.7188 | | | | |
| | 0.7125 | | | | |
| | 0.7201 | | | | |
| | 0.7323 | | | | |
| 6+6 | 0.8525 | 0.8652 | 0.0155 | 1.79 | 101.53 |
| | 0.8783 | | | | |
| | 0.8897 | | | | |
| | 0.8612 | | | | |
| | 0.8646 | | | | |
| | 0.8713 | | | | |
| | 0.8389 | | | | |

Table 20 Accuracy validation of analytical method of zinc oxide combined with calcium carbonate

| TiO$_2$+CaCO$_3$ Conc.(%w/w) | Absorption | Mean | SD | % RSD | % recovery |
|---|---|---|---|---|---|
| 4+4 | 0.1644 | 0.1667 | 0.0030 | 1.83 | 99.23 |
| | 0.1701 | | | | |
| | 0.1672 | | | | |
| | 0.1648 | | | | |
| | 0.1657 | | | | |
| | 0.1715 | | | | |
| | 0.1634 | | | | |
| 5+5 | 0.2061 | 0.2135 | 0.0040 | 1.89 | 98.84 |
| | 0.215 | | | | |
| | 0.2141 | | | | |
| | 0.2159 | | | | |
| | 0.2111 | | | | |
| | 0.2201 | | | | |
| | 0.2122 | | | | |
| 6+6 | 0.2675 | 0.2674 | 0.0050 | 1.87 | 100.68 |
| | 0.2662 | | | | |
| | 0.2649 | | | | |
| | 0.2587 | | | | |
| | 0.2722 | | | | |
| | 0.2757 | | | | |
| | 0.2666 | | | | |

## 3. Precision

Precision of titanium dioxide and zinc oxide combined with calcium carbonate were assessed within and between run precision. For titanium dioxide combined with calcium carbonate, within run precision was assessed with total concentrations of standard samples at 6, 10 and 14%w/w, the ratio of the combination was 1:1. The percentage relative standard deviation (%RSD) for the total concentrations of 6, 10 and 14%w/w were 1.92, 1.47 and 1.61, respectively. Between run precision was validated with total concentration of standard sample at 10%w/w, with the ratio of the combination 1:1, on 3 days. The percentage of RSD for day 1, day 2 and day 3 were 1.15, 1.15 and 1.57, respectively. The results were explained in Table 21 and Table 22.

For zinc oxide combined with calcium carbonate, within run precision was assessed with total concentrations of standard samples at 6, 10 and 14%w/w, the ratio of the combination was also 1:1. The percentage of RSD for the total concentrations of 6, 10 and 14%w/w were 1.73, 1.89 and 1.54 respectively. Between run precision was validated with total concentration of standard sample at 10%w/w, with the ratio of the combination 1:1, on 3 days. The percentage of RSD for day 1, day 2 and day 3 were 1.35, 1.02 and 1.13, respectively. The results were explained in Table 23 and Table 24.

Table 21 Within run precision of titanium dioxide combined with calcium carbonate

| Concentration (%w/w) | Absorption | Mean | SD | % RSD |
|---|---|---|---|---|
| 6 | 0.4333 | 0.4307 | 0.0083 | 1.92 |
| | 0.4278 | | | |
| | 0.4218 | | | |
| | 0.4357 | | | |
| | 0.4451 | | | |
| | 0.4298 | | | |
| | 0.4216 | | | |
| 10 | 0.7300 | 0.7198 | 0.0106 | 1.47 |
| | 0.7261 | | | |
| | 0.6990 | | | |
| | 0.7188 | | | |
| | 0.7125 | | | |
| | 0.7201 | | | |
| | 0.7323 | | | |
| 14 | 0.9888 | 0.9849 | 0.0158 | 1.61 |
| | 0.9511 | | | |
| | 0.9796 | | | |
| | 0.9908 | | | |
| | 0.9912 | | | |
| | 0.9856 | | | |
| | 1.0070 | | | |
| Mean | | 0.7116 | 0.0116 | 1.67 |

Table 22 Between run precision of titanium dioxide combined with calcium carbonate

| day | Absorption | Mean | SD | % RSD |
|-----|-----------|------|------|-------|
| 1 | 0.7259 | 0.7195 | 0.0083 | 1.15 |
| | 0.7101 | | | |
| | 0.7224 | | | |
| 2 | 0.7278 | 0.7193 | 0.0083 | 1.15 |
| | 0.7189 | | | |
| | 0.7112 | | | |
| 3 | 0.7200 | 0.7128 | 0.0112 | 1.57 |
| | 0.7186 | | | |
| | 0.6999 | | | |
| Mean | | 0.7172 | 0.0093 | 1.29 |

Table 23 Within run precision of zinc oxide combined with calcium carbonate

| Concentration (%w/w) | Absorption | Mean | SD | % RSD |
|:---:|:---:|:---:|:---:|:---:|
| 6 | 0.1217 | 0.1240 | 0.0021 | 1.73 |
| | 0.1255 | | | |
| | 0.1256 | | | |
| | 0.1246 | | | |
| | 0.1232 | | | |
| | 0.1265 | | | |
| | 0.1208 | | | |
| 10 | 0.2061 | 0.7213 | 0.0040 | 1.89 |
| | 0.215 | | | |
| | 0.2141 | | | |
| | 0.2159 | | | |
| | 0.2111 | | | |
| | 0.2201 | | | |
| | 0.2122 | | | |
| 14 | 0.3222 | 0.3276 | 0.0050 | 1.54 |
| | 0.3272 | | | |
| | 0.3352 | | | |
| | 0.3198 | | | |
| | 0.3298 | | | |
| | 0.3265 | | | |
| | 0.3325 | | | |
| Mean | | 0.3910 | 0.0037 | 1.72 |

Table 24 Between run precision of zinc oxide combined with calcium carbonate

| day | Absorption | Mean | SD | % RSD |
|---|---|---|---|---|
| 1 | 0.2101 | 0.2133 | 0.0029 | 1.35 |
| | 0.2157 | | | |
| | 0.2141 | | | |
| 2 | 0.2155 | 0.2176 | 0.0022 | 1.02 |
| | 0.2179 | | | |
| | 0.2135 | | | |
| 3 | 0.2200 | 0.2177 | 0.0025 | 1.13 |
| | 0.2151 | | | |
| | 0.2181 | | | |
| Mean | | 0.2162 | 0.0025 | 1.17 |

## 4. Specificity

The comparisons were carried out between 5%w/w titanium dioxide or zinc oxide combined with 5%w/w calcium carbonate dispersion in dimethicone oil and dimethicone oil alone. The absorption spectra of both physical sunscreen combinations were different from individual dimethicone oil which was no peak presented. There was no other peak interrupt the major peak of both combinations. The graphs of titanium dioxide and zinc oxide combined with calcium carbonate was shown in Figure 48 and 49, respectively. The data was presented in Table 25.
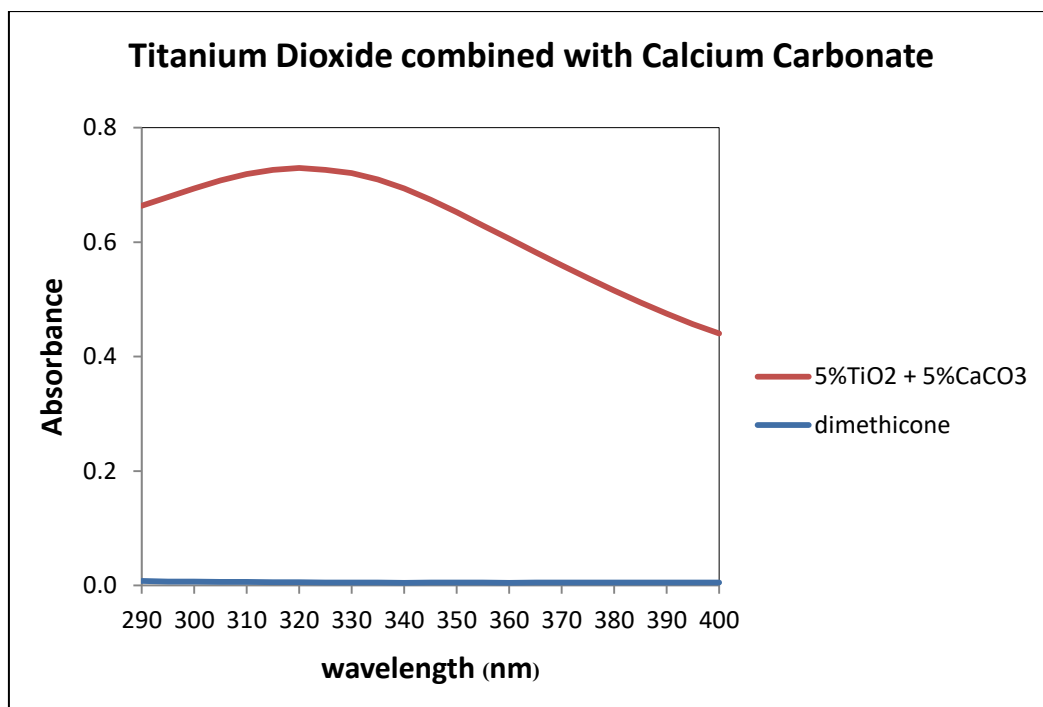
Figure 48 Specificity validation method for absorbance spectra of titanium dioxide
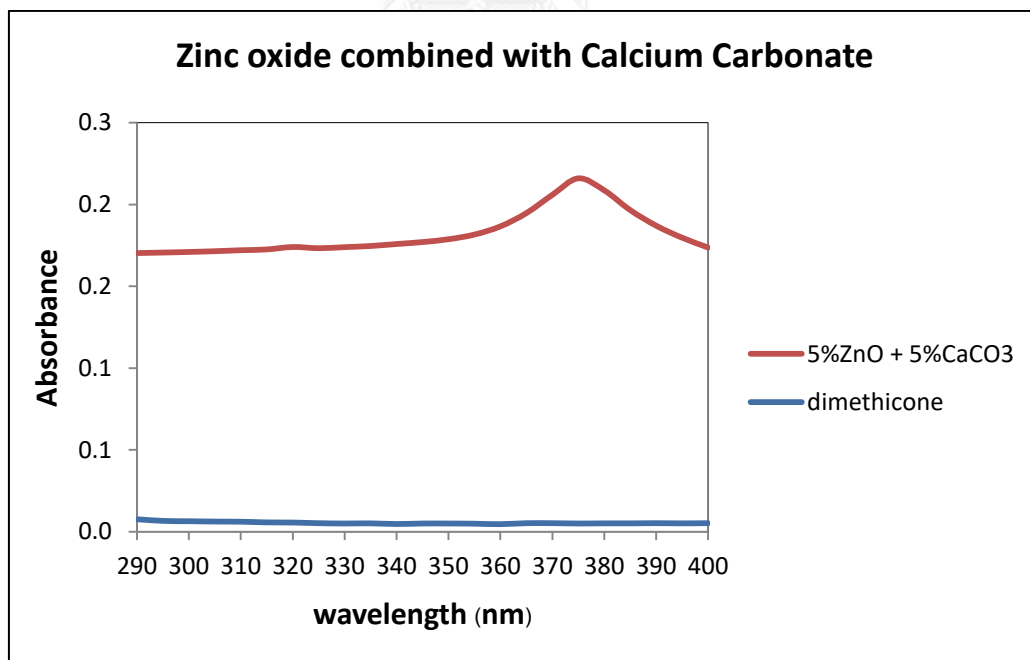combined with calcium carbonate compared with dimethicone alone



Figure 49 Specificity validation method for absorbance spectra of zinc oxide
combined with calcium carbonate compared with dimethicone alone

Table 25 Specificity validation of the absorption values of both physical sunscreens combined with calcium carbonate and dimethicone oil

| Wavelength (nm) | $TiO_2 + CaCO_3$ | | $ZnO + CaCO_3$ | | Dimethicone oil | |
|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD |
| 290 | 0.6635 | 0.0240 | 0.1703 | 0.0051 | 0.0076 | 0.0005 |
| 295 | 0.6787 | 0.0248 | 0.1706 | 0.0050 | 0.0066 | 0.0002 |
| 300 | 0.6938 | 0.0254 | 0.1710 | 0.0049 | 0.0064 | 0.0004 |
| 305 | 0.7077 | 0.0260 | 0.1715 | 0.0048 | 0.0062 | 0.0003 |
| 310 | 0.7189 | 0.0266 | 0.1721 | 0.0045 | 0.0061 | 0.0003 |
| 315 | 0.7261 | 0.0263 | 0.1726 | 0.0044 | 0.0057 | 0.0005 |
| 320 | 0.7296 | 0.0257 | 0.1740 | 0.0042 | 0.0056 | 0.0002 |
| 325 | 0.7264 | 0.0245 | 0.1733 | 0.0040 | 0.0052 | 0.0007 |
| 330 | 0.7206 | 0.0226 | 0.1740 | 0.0039 | 0.005 | 0.0005 |
| 335 | 0.7096 | 0.0206 | 0.1746 | 0.0039 | 0.0051 | 0.0005 |
| 340 | 0.6941 | 0.0180 | 0.1759 | 0.0038 | 0.0047 | 0.0007 |
| 345 | 0.6744 | 0.0151 | 0.1771 | 0.0040 | 0.005 | 0.0005 |
| 350 | 0.6524 | 0.0126 | 0.1788 | 0.0039 | 0.005 | 0.0008 |
| 355 | 0.6292 | 0.0100 | 0.1816 | 0.0039 | 0.0049 | 0.0004 |
| 360 | 0.6062 | 0.0079 | 0.1866 | 0.0042 | 0.0046 | 0.0006 |
| 365 | 0.5823 | 0.0064 | 0.1948 | 0.0048 | 0.0052 | 0.0005 |
| 370 | 0.5598 | 0.0060 | 0.2059 | 0.0056 | 0.0052 | 0.0007 |
| 375 | 0.5371 | 0.0064 | 0.2160 | 0.0058 | 0.005 | 0.0005 |
| 380 | 0.5154 | 0.0076 | 0.2087 | 0.0045 | 0.0051 | 0.0005 |
| 385 | 0.4948 | 0.0089 | 0.1966 | 0.0035 | 0.0051 | 0.0004 |
| 390 | 0.4751 | 0.0103 | 0.1871 | 0.0030 | 0.0052 | 0.0006 |
| 395 | 0.4566 | 0.0115 | 0.1797 | 0.0028 | 0.0051 | 0.0005 |
| 400 | 0.4402 | 0.0126 | 0.1737 | 0.0027 | 0.0052 | 0.0004 |

# VITA

Miss Wachiraphon Yuensook was born on April 2, 1992. She received the Bachelor Degree in Applied Thai Traditional Medicine (B.ATM.), in 2013 from the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. She presented a poster titled "Effect of Calcium Carbonate Nanoparticles on in vitro Photoprotection Efficiency of Physical Sunscreens" in 13th National Kasetsart University Kamphaeng Saen on December 8-9, 2016 at Kasetsart University Kamphaeng Saen, Nakhon Pathom, Thailand.

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