

CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Acetone (Analytical grade, Lot No. 10090110, RCI Labscan, Bangkok, Thailand)
2. Acetonitrile (HPLC grade, Lot No. 13050204, RCI Labscan, Bangkok, Thailand)
3. Benzophenone-3; Eusolex[®] 4360 (Cosmetic grade, Lot No. K43577876, Merck, Darmstadt, Germany)
4. Bis-ethylhexyloxyphenol methoxyphenyltriazine (BEMT); Tinosorb[®] S (Cosmetic grade, Lot No. 0100083788, BASF, Ludwigshafen, Germany).
5. Butylmethoxy dibenzoylmethane; Eusolex[®] 9020 (Cosmetic grade, Lot No. 5844E270, Merck, Darmstadt, Germany)
6. Ethyl acetate (HPLC grade, Lot No. 11100284, RCI Labscan, Bangkok, Thailand)
7. Ethylhexyl dimethyl PABA; Eusolex[®] 6007 (Cosmetic grade, Merck, Darmstadt, Germany)
8. Ethylhexyl methoxycinnamate; Uvinul[®] MC 80 (Cosmetic grade, Lot No. 57866236W0, BASF, Ludwigshafen, Germany).
9. Ethylhexyl salicylate; Eusolex[®] OS (Cosmetic grade, Lot No. K93230849, Merck, Darmstadt, Germany)
10. Ethylhexyl triazone; Uvinul[®] T 150 (Cosmetic grade, Lot No. 113030P050, BASF, Ludwigshafen, Germany).
11. Homosalate; Eusolex[®] HMS (Cosmetic grade, Lot No. K41236212, Merck, Darmstadt, Germany)
12. Propan-2-ol (Analytical grade, Lot No. 12060032, RCI Labscan, Bangkok, Thailand)
13. Methanol (HPLC grade, Lot No. 13040206, RCI Labscan, Bangkok, Thailand)

14. Methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT); Tinosorb[®] M (Cosmetic grade, Lot No. 0005787740, BASF, Ludwigshafen, Germany).

15. Octocrylene; Eusolex[®] OCR (Cosmetic grade, Lot No. 5377E144, Merck, Darmstadt, Germany)

16. Sterile water for injection (A.N.B. Laboratories, Bangkok, Thailand)

Apparatus

1. Analytical balance (AT 201 FACT, Mettler Toledo, Bangkok, Thailand)

2. C-18 Reverse phase HPLC column (Luna[®], 5 μ m C18, 250 x 4.60 mm, Phenomenex, Torrance, USA) with guard cartridge (KJO-4282, Phenomenex, Torrance, USA)

3. Disposable syringe, 1 mL (Nipro[®], Ayutthaya, Thailand)

4. High performance liquid chromatography; HPLC (Shimadzu, Kyoto, Japan) consisting of a SCL-10AVP system controller, dual LC-10AVP pumps, CTO-10ASVP column oven, SPD-10ASVP UV-VIS detector, SIL-20ACHT auto-sampler and Class VP software Version 6.12 SP3 (Shimadzu, Kyoto, Japan)

5. High performance liquid chromatography; HPLC (Shimadzu, Kyoto, Japan) consisting of a LC-20AT system pump and controller, CTO-10ASVP column oven, SPD-20AUV-VIS detector, SIL-10AD VP auto-sampler and LC solution software Version 1.21 SP1 (Shimadzu, Kyoto, Japan)

6. Nylon membrane filters (0.45 μ m, Vertical[®], Bangkok, Thailand)

7. Sonication bath (Transsonic, 829/H, Elma[®], Lebanon)

8. SPF 290s analyzer (SPF-290S, Optometrics Corporation, Massachusetts, USA)

9. Syringe filter (0.45 μ m Nylon membrane, VertiClean[™], Vertical Chromatography Co., Ltd., Nonthaburi, Thailand)

10. Vortex (Scientific industries, Alabama, USA)

Methodology

The studies shown in this thesis were divided into three parts.

Part 1: A Survey of UV filters in sunscreen products

A survey of UV filters used in commercially available sunscreen products was performed in local hypermarkets in Thailand during August to September 2012. The observation was limited in the primary sunscreen products. Cosmetic products which the major purpose were not claimed to protect skin from UV radiation but also contain UV filters were not included in this study. The UV protection efficacy of sunscreen products displayed on Sun Protection Factor (SPF) and protection factor of UVA (PA) were recorded.

Part 2: The development of a HPLC method

1. Determination of a suitable solvent to dissolve the UV filters

To perform an assay, it is necessary to find a suitable solvent to dissolve the UV filters selected based on the survey as described above. A variety of solvents or solvent mixture were tested for their ability to dissolve the UV filters at concentration 1 mg/mL. A vortex mixer and/or an ultrasonic bath were used to enhance the dissolution of the UV filters. A suitable solvent was chosen based on its ability to give optically clear solutions without precipitates.

2. Preparation of mixed standard stock solution

A standard stock solution of UV filters was prepared at concentration 1 mg/mL in a suitable solvent according to the results of the “determination of a suitable solvent to dissolve the UV filters”. The stock solution was sonicated in an ultrasonic bath until completely solubilised. Then it was filtered through a syringe filter (0.45 µm nylon membrane, VertiClean™, Vertical Chromatography Co., Ltd., Nonthaburi, Thailand) and injected into the high performance liquid chromatography (HPLC) system.

3. Development and optimization of HPLC method

The development and optimization of HPLC analyses for all UV filters were performed to determine optimum separating conditions for the selected UV filters according to the survey by using an isocratic and gradient methods.

3.1 Isocratic elution mode

A Shimadzu (Kyoto, Japan) HPLC system consisting of a SCL-10AVP system controller, dual LC-10AVP pumps, a CTO-10ASVP column oven and a SIL-20AHT auto-sampler was used in the analysis. Ultraviolet detection was achieved using a SPD-10ASVP UV-VIS detector (Shimadzu, Kyoto, Japan). A maximum absorption wavelength of the all UV filters at 325 nm was used in the analysis. The analysis of data was processed by using Class VP software Version 6.12 SP3 (Shimadzu, Kyoto, Japan). The separation was performed using Luna[®] C18 Column, 250 x 4.6 mm, i.d. 5 μ m) (Phenomenex, USA) fitted with a guard cartridge (Phenomenex, USA). An injection volume was 20 μ l.

Initially, the method development was started by an isocratic elution mode. A various parameters including types of mobile phase, flow rate and column temperature were investigated. The effect of mobile phase effect was studied by varying types and compositions of organic solvents, while the column temperature and flow rate was fixed at 25°C and 1.0 mL/min, respectively. The development **was started** with the interesting mobile phase system selected from the study of Gaspar and Maia Campos (2006) which composed of methanol: water (88:12, v/v). The condition for HPLC analysis was further optimized by varying the column temperature at 25, 30 and 35°C. After the effect of flow rate was investigated between 1.0 and 1.5 mL/min. The optimum separating conditions were selected based on the total analysis time and the resolution (R_s) value. The acceptable criteria for R_s value were greater than or equal to 1.5 (Dong, 2006).

The mobile phase preparation was prepared in volume ratios (v/v) by measuring a desire volume of each solvent into a bottle. All solvents used for mobile phase were HPLC grade. The solvent was mixed together and shaken thoroughly, filtered through the nylon membrane filters (0.45 μ m, Vertical[®], Bangkok, Thailand) by vacuum filtration and degassed by an ultrasonicator.

3.2 Gradient elution mode

For the gradient mode, a Shimadzu (Kyoto, Japan) HPLC instrument was used in the analysis. The system consists of a LC-20AT controller, LC-20AT low pressure gradient system quaternary pump, a CTO-10ASVP column oven, a SIL-10AD VP auto-sampler and a PD-20AUV-VIS detector was used in the separation. The

detection wavelength was 325 nm. The analysis of data was processed by using LC solution software Version 1.21 SP1 (Shimadzu, Kyoto, Japan). Luna[®] C18 Column (250 x 4.6 mm, i.d. 5 µm) was used with an injection volume 20 µL.

Although gradient elution requires more complex and expensive equipment, this technique may be benefit for a complex sample containing compounds with wide range of polarities. In this study, the optimization was performed by gradually changing type and ratio of organic modifier with a constant controlling of the suitable flow rate and column temperature. Again, the optimum separating conditions were selected based on the total analysis time and the resolution (R_s) value. The acceptable criteria for R_s value were greater than or equal to 1.5 (Dong, 2006).

4. Method validation

The method validation was performed following ICH Q2 (R1) guideline in Validation of Analytical Procedures (International Conference on Harmonisation, 2013). The parameters including linearity of calibration, accuracy, precision, specificity, limit of detection (LOD) and limit of quantitation (LOQ) were investigated.

4.1 Linearity of calibration curve

Linearity is the range between the instrument response and known concentration of substances. In this study, the instrument response, i.e. peak areas were plotted in the Y-axis and the substance concentrations were plotted in the X-axis. The linear relationship between the instrument response and known concentration of substances was described by the linear equation, $y = mx + c$, where y is response peak areas (AU), m is the slope of the regression line, x is the concentration of substance (µg/mL) and c is the intercept with the y-axis of calibration curve.

Linearity was performed by diluting mixed standard stock solution (see Section 3) to six levels of concentration between 5 – 100 µg/mL using a suitable solvent according to the results of the “determination of a suitable solvent to dissolve the UV filters”. The experiment was performed in triplicate, the R_s value for each peak of UV filters is expected to be greater than or equal to 1.5 and the correlation coefficient (r) is required at $r > 0.99$ (Dong, 2006). The residual sum of squares (RSS), a measure of the discrepancy between the observed value and the predicted, was computed. The linear regression analysis was assessed by using one-way ANOVA F -test and t -test at 95% confidence level.

4.2 Accuracy

Accuracy is the value indicating the closeness of agreement between the true value (spiked concentration) and the value found using the experimental procedure (investigated concentration). The accuracy was measured using six determinations per concentration at three different concentrations. It is expressed as percent recovery of known amount of the analyte spiked into the sample. The percent accuracy was calculated using equation (1). The acceptable range was 95-105% (The European Cosmetics Association, 2011).

$$\% \text{ Accuracy or } \% \text{ Recovery} = (\text{Investigated conc.} / \text{Spiked conc.}) \times 100 \quad \dots\dots\dots (1)$$

4.3 Precision

The precision of the developed method describes the closeness of the assay values obtained from repeated analyzes. The precision of the developed method was proofed by intra-day (repeatability) and inter-day variation in analysis. Intra-day and inter-day were performed using minimum, middle and high concentrations of the validation range (include the LOQ). Regarding the intra-day precision, the triplicate analysis of three determinations and three different concentrations at 10, 50 and 90 $\mu\text{g/mL}$ were performed within one day. Inter-day precision was determined across three consecutive days. The intra- and inter-day variation in the peak area of analytes was calculated in the terms of coefficient of variation (C.V.) using equation 2. The acceptable value of precision required at lower than 5% (Ermer and Miller, 2005).

$$\% \text{ CV} = (\text{Standard deviation} / \text{Mean}) \times 100 \dots\dots\dots (2)$$

4.4 Specificity

Specificity is the parameter indicates the interference of substance in the assay including impurities, degradants, matrix, etc. Specificity was determined by comparing the results obtained from the HPLC analysis of the blank base cream and the spiked samples (UV filters + base cream).

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

The LOD and LOQ are described about the sensitivity of determination process, based on the signal to noise ratio. These values were determined by further diluting mixed standard solution from 5 $\mu\text{g/mL}$ (the lowest concentration of the calibration curve) to various lower concentrations. The lowest amount of analytes in a sample that could be detected but not quantitated, based on a signal to noise ratio of 3 to 1, was indicated by the LOD (Dong, 2006). The LOQ represented the lowest amount of analytes in a sample that could be quantitatively determined with the specified degree of accuracy and precision, based on a signal to noise ratio of 10 to 1 (Dong, 2006).

Part 3: Validation the applicability of the developed method to various sunscreen samples

The sunscreen samples tested were standard sunscreens, formulated sunscreens and commercial sunscreens.

1. Standard sunscreens

The standard sunscreens were sunscreen formulations published as reference formulations for determinations of Sun Protection Factor (SPF) or UVA protection factor (UVA-PF) in various guidelines such as USFDA and COLIPA. These formulations gave detail of ingredients and the preparation method. In this study, the standard sunscreen S1 (SPF 5.1 ± 0.38) and S2 (SPF 16.6 ± 1.22) were selected and SPF value was measured by SPF-290 analyzer (Optometrics Corporation, USA). The ingredients are shown in Table 9 and 10, respectively.

Table 9 Ingredients of the standard S1 sunscreen formulation, SPF 5.1 ± 0.38
(COLIPA, 2006)

Ingredients	Master formula (%w/w)
Lanolin	5.0
Homosalate	8.0
Petrolatum	2.5
Stearic Acid	4.0
Propyl paraben	0.05
Methyl paraben	0.10
Disodium Edetate	0.05
Propylene Glycol	5.0
Triethanolamine	1.0
Purified water	74.30

Table 10 Ingredients of the standard S2 sunscreen formulation, SPF 16.6 ± 1.22
(COLIPA, 2006)

Ingredients	Master formula (%w/w)
Lanolin	4.5
Cocoa butter	2.0
Glyceryl stearate	3.0
Stearic acid	2.0
Ethylhexyl dimethyl PABA	7.0
Benzophenone-3 (Oxybenzone)	3.0
Water	71.6
Sorbitol	5.0
Triethanolamine	1.0
Methyl paraben	0.3
Propyl paraben	0.1
Benzyl Alcohol	0.5

2. Formulated sunscreens

The formulated sunscreens were sunscreen formulations prepared in-house. Types of UV filters added in the formulated sunscreens were based on the results of the survey. In this study, a single and combination of UV filters were added into base cream. The formulations containing single UV filters were prepared at moderate and **high levels** of concentration regulated by Thai FDA. For the formulations containing combination of UV filters, various types, number and amount of UV filters were added. The ingredients of the base cream are shown in Table 11.

Table 11 Ingredients of base cream used to prepare formulated sunscreens
(Kuntared and Hiranyaeakkaphap, 2012)

Ingredients	Master formula (%w/w)
Cetylstearyl alcohol	2.00
Glycerylmonostearate	2.00
Stearic acid	2.00
Isopropyl myristate	7.04
Glycerin	2.00
Carbomer 934	0.08
Triethanolamine (TEA)	0.50
Tetrasodium EDTA	0.04
Sodium lauryl sulfate (SLS)	0.08
Methyl paraben	0.20
Propyl paraben	0.04
Water	68.0

3. Commercial sunscreens

The sunscreens available commercially were also included in the validation of the applicability of the method developed. A various commercial sunscreen products were selected from the local shops and department stores. The products selected were primary sunscreen which was the products claimed as the majority for protection from UV radiation, with SPF ranging from 15 to at least 50.

4. Sample preparation for HPLC analysis

The sunscreen samples including standard sunscreens, formulated sunscreens and commercial sunscreens were prepared for HPLC analysis by accurately weighing the sample (40 mg) into 10 mL volumetric flask and dissolving in suitable solvents. The sample solutions were sonicated until completely dissolved, filtered through 0.45 μm syringe filter (VertiCleanTM, Vertical Chromatography Co., Ltd., Nonthaburi, Thailand) prior to inject into the developed HPLC system. The peak area of the analyzed compounds was obtained from integration of chromatogram analysis and the amount of each UV filters was calculated by the linear equation ($y = mx + c$) of each standard UV filters obtained from the calibration curves.