CHAPTER IV

RESULTS AND DISCUSSION

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. Thirty three brands of primary sunscreen products were observed. They were manufactured in many countries, USA, Australia, Poland, Taiwan and Thailand. The UV filters found in those products were 4-methylbenzylidene camphor (4-MBC), benzophenone-3 (BZ-3), benzophenone-4 (BZ-4), bis-ethylhexyloxyphenol methoxyphenyltriazine (BEMT), butylmethoxy dibenzoylmethane (BMDBM), diethylaminohydroxybenzoyl hexylbenzoate (DAHB), drometrizoletrisiloxane (DTS), ethylhexyl methoxycinnamate (EHMC), ethylhexyl salicylate (EHS), ethylhexyl triazone (EHT), homosalate (HMS), methylene bis-benzotriazolyltetramethyl butylphenol (MBBT), octocrylene (OCR), phenylbenzimidazole sulfonic acid (PBSA), terephthaylidenedicamphor sulfonic acid (TDSA), titanium dioxide, zinc oxide (Table 12). Among the thirty-three sunscreen products, the top three uses of UV filters were EHMC, titanium dioxide and BMDBM, respectively. On the other hand, BZ-4, DAHB, DTS, EHT and TDSA were not widely used UV filters. Each of them was found in one brand. From the survey, although the following UV filters:4-MBC, BZ-4, DTS, PBSA and TDSA were found, they were not included in this study. The reasons are as follows. The safety issue of 4-MBC has recently been questioned because it has been reported to be absorbed into systemic following topical application (Janjua, et al., 2004). In US, Australia and Canada this filter is not approved as an active ingredient for sunscreen (Kumar and Gupta, 2013). It is allowed be used as an inactive ingredient in some products. In Europe, many reports have shown toxicity to thyroid gland and reproductive systems (The SCCNFP, 2004) and thus it is suggested not to be used in sunscreen. The BZ-4 has been reported on side effects and human toxicity which showed significantly positive patch test on contact dermatitis investigation (Hughes and Stone, 2007). It is rarely used in the present and the results of this survey found BZ-4 in only one commercial brand. PBSA has been

reported to act as a photosentizer when it was exposed to UV radiation (Bastien, et al., 2010). DTS and TDSA are UV filters which are patented by L'Oreal under trade name Mexoryl XL and Mexoryl SX, respectively. Thus, these two UV filters are limited used only for L'Oreal's products. For titanium dioxide and zinc oxide, they are physical UV filters which not absorb UV radiation and thus cannot be detected by HPLC coupled with UV-VIS absorbance detector. Thus they were also not included in this study. For ethylhexyl dimethyl PABA (ED-PABA), based on the survey, it was found to be not used in any commercial products. However, it is the UV filter contained in reference sunscreen formulation P2 which regulated by the Cosmetic, Toiletry and Fragrance Association or CTFA (COLIPA, 2006) for testing the efficiency of sunscreen products and verify labeling on accurately test results. Consequently, ED-PABA was included in this study. In conclusion, the UV filters which were aimed to be assayed in this study were BZ-3, BEMT, BMDBM, ED-PABA, EHMC, EHS, EHT, HMS, MBBT, and OCR. The chemical name, trade name, chemical structure and the maximum concentration of these ten UV filters are shown in Table 13. Interestingly, the results of this survey found that the frequently used UV filters observed are similar to those observed by Kerr (2010) who surveyed the UV filters used in 337 sunscreen products with a median sun protection factor of 30. This study was performed in Dundee, UK, in 2010 and was compared with the survey data performed in 2007 by Wahie, et al. (2007) and indicated as percentage change. The frequent used UV filters observed are tabulated in Table 14. Surprisingly, the UV filters observed in Thailand according to this study were in the top rank of what were observed in Dundee, UK.

Table 12 The frequently used UV filters obtained from a survey of sunscreen products in Thailand

Sunscreen Brands	UV filters																	
	Chemical															Physical		
	4-MBC	BZ-3	BZ-4	BEMT	BMDBM	DAHB	DTS	EHS	EHT	HMS	MBBT	OCR	EHMC	PBSA	TDSA	titanium	dioxide	zinc oxide
Amela-Ex: UV Rescue Sun Block SPF 60 PA++++	-	-	-	-	✓	-	-	-	-	-	-	✓	-	-	-	✓		-
Banana Boat: SPORT Sunscreen SPF 110 PA+++	-	✓	-	-	✓	-	-	-	-	-	-	✓	-	-	-	-		-
Banana Boat: Ultra defense Broad Spectrum Sunblock SPF 50	-	-	✓	-	✓	-	-	✓	-	✓	-	✓	-	-	-	-		-
Biore: UV Aqua Rich SPF 50+ PA+++	-	-	-	-	-	✓	-	-	-	-	-	-	✓	-	-	-		-
Biore: UV Perfect Face Milk SPF50+ /PA+++	-	-	-	-	-	-	-	-	-	-	-	-	✓	-	-	-		✓
Cancer Council: Ultra Sunscreen SPF 50 PA+++	-	-	-		✓	-	-	-	-	-	✓	✓	✓	-	-	-		-
C'Care: Sun Block Body lotion SPF 50	-	✓	-	-	-	-	-	✓	-	✓	-		✓	-	-	✓		-
Cetaphil: Defense SPF50 UVA&UVB protection	-	✓	-	-	-	-	-	✓	-	-	-	✓	✓	-	-	✓		-
Concept: Ultimate Sun Protection SPF 50 PA+++	-	-	-	-	✓	-	-	-	-	✓	-	-	✓	-	-	✓		-

Table 12 (Cont.)

Sunscreen Brands	UV filters																	
	Chemical															Physical		
	4-MBC	BZ-3	BZ-4	BEMT	BMDBM	DAHB	DTS	EHS	EHT	HMS	MBBT	OCR	EHMC	PBSA	TDSA	titanium dioxide		zinc oxide
Cute Press: Milk UV Expert Protection SPF50 PA+++	-	✓	-	✓	-	-	-	✓	-	-	-	-	✓	-	-	✓		-
Eucerin: Daily protection SPF 30 Face Lotion	-	-	-	-	-	-	-	✓	-	-	-	-	✓	✓	-	✓		✓
Eucerin: Kids Sun Lotion SPF 50+ UVB & UVA protection	-	-	-	✓	✓	-	-	-	-	-	-	✓	-	-	-	✓		-
Eucerin: Sun Crème for Face SPF 50+ UVB & UVA protection	-	-	-	✓	✓	-	-	✓	-	✓	-	✓	-	-	-	✓		-
Eucerin: Sun Spray Transparent SPF 50 UVB & UVA protection	-	-	-	✓	✓	-	-	✓	-	✓	-	✓		-	-	-		-
Faris by Naris: Perfect Protection milky SPF 50	-	-	-	-	-	-	-	-	-	-	-	-	✓	-	-	✓		✓
Garnier: UV Protect SPF50 PA+++	-	✓	-	-	-	-	✓	-	-	-	-	-	✓		✓	✓		-

Table 12 (Cont.)

Sunscreen Brands	UV filters																	
	Chemical															Physical		
	4-MBC	BZ-3	BZ-4	BEMT	BMDBM	DAHB	DTS	EHS	EHT	HMS	MBBT	OCR	EHMC	PBSA	TDSA	titanium	dioxide	zinc oxide
KA:UV Whitening Color Pastel SPF 50 PA+++	-	✓	-	-	-	-	-	✓	-	-	-	-	✓	-	-	-	-	
La Roche-Posay: Anthelios 60 Ultralight Face	-	✓	-	-	✓	-	-	✓	-	✓	-	✓	-	-	-	-	-	
Minus-sol: Facial Sun Protection SPF 30+	✓	-	-	-	-	-	-	-	-	-	-	✓	✓	-	-	✓	-	
Minus-Sun: Facial Sun Protection SPF 40 PA+++	-	-	-	-	-	-	-	-	-	-	-	-	✓	-	-	✓	✓	
Mistene: White image SPF 60 PA+++	✓	-	-	-	-	-	-	-	-	-	-	✓	✓	-	-	✓	-	
Neutrogena: Ultra Sheer Dry Touch SPF 45	-	✓	-	-	✓	-	-	✓	-	✓	-	✓	-	-	-	-	-	
Nivea: Sun Face Moisturizing Immediate Collagen SPF 50+	-	✓	-	✓	✓	-	-	✓	-	✓	-	-	✓	✓	-	✓	-	
Nivea: Sun Spray Immediate Collagen SPF 30 PA+++	-	-	-	✓	✓	-	-	-	✓	-	-	-	✓	-	-	-	-	
Oriental Princess: Natural Sunscreen Extra Protection For Face SPF30	-	-	-	-	✓	-	-	✓	-	-	-	-	✓	-	-	-	-	

Table 12 (Cont.)

Sunscreen Brands	UV filters																	
	Chemical															Physical		
	4-MBC	BZ-3	BZ-4	BEMT	BMDBM	DAHB	DTS	EHS	EHT	HMS	MBBT	OCR	EHMC	PBSA	TDSA	titanium	dioxide	zinc oxide
Provamed: Physical Sunscreen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	✓		✓
Smooth E : Physical Sunscreen	-	-	-	-	-	-	-	-	-	-	-	-	-	-		✓		✓
Spectraban: Sensitive SPF 30 PA+++	-	-	-	-	-	-	-	-	-	-	✓		✓	-	-	-		-
Spectraban: UVA& UVB Facial sunblock	✓	-	-	-	✓	-	-	-	-	-	-	-	✓	-	-	-		✓
Vaseline: Healthy Sunblock	-	-	-	-	-	-	-	-	-	-	-	-	✓	-	-	-		✓
Vichy: Capital Soleil SPF 60 Soft Sheer Sun Lotion	-	✓	-	-	✓	-	-	✓	-	✓	-	✓	-	-	-	-		-
Vitara: Facial sunscreen SPF 40 UVA & UVB Protection	-	✓	-	-	-	-	-	✓	-	✓	-	-	✓	-	-	✓		-
Za Cosmetics: Power Block UV SPF 40 PA+++	-	-	-	-	-	-	-	-	-	-	-	-	✓	-	-	✓		✓

Table 13 The chemical name, trade name, chemical structure and maximum concentration of the ten UV filters by Food and Drug Administration, Thailand

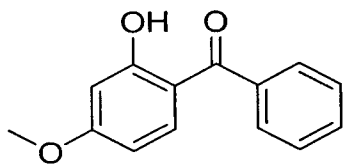
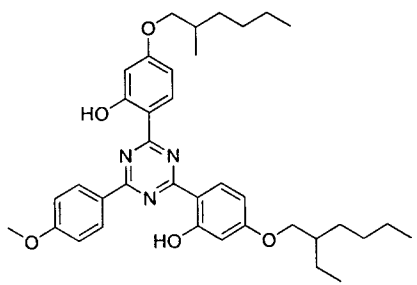
INCI name (The name used in this study)	Chemical name	Chemical Structure	Appearance	% Allowed in Thailand
Benzophenone-3; BZ-3 (CAS No. 131-57-7)	2-Hydroxy-4-methoxybenzophenone; Oxybenzone		Pale yellow powder	10
Bis-Ethylhexyloxyphenol methoxyphenyltriazine; BEMT (CAS No. 187393-00-6)	2,4-bis-[4-(2-Ethylhexyloxy)-2-hydroxy-phenyl)-6-(4-methoxyphenyl)-1,3,5-triazine]; Anisotriazine		White powder	10

Table 13 (Cont.)

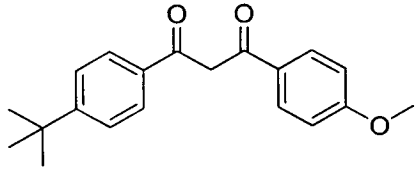
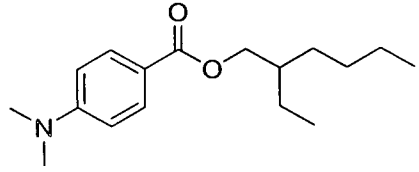
INCI name (The name used in this study)	Chemical name	Chemical Structure	Appearance	% Allowed in Thailand
Butylmethoxy-dibenzoyl methane; BMDBM (CAS No. 70356-09-1)	4-tert.-Butyl-4'-methoxy-dibenzoyl-methane; 1-(4-(1,1-Dimethylethyl)-phenyl)-3-(4-methoxy-phenyl)-propan-1,3-dione; Avobenzone		White powder	5
Ethylhexyl dimethyl PABA; ED-PABA (CAS No. 21245-02-3)	2-Ethylhexyl-4-dimethylaminobenzoate; Octyldimethyl PABA; Padimate-O		Colorless liquid	8

Table 13 (Cont.)

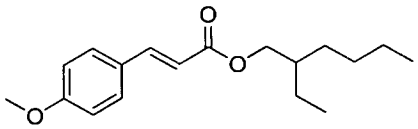
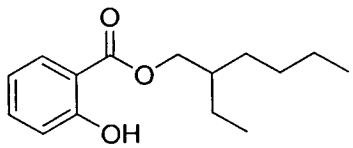
INCI name (The name used in this study)	Chemical name	Chemical Structure	Appearance	% Allowed in Thailand
Ethylhexyl methoxy-cinnamate; EHMC (CAS No.5466-77-3)	2-Ethylhexyl-4-methoxycinnamate;2-ethyhexyl ester; Octinoxate; Octylmethoxycinnamate		Colorless to light yellow liquid	10
Ethylhexyl salicylate; EHS (CAS No. 118-60-5)	2-Ethylhexyl-2-hydroxybenzoate; 2-Ethylhexylsalicylate; Octylsalicylate (OS)		Colorless liquid	5

Table 13 (Cont.)

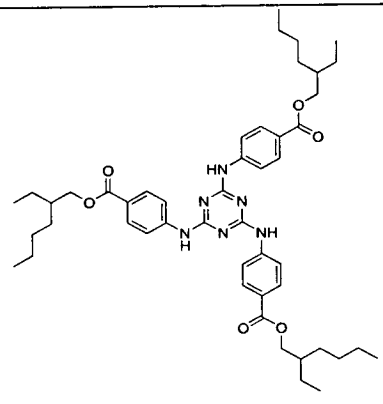
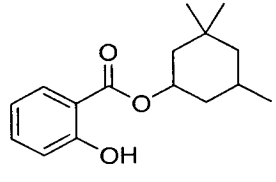
INCI name (The name used in this study)	Chemical name	Chemical Structure	Appearance	% Allowed in Thailand
Ethylhexyl triazone; EHT (CAS No. 8812-99-0)	2,4,6-Trianilino-(4-carbo-2'-ethylhexyl-1'-oxy)-1,3,5-triazine; 2,4,6-Trianilino(p-carbo-2'-ethylhexyl-1'-oxy)-1,3,5-triazine; Octyltriazone (OT)		White powder	5
Homosalate; HMS (CAS No. 118-56-9)	3,3,5-Trimethylcyclohexyl-2-hydroxybenzoate; Homomenthyl salicylate		Colorless liquid	10

Table 13 (Cont.)

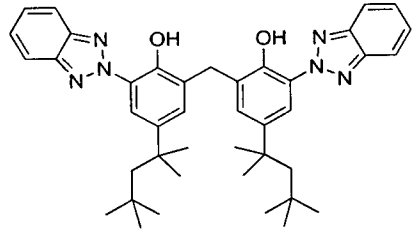
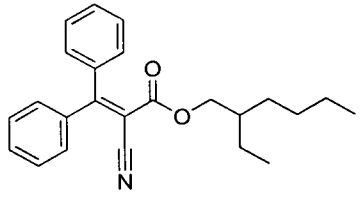
INCI name (The name used in this study)	Chemical name	Chemical Structure	Appearance	% Allowed in Thailand
Methylene bis-benzotriazolyl tetramethyl-butylphenol; MBBT (CAS No. 103597-45-1)	2,2'-Methylen-bis-(6-(2H-benzotriazol-2-yl)-4- (1,1,3,3-tetramethyl butyl)phenol); Bisoctyltriazole		White semi-solid or white suspension	10
Octocrylene; OCR (CAS No. 6197-30-4)	2-Ethylhexyl-2-cyano-3,3-diphenylacrylate		Colorless liquid	10

Table 14 Frequency of ultraviolet (UV) filters present in products in the current 2010 survey (Kerr, 2010)

UV filter (INCI name)	Absorption spectrum	Filter present in percentage of products		
		Kerr (2010)	Wahie, et al. (2007)	Percentage change
Butylmethoxy dibenzoylmethane (BMDBM)	UVA	96.4	73.4	23
Octocrylene (OCR)	UVB, UVA	90.5	36.4	54.1
Bis-ethylhexyloxyphenol methoxy-phenyltriazine (BEMT)	UVB, UVA	58.5	15.9	42.6
Ethylhexyl salicylate (EHS)	UVB	32.6	20.8	11.8
Diethylhexylbutamidotriazone	UVB	32	10.4	21.6
Methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT)	UVB, UVA	32	7.8	24.2
Ethylhexylmethoxycinnamate (EHMC)	UVB	17.8	53.6	-35.8
Ethylhexyltriazone (EHT)	UVB	16	14.9	1.1
Homosalate (HMS)	UVB	15.7	2.3	13.4
Benzophenone-3 (BZ-3)	UVB, UVA	15.1	16.9	-1.8
Terephthalylidenedicamphor sulfonic acid (TDSA)	UVA	14.2	14.6	-0.4
Drometrizoletrisiloxane (DTS)	UVB, UVA	13.4	11	2.4
Phenylbenzimidazole sulfonic acid (PBSA)	UVB	5.6	4.9	0.7
Diethylaminohydroxybenzoyl hexyl benzoate (DAHB)	UVA	5	1	4

Table 14 (Cont.)

UV filter (INCI name)	Absorption spectrum	Filter present in percentage of products		
		Kerr (2010)	Wahie, et al. (2007)	Percentage change
Polysilicone-15	UVB	3.3	2.6	0.7
4-Methylbenzylidene camphor (4-MBC)	UVB	1.2	25.3	-24.1
Isoamyl-p-methoxycinnamate	UVB	0.9	0	0.9
Disodium phenyl dibenzimidazole-tetrasulfonate	UVA	0.9	1.9	-1
Titanium dioxide	UVB, UVA, visible	49	45.1	3.9

Part 2: The development of a HPLC method

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

As the polarity properties of the selected UV filters are different. Therefore, a solvent which is able to solubilize all ten UV filters must be determined. The determinations were performed by fixing the concentration of each UV filters at 1 mg/mL. Initially, the study was proposed to use a single solvent to dissolve all ten filters. It was found that when using acetonitrile, ethanol, ethyl acetate, isopropanol, or methanol, the polar UV filters (i.e., BZ-3, BMDBM, ED-PABA, EHMC, EHS, HMS and OCR) could be dissolved (Table 15). However, non-polar filters (i.e., BEMT, EHT, and MBBT) could be dissolved only by ethyl acetate. Ethyl acetate is often used in dissolving and extraction process. It is a good solvent to dissolve a non-polar UV filters. However, it is a rapidly evaporated solvent due to its low boiling point and may not suitable for sample preparation. In addition, ethyl acetate is immiscible with water and thus hydrophobic UV filters are possible to precipitate in mobile phase containing high amount of water. It is necessary to use a mixed solvent which has different solubilizing characteristics compared to ethyl acetate to defer the evaporation rate and polarity. Acetonitrile was gained interesting due to its high polarity as compare to ethyl acetate

and it can dissolve all polar UV filters. The solubilization study was again performed using a mixed solvent of ethyl acetate and acetonitrile. The ratio of ethyl acetate to acetonitrile was titrated from 100% to 50%. All ten UV filters were successfully dissolved when using a mixture of 50% ethyl acetate and 50% acetonitrile. Therefore, this mixed solvent was chosen for sample preparation. A result of variety solvents tested in solvent determination for dissolve the UV filters is shown in Table 15.

Table 15 A varieties of solvents tested to solubilize the ten UV filters

UV filters	Solvents					
	Acetonitrile	Ethanol	Methanol	Isopropanol	Ethyl acetate	Acetonitrile and ethyl acetate (1:1)
BZ-3	✓	✓	✓	✓	✓	✓
BEMT	X	X	X	X	✓	✓
BMDBM	✓	✓	✓	✓	✓	✓
ED-PABA	✓	✓	✓	✓	✓	✓
EHMC	✓	✓	✓	✓	✓	✓
EHS	✓	✓	✓	✓	✓	✓
EHT	X	X	X	✓	✓	✓
HMS	✓	✓	✓	✓	✓	✓
MBBT	X	X	X	X	✓	✓
OCR	✓	✓	✓	✓	✓	✓

2. Development and optimization of HPLC method

The method development can be divided into isocratic and gradient modes. In C18 reverse-phase column, the separation is based on the partition of substance molecules between the mobile phase and the stationary phase. The factors affecting the separation in the reverse phase include the properties of substance, elution strength of organic solvent used for mobile phase, temperature, pH of mobile phase and flow rate. The experimental was started by isocratic mode. Initially, the development was

proposed to find a suitable mobile phase to separate all ten UV filters by a various type of organic solvent was considered. Composition of methanol, acetonitrile, or water is generally used as a mobile phase on reverse phase separation. However, the other types of solvent were also investigated in this study in order to obtain various elution strengths to elute ten UV filters which have various polarity properties. The elution strength of the mobile phase refers to the power or strength of the solvent in order to elute the substances from the column. A solvent which has a high elution strength provides fast elution. In reverse phase HPLC, an order of organic solvent strength can be tetrahydrofuran < acetone < isopropanol < ethyl acetate < acetonitrile < methanol < water. In addition, the method could optimized by adjusting a variety of the pH, column temperature, and flow rate of mobile phase. The criterias used for a selection were the resolution ($R_s > 1.5$) and suitable total analysis time.

2.1 Isocratic elution mode

The objective of the study was to develop simultaneous separating conditions for ten UV filters. The HPLC column containing C18 as a stationary phase was used for the separation of ten UV filters. Therefore, the modification of the mobile phase compositions was applied as the strategies to gain the sufficient separation of all analytical substances. Besides the effects of column temperature and flow rate were subsequently investigated. The optimized condition was selected based on a baseline separation of investigated peaks as well as an acceptable analysis time. The resolution (R_s) value of 1.5 or greater between two adjacent peaks is acceptable for the baseline separation.

Effects of mobile phase composition

The employed C18-column is a non-polar stationary phase, so the alteration of the solvent strength such as the modification of types and amounts of organic solvents in the mobile phase is one of the convenient ways to adjust the retention and the resolution of the analytes. In this study, the column temperature and flow rate were fixed at 25°C and 1.0 mL/min, respectively. The total analysis time for UV filters and resolution value were observed. The development of HPLC method was started using methanol and water (88:12, v/v) as a mobile phase (Chisvert, et al., 2001). It was observed that after analysis times of 120 min, only seven polar UV filters, i.e., BZ-3, OCR, BMDBM, ED-PABA, EHMC, EHS and HMS were eluted (Figure 6). The

hydrophobic UV filters including EHT, MBBT, and BEMT were not detected within 120 min. In addition, the HPLC peaks of BMDBM and ED-PABA were co-eluted. As the C18 column was used, non-polar compounds are preferably retained in the reverse phase column. Therefore, a mobile phase with higher elution strength is required.

Methanol and acetonitrile are the most commonly used organic solvents for mobile phases in reversed-phase chromatography. In an appropriate amount, they are both miscible with water. However, methanol is a protic solvent while acetonitrile is aprotic. Therefore, methanol is strongly associated with water while acetonitrile does not. In general, the acetonitrile-based mobile phase has a higher elution strength compared to methanol and thus it was selected to replace methanol. As a result, the composition of mobile phase was changed to acetonitrile: water (88:12, v/v). It was observed that the same group of seven UV filters including BZ-3, OCR, BMDBM, ED-PABA, EHMC, EHS and HMS was eluted (Figure 7). Using the acetonitrile instead of methanol at the same ratio, peaks of BMDBM and ED-PABA could be separated but the peak of BMDBM was co-eluted with OCR peak. However, the HPLC peaks of EHT, MBBT, and BEMT were still not observed within analysis times of 120 min. The results suggest that the elution strength of the mobile phase containing acetonitrile: water (88:12, v/v) still does not sufficient for EHT, MBBT, and BEMT.

In order to increase the solvent strength of the mobile phase, ethyl acetate was used to replace water. The mobile phase consisting of acetonitrile and ethyl acetate (90:10, v/v) could successfully elute ten UV filters within 40 min. In addition, all seven UV filters in the first group of chromatogram including BZ-3, OCR, BMDBM, ED-PABA, EHMC, EHS and HMS were eluted separately (Figure 8). Peaks of EHT, MBBT, and BEMT were observed and defined as the second group of the chromatogram. However, the peaks of MBBT and BEMT were co-eluted (Figure 8) and the R_s of UV filters in the first group of chromatogram was found to be lower than 1.5. The elution strength of the mobile phase was then reduced in order to let the analytes stay longer in the column and improve the peak resolution. Therefore the ratio of ethyl acetate was gradually decreased from 10% to 5% (v/v) while the amount of acetonitrile was raised from 90% to 95% (v/v) to make the total volume ratio of 100% (v/v) of the mobile phase.

Using the mobile phase containing acetonitrile: ethyl acetate at ratio 95:5 (v/v), all seven UV filters in the first group of chromatogram were still eluted separately (Figure 9, however the resolution value of some peaks is lower than 1.5. Fortunately, the separation of MBBT and BEMT was achieved with the R_s higher than 1.5 (Figure 9). To improve the resolution of the hydrophilic UV filters, water was added into the mobile phase of acetonitrile: ethyl acetate (95:5, v/v). Using acetonitrile: ethyl acetate: water (95:5:2, v/v/v) (Figure 10) and acetonitrile: ethyl acetate: water (95:5:4, v/v/v) (Figure 11) could improve the resolution of hydrophillic substances into the acceptable range ($R_s \geq 1.5$) (Table 16). However, the total analysis time of UV filters increased with the increasing of the amount of water in the mobile phase indicating the elution strength is decreased. The use of acetonitrile: ethyl acetate: water (95:5:2, v/v/v) showed a 28 min shorter analysis time than the use of acetonitrile: ethyl acetate: water (95:5:4, v/v/v) (Table 16). Therefore, the mobile phase employing acetonitrile: ethyl acetate: water (95:5:2, v/v/v) was chosen for further studies.

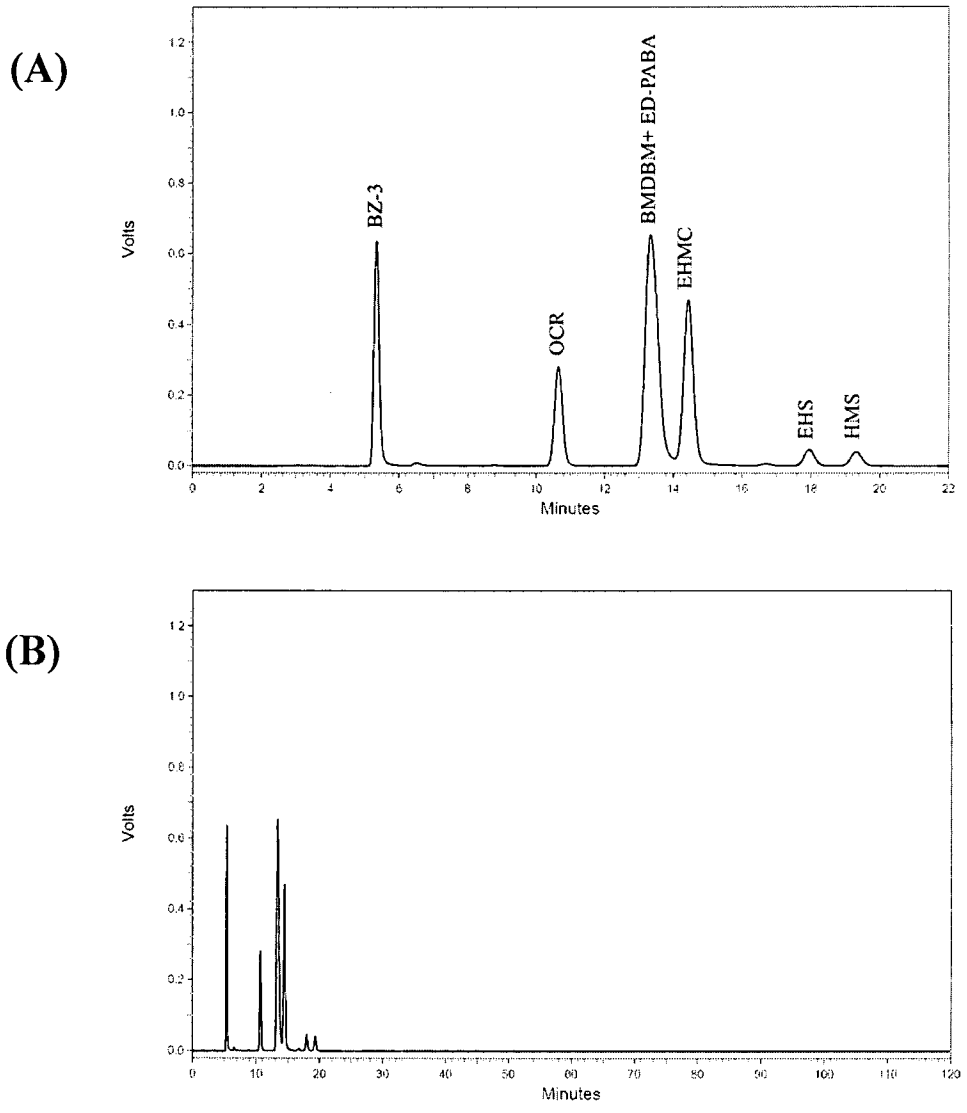


Figure 6 Chromatogram of ten UV filters detected at 325 nm. Chromatography was performed under isocratic mode of methanol: water (88:12, v/v) at a flow rate 1.0 mL/min and an injection volume 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used with a temperature control at 25 °C. A: enlarged scale of the first 22 min and B: chromatograms of the whole assay.

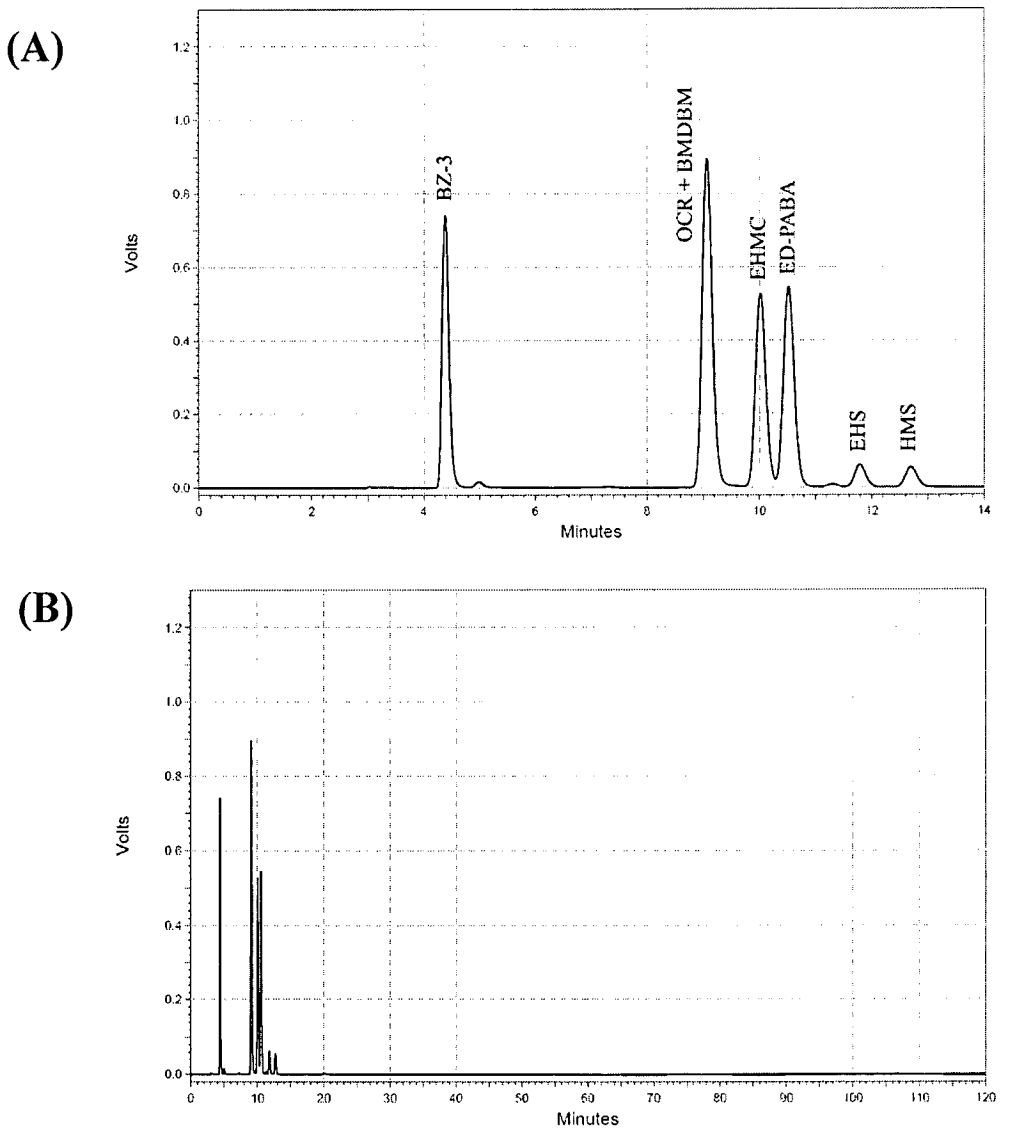


Figure 7 Chromatogram of ten UV filters detected at 325 nm. Chromatography was performed under isocratic mode of acetonitrile: water (88:12, v/v) at a flow rate 1.0 mL/min and an injection volume 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used with a temperature control at 25 °C. A: enlarged scale of the first 14 min and B: chromatograms of the whole assay.

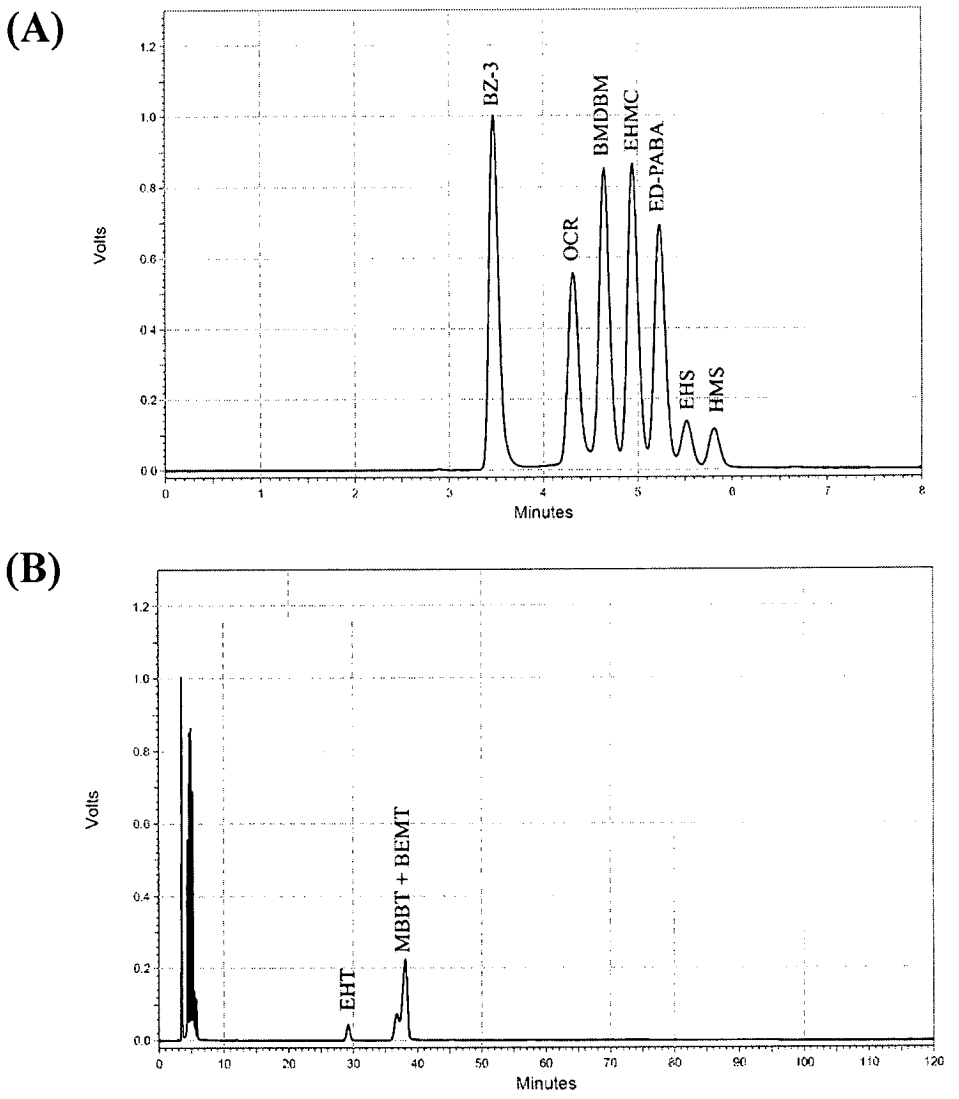


Figure 8 Chromatogram of ten UV filters detected at 325 nm. Chromatography was performed under isocratic mode of acetonitrile: ethyl acetate (90:10, v/v) at a flow rate 1.0 mL/min and an injection volume 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used with a temperature control at 25 °C. A: enlarged scale of the first 8 min and B: chromatograms of the whole assay.

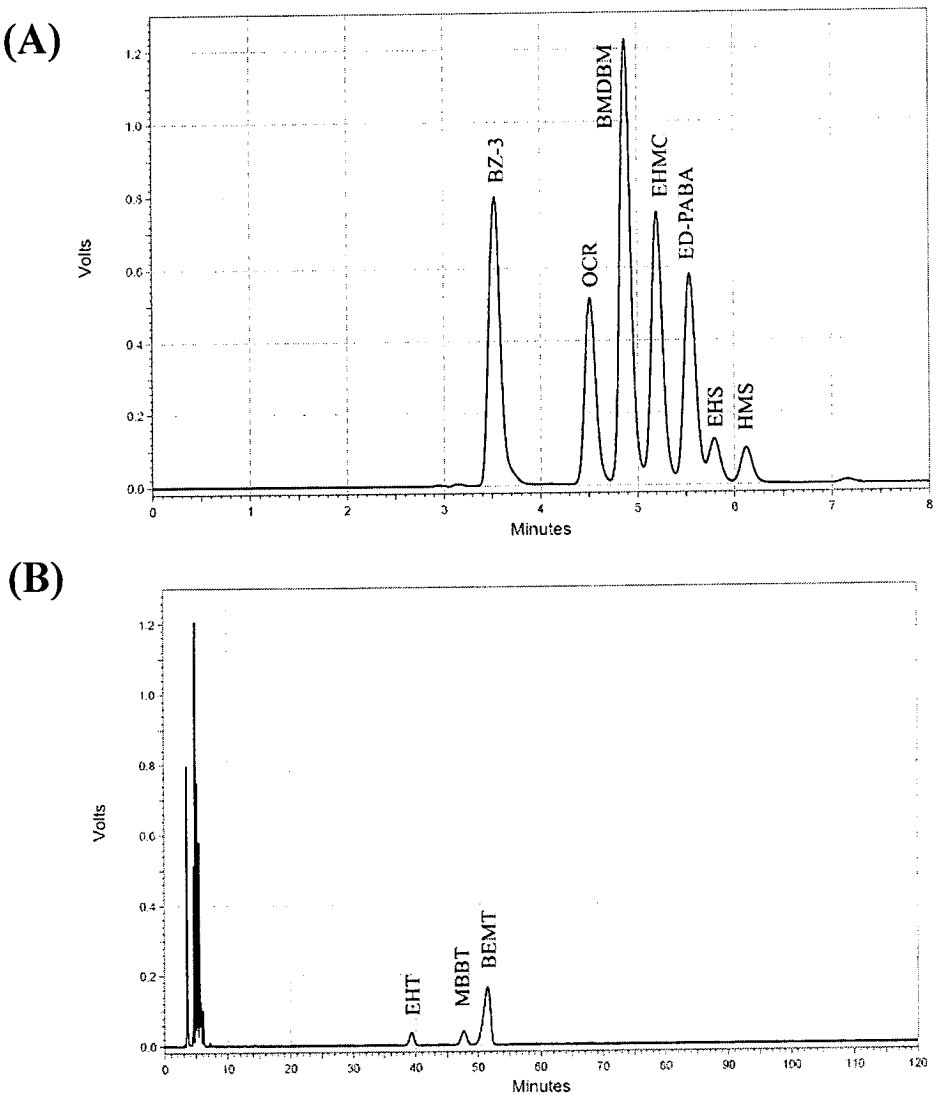


Figure 9 Chromatogram of ten UV filters detected at 325 nm. Chromatography was performed under isocratic mode of acetonitrile: ethyl acetate (95:5, v/v) at a flow rate 1.0 mL/min and an injection volume 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used with a temperature control at 25 °C. A: enlarged scale of the first 8 min and B: chromatograms of the whole assay.

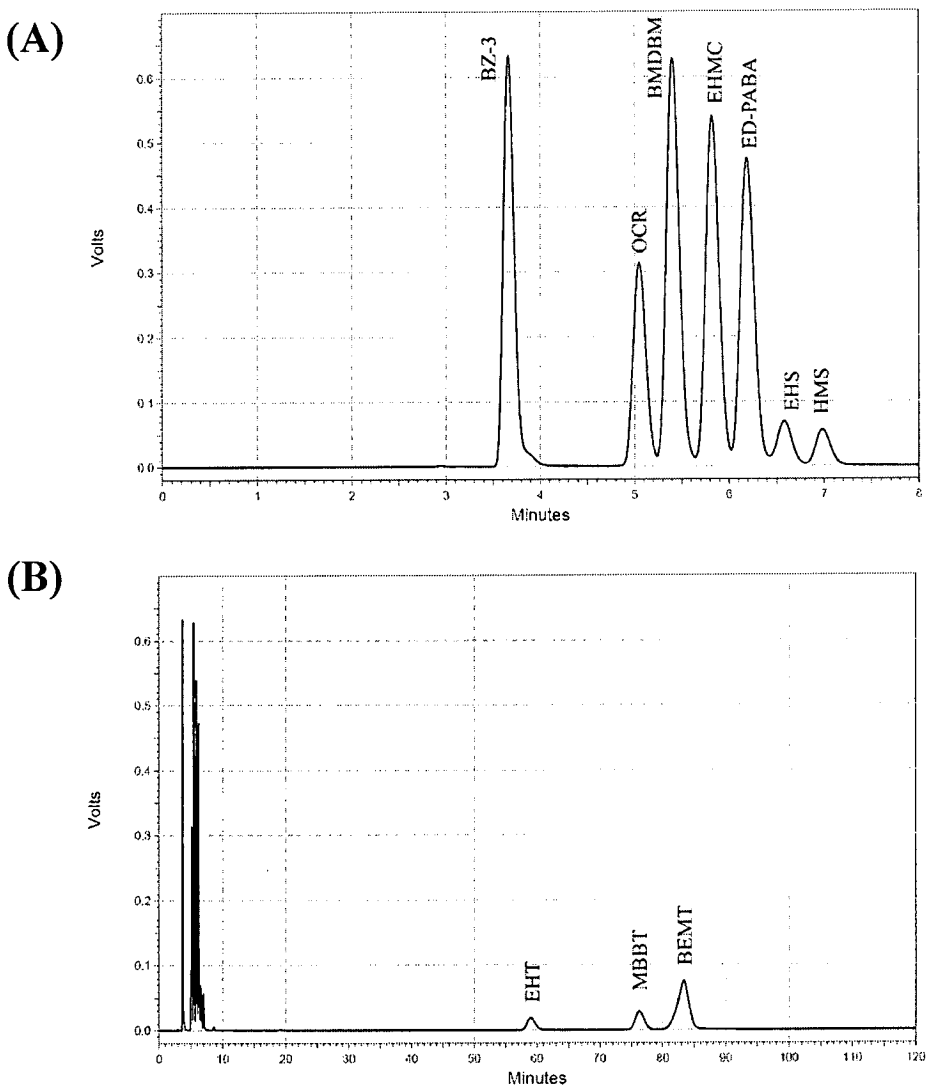


Figure 10 Chromatogram of ten UV filters detected at 325 nm. Chromatography was performed under isocratic mode of acetonitrile: ethyl acetate: water (95:5:2, v/v/v) at a flow rate 1.0 mL/min and an injection volume 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used with a temperature control at 25 °C. A: enlarged scale of the first 8 min and B: chromatograms of the whole assay.

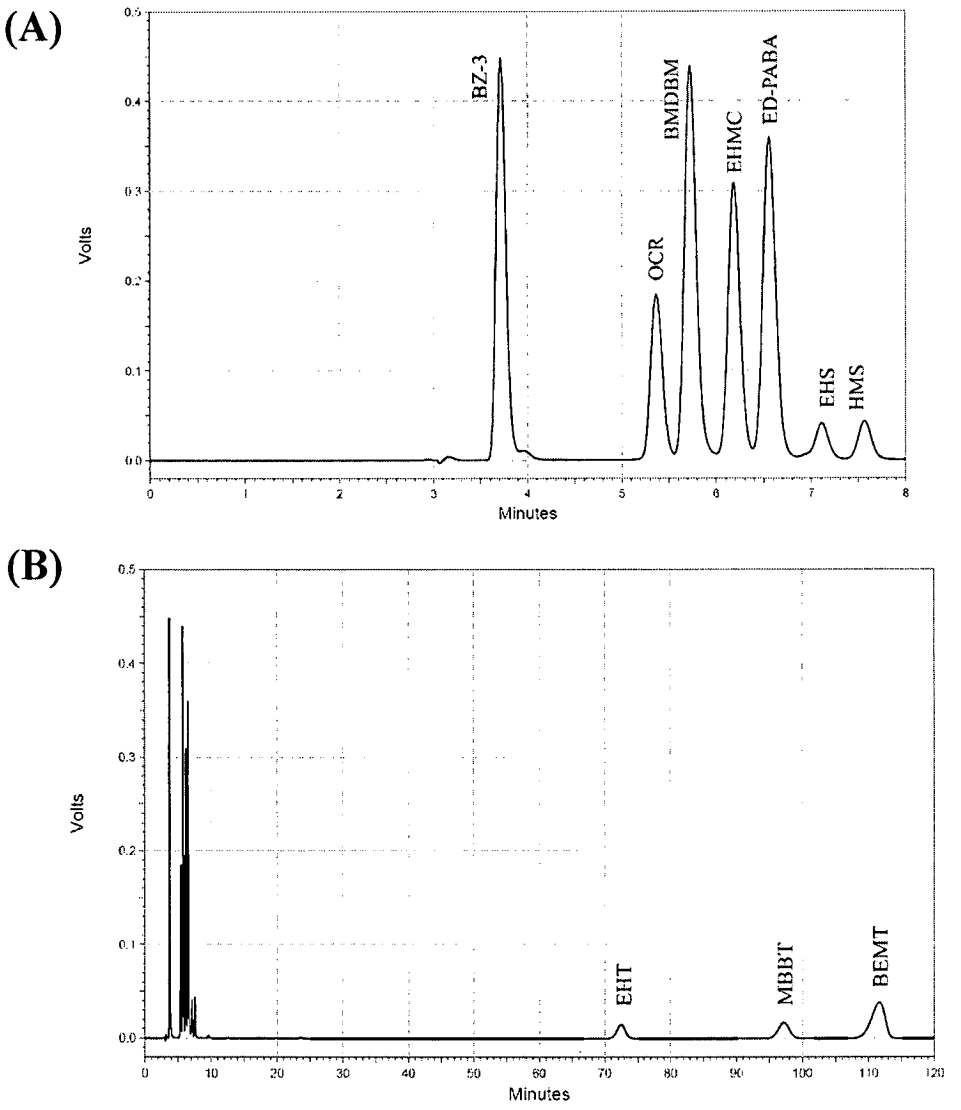


Figure 11 Chromatogram of ten UV filters detected at 325 nm. Chromatography was performed under isocratic mode of acetonitrile: ethyl acetate: water (95:5:4, v/v/v) at a flow rate 1.0 mL/min and an injection volume 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used with a temperature control at 25 °C. A: enlarged scale of the first 8 min and B: chromatograms of the whole assay.

Table 16 The retention time and resolution of ten UV filters using the mobile phase; acetonitrile: ethyl acetate: water at the ratio of 95:5:2 and 95:5:4 (v/v/v)

UV filters	Retention time: t_R (min)		Resolution: R_s	
	95:5:2	95:5:4	95:5:2	95:5:4
BZ-3	3.59	3.71	-	-
OCR	4.94	5.37	6.38	7.72
BMDBM	5.29	5.72	1.51	1.53
EHMC	5.69	6.19	1.76	1.95
ED-PABA	6.05	6.56	1.50	1.51
EHS	6.44	7.11	1.57	2.18
HMS	6.83	7.56	1.54	1.74
EHT	58.87	72.50	49.15	50.79
MBBT	76.37	97.78	7.97	9.16
BEMT	83.86	111.68	2.65	4.18

Effects of column temperature

After obtaining the optimum composition of mobile phase, the effects of column temperature were then investigated by varying column temperature at 25, 30 and 35 °C. The resolution value and the retention time for each UV filters were observed.

The chromatograms showing the effects of the column temperature are shown in Appendix A, The results showed that the total analysis time was decreased when the column temperature was increased. However, the resolution values of almost all UV filters were also decreased with an increasing of the column temperature (Table 17). As increasing column temperature, the viscosity of the mobile phase decreases resulting in the reduction of column back pressure. In addition, high temperature affects driving force of molecules. Molecules can move faster at higher temperature than at lower temperature. Thus, UV filters were eluted faster. However, an increase in temperature may cause overlapping of some peaks resulting in poor resolution. In this study, a column temperature controlled at 25°C provided a good separation with R_s values

greater than 1.5. As a result, the column temperature used in the analysis of UV filters was kept at 25°C.

Table 17 The effect of column temperature on the retention time and resolution of ten UV filters analysed using acetonitrile: ethyl acetate: water, 95:5:2 (v/v/v) as a mobile phase

UV filters	Retention time: t_R (min)			Resolution: R_s		
	25 °C	30 °C	35 °C	25 °C	30 °C	35 °C
BZ-3	3.59	3.58	3.58	-	-	-
OCR	4.94	4.90	4.84	6.38	6.23	6.19
BMDBM	5.29	5.24	5.17	1.51	1.48	1.47
EHMC	5.69	5.64	5.55	1.76	1.73	1.70
ED-PABA	6.05	5.99	5.89	1.50	1.46	1.44
EHS	6.44	6.37	6.27	1.57	1.57	1.53
HMS	6.83	6.76	6.64	1.54	1.52	1.48
EHT	58.87	54.72	49.90	49.15	49.27	46.75
MBBT	76.37	71.90	66.61	7.97	8.54	8.02
BEMT	83.86	78.07	71.34	2.65	2.42	2.25

Effects of mobile phase flow rate

Flow rate is one of the factors affecting the separation of analytes. Therefore, the effect of flow rate on the elution time for analytes and resolution value were also studied. Rate of flow was investigated at 1.0 and 1.5 mL/min. At the flow rate of 1.5 mL/min, UV filters were eluted faster than at 1.0 mL/min because of less interaction between substances and the stationary phase. Although increasing flow rate of mobile phase could reduce total analysis time, it also reduced the resolution of some UV filters. The R_s values of some analytes at flow rate 1.5 mL/min fell out of the acceptable range as shown in Table 18. The chromatograms showing the effects of the mobile phase flow rate are shown in Appendix B.

Table 18 Effects of flow rate on the retention time and resolution of ten UV filters analysed using acetonitrile: ethyl acetate: water, 95:5:2 (v/v/v) as a mobile phase.

UV filters	Retention time: t_R (min)		Resolution: R_s	
	1.0 mL/min	1.5 mL/min	1.0 mL/min	1.5 mL/min
BZ-3	3.59	2.55	-	-
OCR	4.94	3.68	6.38	7.19
BMDBM	5.29	3.93	1.51	1.44
EHMC	5.69	4.25	1.76	1.75
ED-PABA	6.05	4.50	1.50	1.37
EHS	6.44	4.88	1.57	1.94
HMS	6.83	5.18	1.54	1.60
EHT	58.87	49.59	49.15	44.79
MBBT	76.37	66.25	7.97	8.08
BEMT	83.86	76.57	2.65	3.82

In conclusion, ten UV filters was simultaneously separated on a C18 reverse phase column under an isocratic elution. The factors including composition of the mobile phase, column temperature and flow rate were investigated for a separation's efficiency. Finally, the mobile phase composed of acetonitrile: ethyl acetate: water at the ratio (95:5:2, v/v/v) was employed for a simultaneous determination of ten UV filters in a single analysis with the flow rate of 1.0 mL/min. The separation was performed at column temperature of 25 °C. A total time spent for a single analysis was 85 min. However, the total run time of the developed isocratic analysis is quite long and may not suitable for bulk product in routine analysis. A gradient elution allowing the change of mobile phase's compositions during the chromatographic run was therefore gained interesting. Theoretically, this mode is suitable for a separation of substances which have significant difference in polarity properties. Hence, a gradient elution mode was subsequently investigated in order to reduce the total analysis time and enhance the separation efficiency of the analysis of UV filters.

2.2 Gradient elution mode

In the isocratic elution mode, the HPLC analysis was performed using constant mobile phase composition. In this section, the mobile phase composition in the gradient elution mode was altering during the analytical run. The initial mobile phase's composition of the gradient system was started using the optimal composition obtained from isocratic mode. The results obtained from the studies of isocratic elution mode showed that the analyzed UV filters can be divided into two groups. The first group refers to polar UV filters that are BZ-3, OCR, BMDBM, EHMC, ED-PABA, EHS and HMS. The second group refers to EHT, MBBT and BEMT which are hydrophobic compounds. The optimized mobile phase from isocratic elution mode comprising of acetonitrile: ethyl acetate: water (95:5:2 v/v/v) was considered for a separation of polar UV filters. However, the total ratio of mobile phase's composition must equal to 100%, so the ratio of the mobile phase was adjusted from 95:5:2 to 92:4:4 (v/v/v), accordingly. For the hydrophobic group, the baseline separation of three hydrophobic UV filters could be achieved by employing the mobile phase containing acetonitrile: ethylacetate (95:5, v/v) in isocratic elution. Thus in the development of gradient HPLC method, these compositions were first applied (System 1, Table 19). The mobile phase of acetonitrile: ethyl acetate: water at ratio 92:4:4 (v/v/v) was used from time 0 min to 8 min of the frist gradient step. Then, the mobile phase was change to acetonitrile: ethylacetate at ratio 95:5 (v/v) during 8.1 min to 15 min. After that (15.1 min to 20 min), the solvent was adjusted to the same composition and ratio as in the initial gradient step to equilibrate the system for next injections. Fortunately, this gradient system could reduce the retention time of EHT from 58.87 min to 11.80 min. The retention time of MBBT and BEMT which were observed in isocratic analysis at 76.37 min and 83.86 min, respectively, were reduced to 12.90 min in gradient elution. However, the co-elution of MBBT and BEMT peaks was obtained (Figure 12). Although the mobile phase of acetonitrile and ethyl acetate (95:5, v/v) was able to separate three hydrophobic filters in isocratic elution mode, it was not satisfied in gradient HPLC method. It is hypothesized that the elution strength of the mobile phase comprising of acetonitrile: ethyl acetate (95:5, v/v) may be too high, resulting in the partition of MBBT and BEMT from the C18 reverse-phase station phase to mobile phase. Thus MBBT and BEMT were co-eluted. Therefore, methanol was selected to replace ethyl acetate (System 2,

Figure 13). Unfortunately, the separation of MBBT and BEMT could still not acheive. In this case, the use of 95% (V/V) of the acetonitrile may lead to a high elution strength of the mobile phase and result in a co-elution of two hydrophobic compounds (MBBT and BEMT). Consequently, during 8.1 min to 15 min, the ratio of acetonitrile in the mobile phase was decreased while the amount of either ethyl acetate and methanol was accordingly increased from 5% to 50% (System 3 and 4, Figure 14 and 15). System 3 could still not be able to separate MBBT and BEMT (Figure 14). Separation of the two peaks could be observed when System 4 was applied (Figure 15). Comparing between ethyl acetate and methanol at the same concentration, the ethyl acetate has higher elution strength than methanol. The excessive powerful may force the hydrophobic substance to elute faster and resulting in co-elution. Whereas the methanol has a dispersive specific characteristic with the stationary phase and substances providing a selectivity in elution by the function of dipole moment and hydrogen bonding (Ermer and Miller, 2005). Using system 4, the resolution of most peaks was found to be greater than 1.5 except the resolution between peak of OCR and BMDBM was found to be 1.27 which was not in the acceptable range ($Rs \geq 1.5$) (Table 20). From the results of isocratic elution, the adjustment of water ratio could improve the resolution of hydrophilic UV filters. Therefore, the ratio of water was further adjusted (System 5) in order to improve the resolution of OCR and BMDBM peaks (Figure 16). It was observed that the resolution of some peaks were increased whereas some peaks were decreased (Table 20). However, the resolution of all peaks reached the acceptable range.

In summary, the baseline separation ($R_s \geq 1.5$) as well as a shorter analysis time of ten UV filters could be acheived by employing the developed gradient system. System 5, the solution containing acetonitrile: ethyl acetate: water (94:4:2, v/v/v) was used as the gradient mobile phase for polar UV filters and the mobile phase consisted of acetonitrile: methanol (50:50, v/v) was applied for hydrophobic UV filters. System 5 was the optimized condition for simulteneous determination of ten UV filters. The identification of each peak of UV filters was confirmed by spiking known reference UV filters in double amount into a mixed stock solution of ten UV filters (which has a final concentration of 1 mg/mL of each UV filter). The results are shown in Figure 17-26. All peaks of UV filters have successfully been identified. Therefore, sytem 5 was further validated for linearity, accuracy, precision, specificity, LOD and LOQ.

Table 19 The gradient time table and the ratio of the organic solvent of developed system

System	Time (min)	Acetonitrile (%)	Ethyl acetate (%)	Water (%)	Methanol (%)
1	0-8	92	4	4	-
	8.1-15	95	5	-	-
	15.1-20	92	4	4	-
2	0-8	92	4	4	-
	8.1-15	95	-	-	5
	15.1-20	92	4	4	-
3	0-8	92	4	4	-
	8.1-15	50	50	-	-
	15.1-20	92	4	4	-
4	0-8	92	4	4	-
	8.1-15	50	-	-	50
	15.1-20	92	4	4	-
5	0-8	94	4	2	-
	8.1-15	50	-	-	50
	15.1-20	94	4	2	-

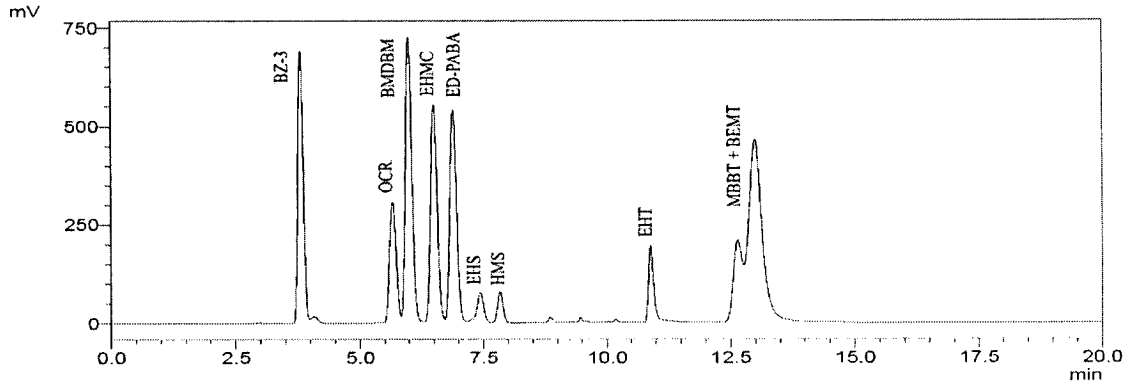


Figure 12 Chromatogram of a mixture UV filters detected at 325 nm. Chromatography was performed under a gradient elution mode complying with the continuous change of acetonitrile, ethyl acetate, and water (system 1) at a flow rate of 1.0 mL/min, and an injection volume of 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used and kept at 25 °C.

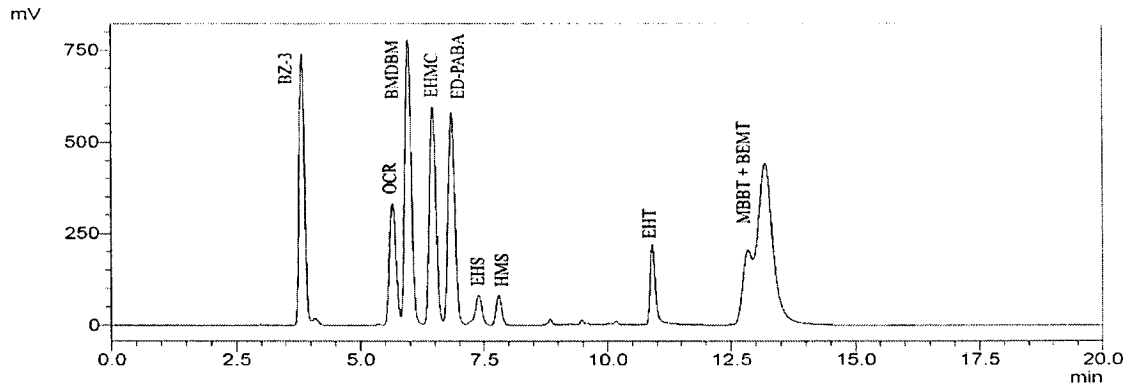


Figure 13 Chromatogram of a mixture UV filters detected at 325 nm. Chromatography was performed under a gradient elution mode complying with the continuous change of acetonitrile, ethyl acetate, water, and methanol (system 2) at a flow rate of 1.0 mL/min, and an injection volume of 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used and kept at 25 °C.

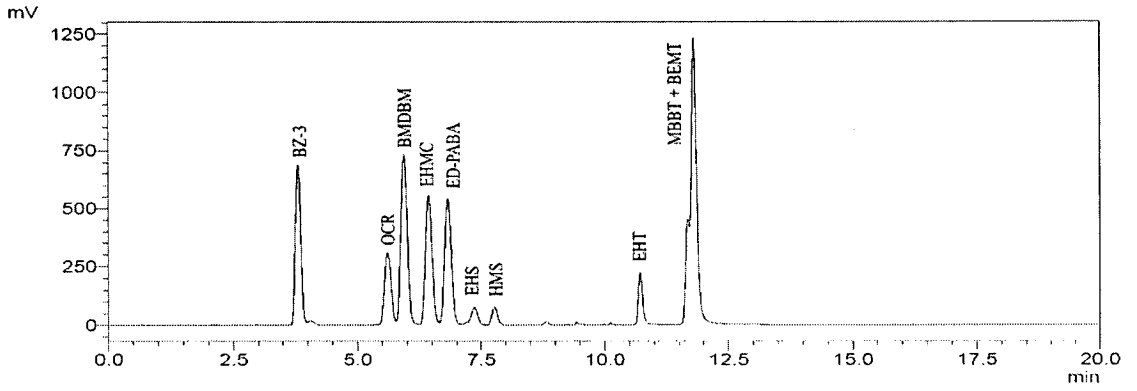


Figure 14 Chromatogram of a mixture UV filters detected at 325 nm. Chromatography was performed under a gradient elution mode complying with the continuous change of acetonitrile, ethyl acetate, and water (system 3) at a flow rate of 1.0 mL/min, and an injection volume of 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used and kept at 25 °C.

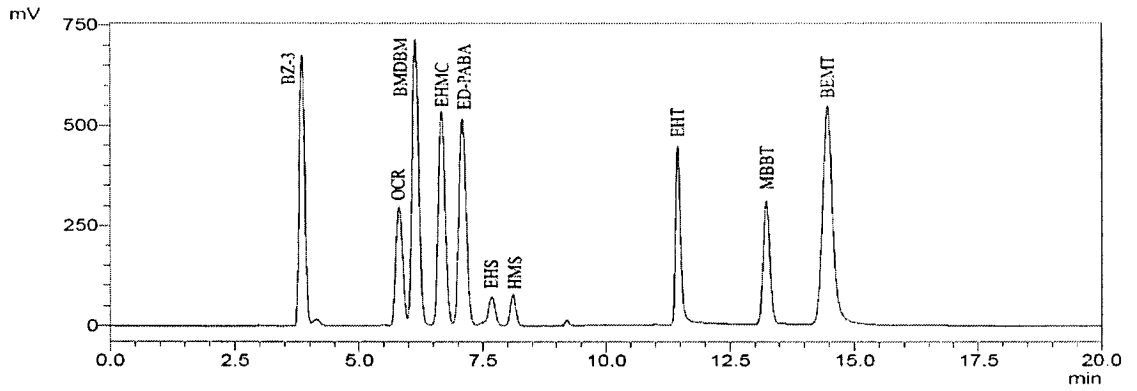


Figure 15 Chromatogram of a mixture UV filters detected at 325 nm. Chromatography was performed under a gradient elution mode complying with the continuous change of acetonitrile, ethyl acetate, water, and methanol (system 4) at a flow rate of 1.0 mL/min, and an injection volume of 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used and kept at 25 °C.

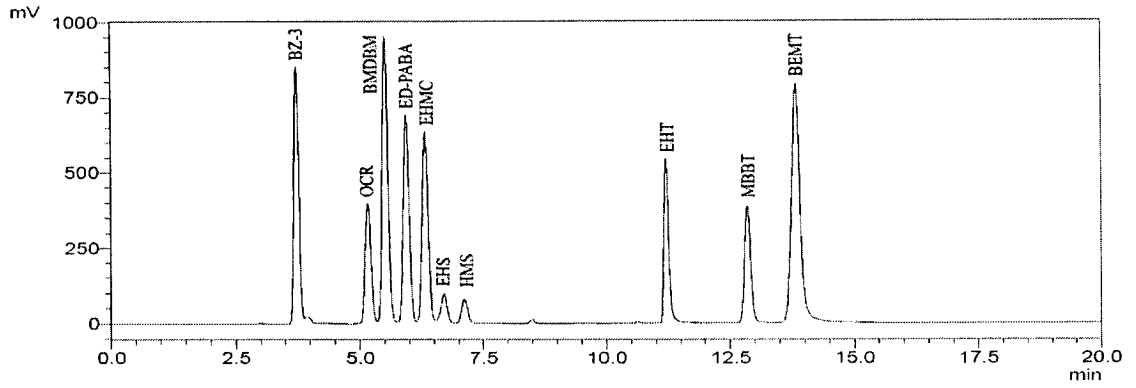


Figure 16 Chromatogram of a mixture UV filters detected at 325 nm. Chromatography was performed under a gradient elution mode complying with the continuous change of acetonitrile, ethyl acetate, water, and methanol (system 5) at a flow rate of 1.0 mL/min, and an injection volume of 20 µL. The reversed phase column C18 (250 x 4.6 mm, 5 µm) was used and kept at 25 °C.

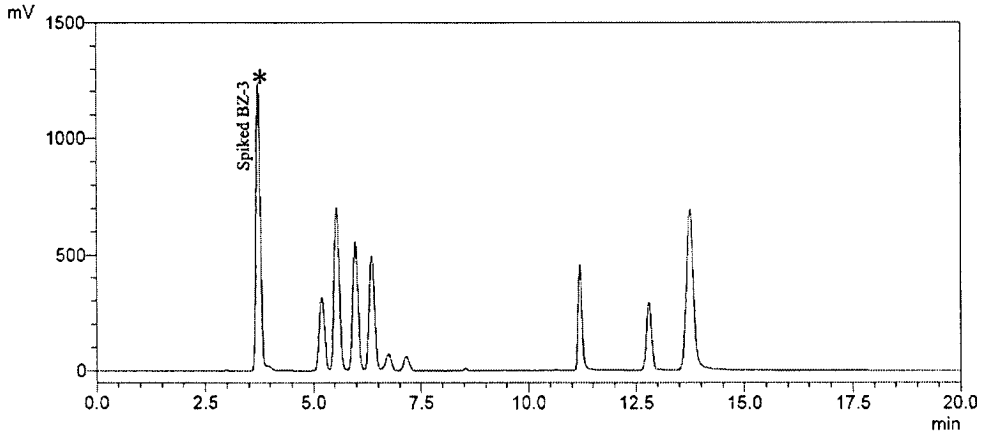


Figure 17 Chromatogram of the spiked standard BZ-3

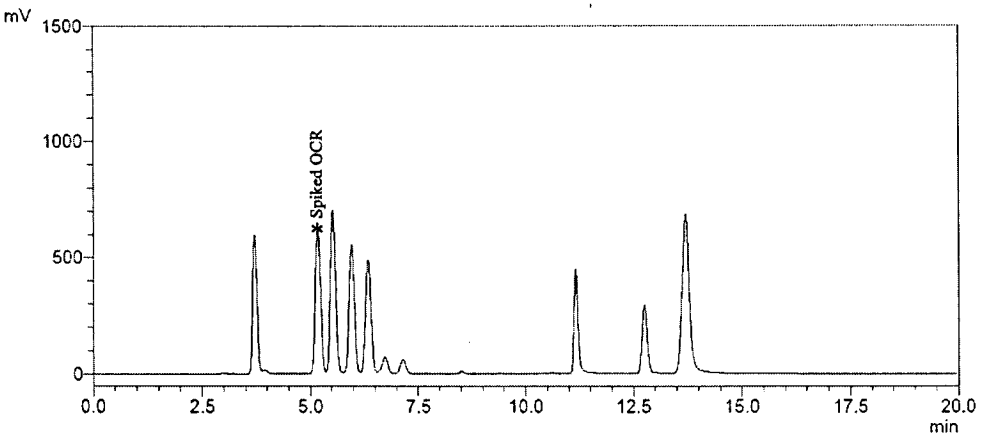


Figure 18 Chromatogram of the spiked standard OCR

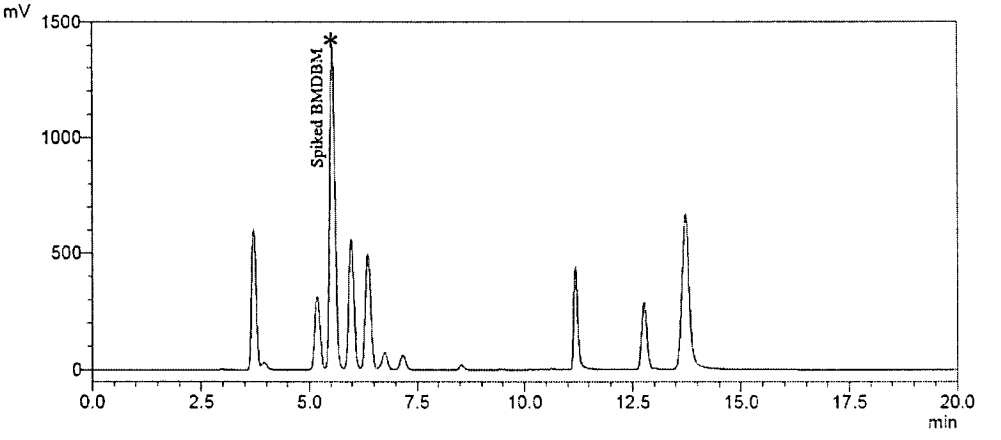


Figure 19 Chromatogram of the spiked standard BMDBM

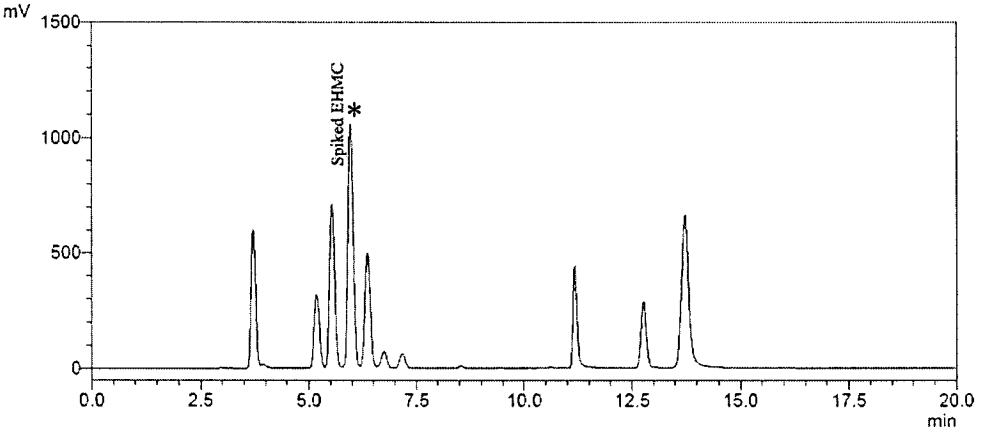


Figure 20 Chromatogram of the spiked standard EHMC

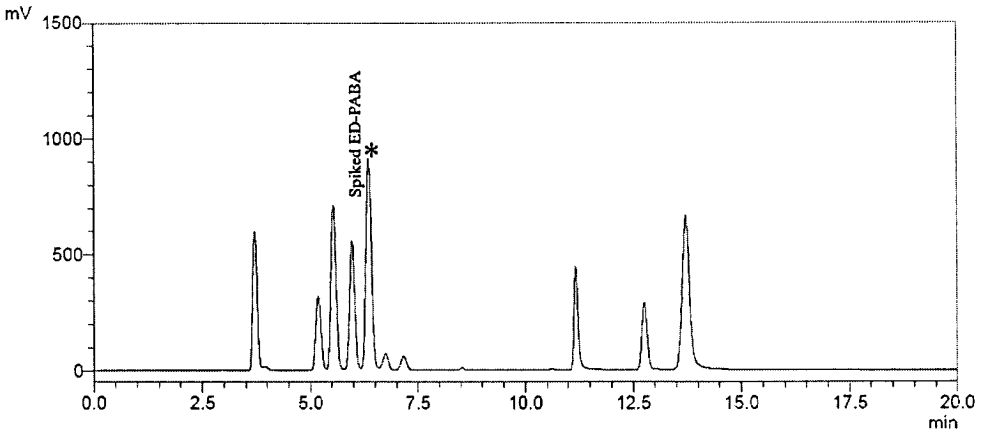


Figure 21 Chromatogram of the spiked standard ED-PABA

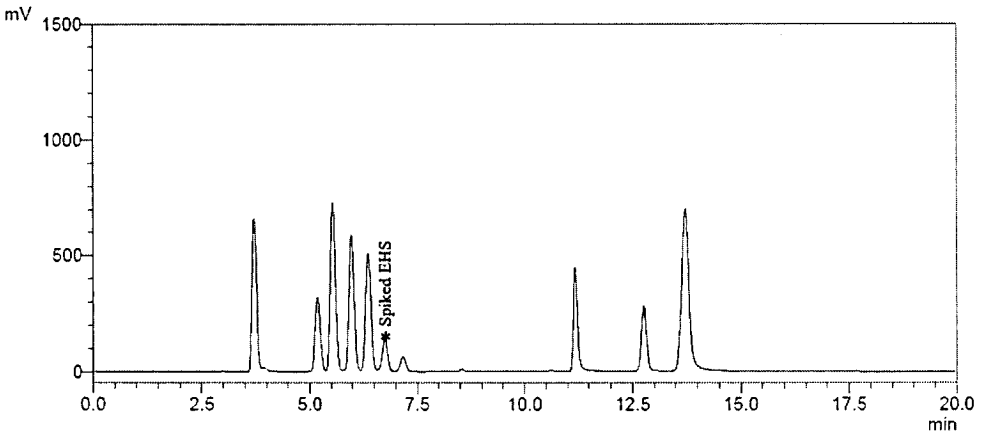


Figure 22 Chromatogram of the spiked standard EHS

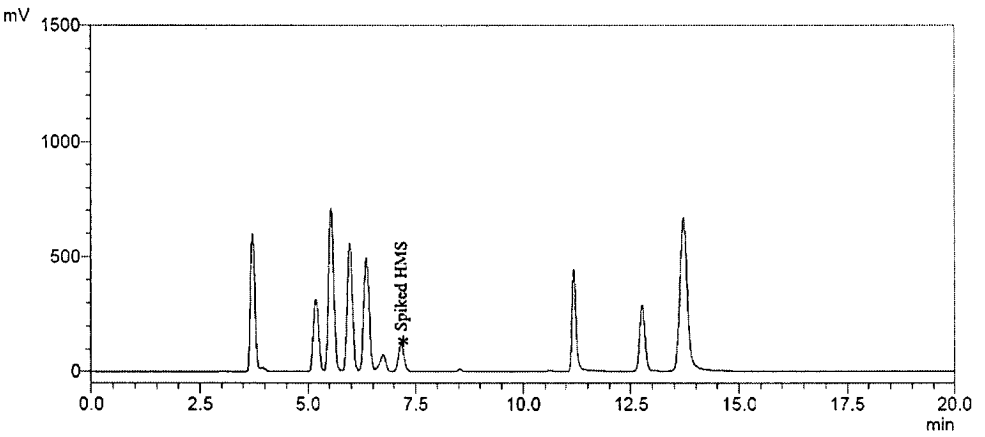


Figure 23 Chromatogram of the spiked standard HMS

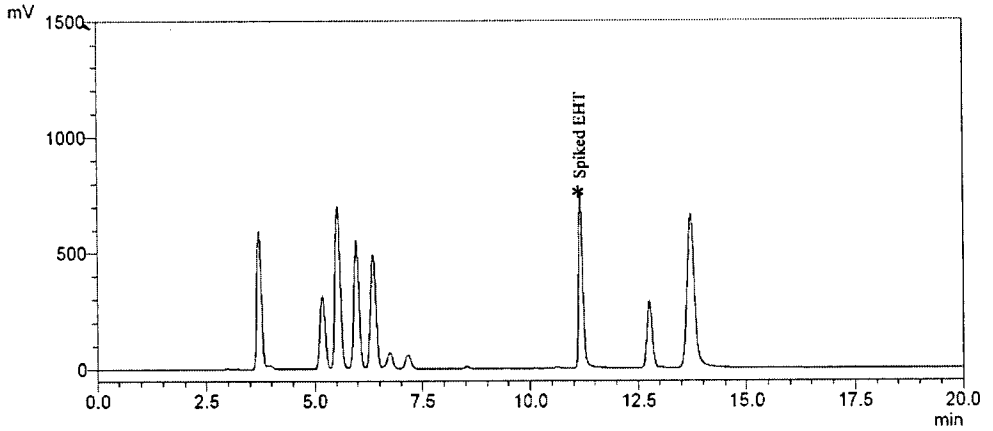


Figure 24 Chromatogram of the spiked standard EHT

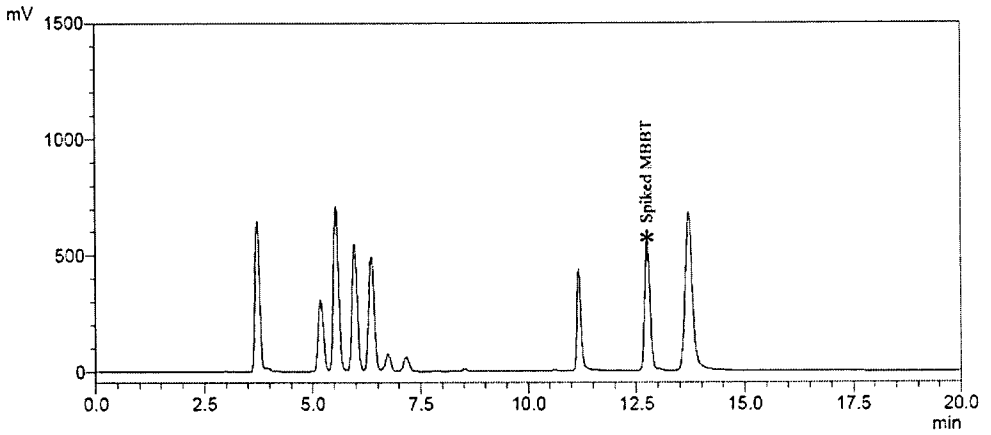


Figure 25 Chromatogram of the spiked standard MBBT

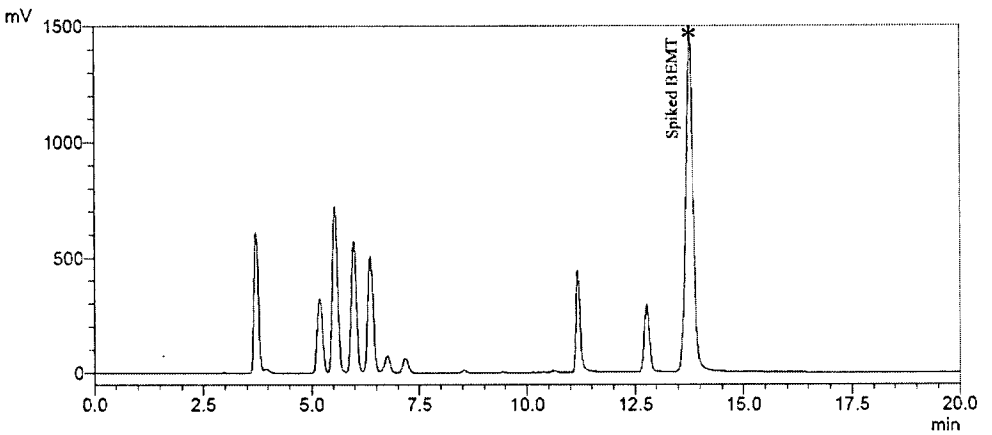


Figure 26 Chromatogram of the spiked standard BEMT

Table 20 The retention time and resolution of the system 4 and 5

UV filters	Retention time: t_R (min)		Resolution: R_s	
	System 4	System 5	System 4	System 5
BZ-3	3.84	3.73	0.00	0.00
OCR	5.80	5.20	8.25	6.98
BMDBM	6.13	5.55	1.27	1.55
EHMC	6.66	5.98	2.04	1.92
ED-PABA	7.09	6.37	1.55	1.66
EHS	7.68	6.76	2.20	1.64
HMS	8.11	7.18	1.78	1.75
EHT	11.45	11.17	16.26	18.91
MBBT	13.23	12.74	8.02	7.65
BEMT	14.46	13.64	3.98	3.43

3. Method validation

The validation of analytical method was aimed to demonstrate the suitability of the analytical procedure for its intended purpose (International Conference on Harmonisation, 2013). The validation was performed under the optimized HPLC analysis of gradient elution at wavelength 325 nm and the flow rate 1.0 mL/min. The compositions of mobile phase as described in system 5 (Table 19) was validated. The parameters including linearity of calibration curve, accuracy, precision, specificity, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated.

3.1 Linearity of calibration curve

Linearity of calibration curve was performed with six concentrations ranging from 5 to 100 µg/mL. The relationship between concentration (x-axis) and response peak areas (y-axis) were observed and showed in linear (Figure 27-36). The correlation coefficient (r) which is the strength of the degree of correlation between the y and x values and can be varies between -1 to 1. The closer to 1 mean the stronger the correlation between x and y. The calibration of all UV filters had the correlation coefficient higher than 0.999 (Table 21). The statistical significance of the regression models of all UV filters performed using ANOVA *F*-test at 95% confidence level are

shown in Appendix C. The results could be concluded that there were significant linear relationships between peak area and concentrations for all UV filters.

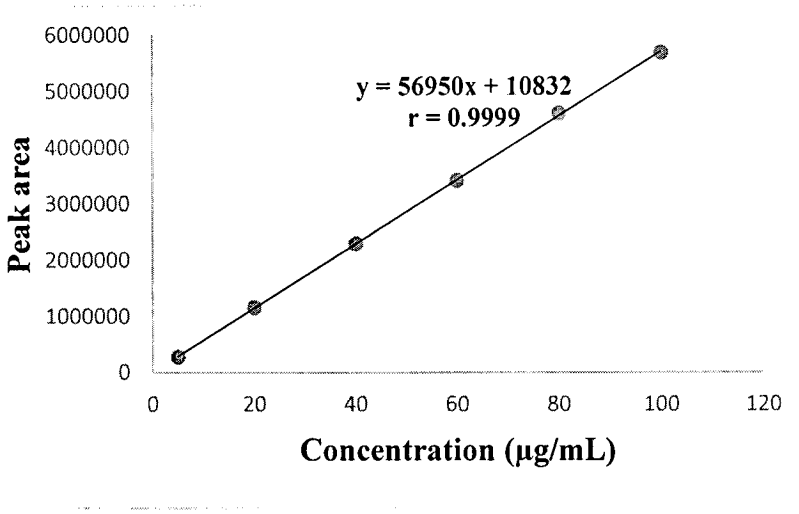


Figure 27 Calibration curve for BZ-3 analysed on reverse phase gradient HPLC using system 5 as mobile phase.

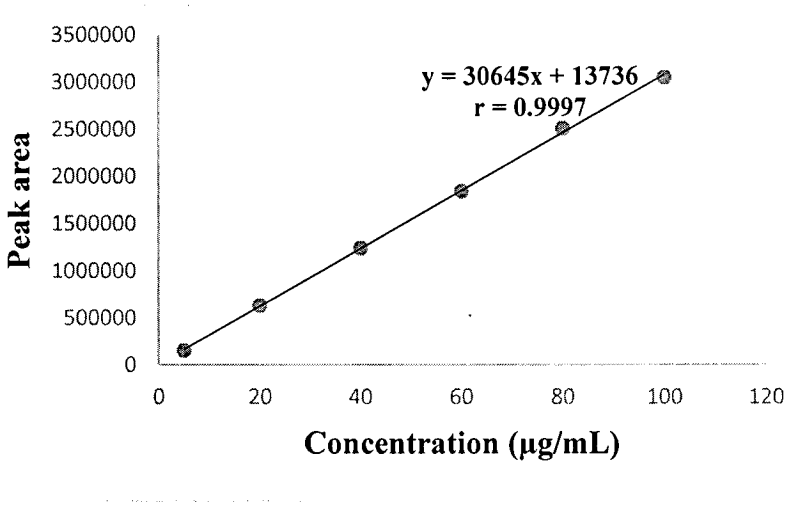


Figure 28 Calibration curve for OCR analysed on reverse phase gradient HPLC using system 5 as mobile phase.

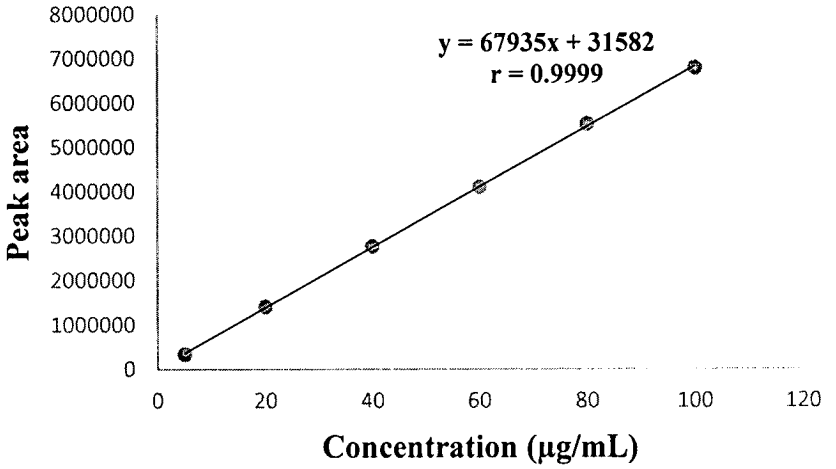


Figure 29 Calibration curve for BMDBM analysed on reverse phase gradient HPLC using system 5 as mobile phase.

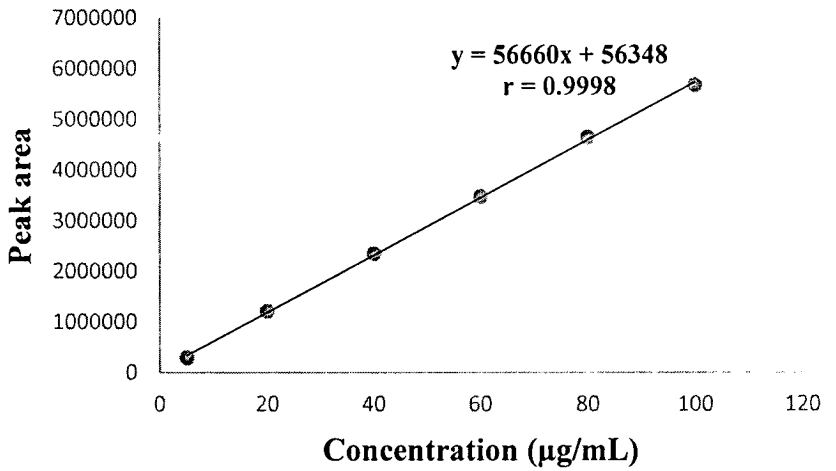


Figure 30 Calibration curve for EHMC analysed on reverse phase gradient HPLC using system 5 as mobile phase.

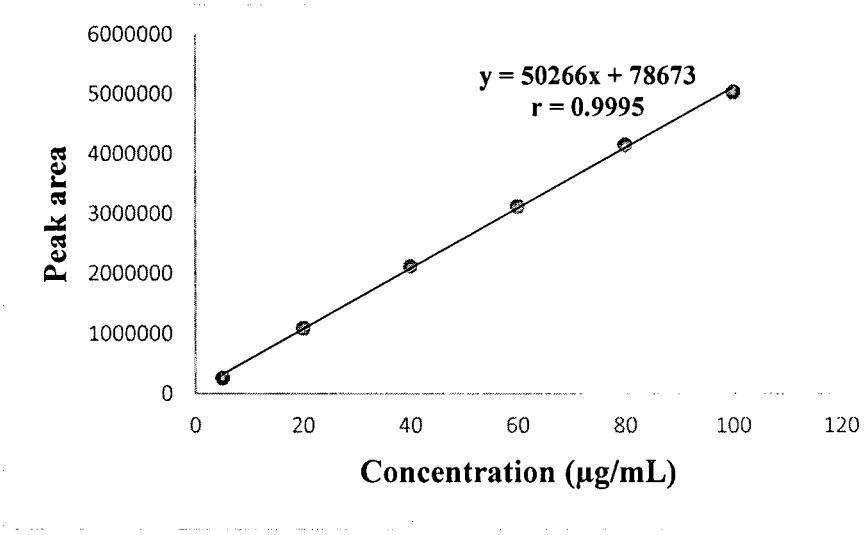


Figure 31 Calibration curve for ED-PABA analysed on reverse phase gradient HPLC using system 5 as mobile phase.

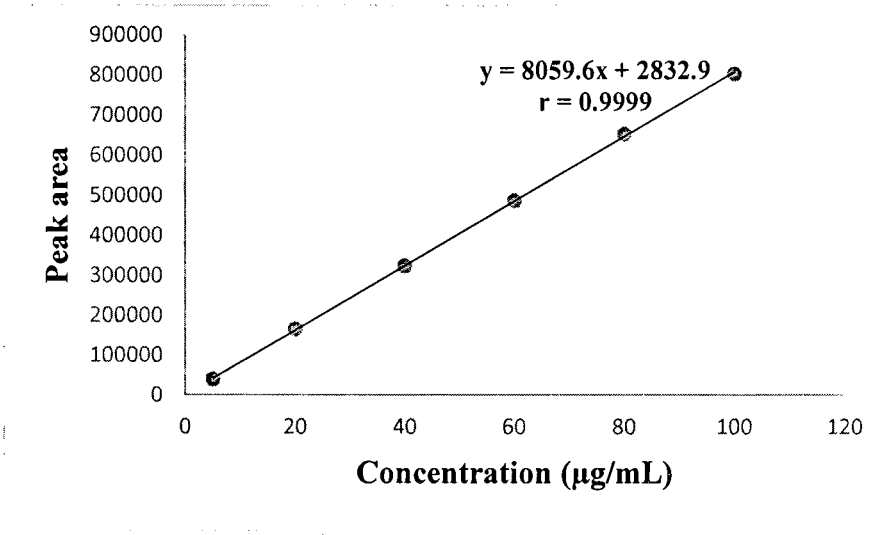


Figure 32 Calibration curve for EHS analysed on reverse phase gradient HPLC using system 5 as mobile phase.

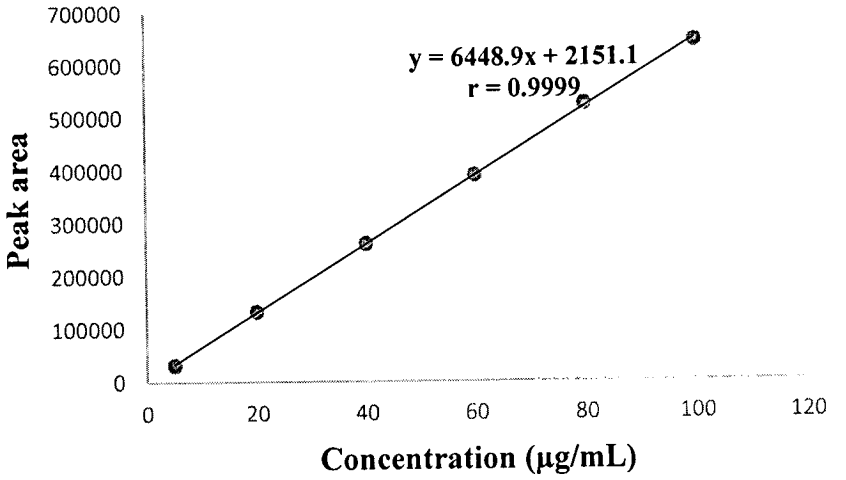


Figure 33 Calibration curve for HMS analysed on reverse phase gradient HPLC using system 5 as mobile phase.

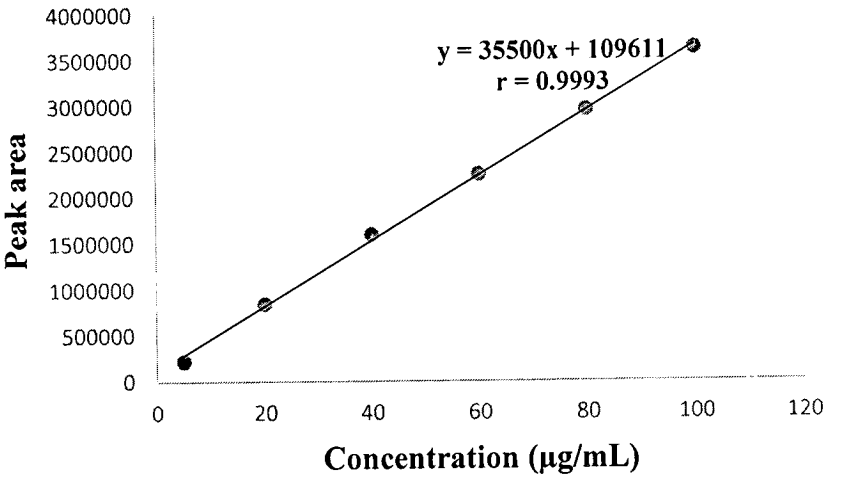


Figure 34 Calibration curve for EHT analysed on reverse phase gradient HPLC using system 5 as mobile phase.

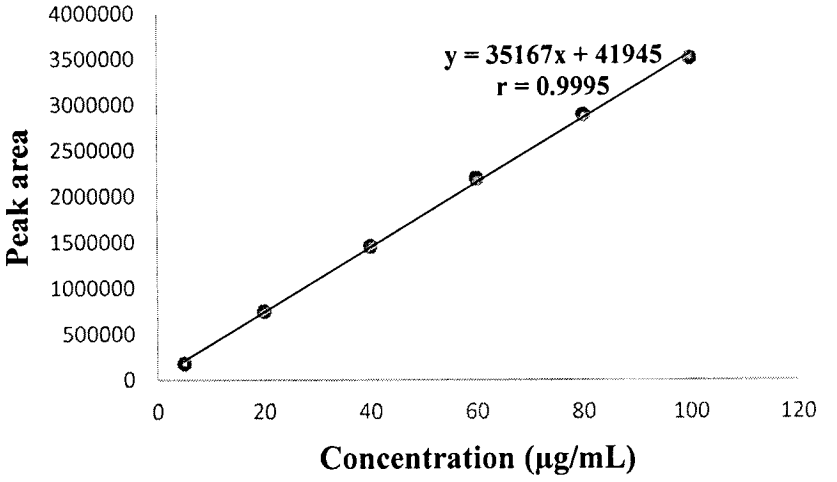


Figure 35 Calibration curve for MBBT analysed on reverse phase gradient HPLC using system 5 as mobile phase.

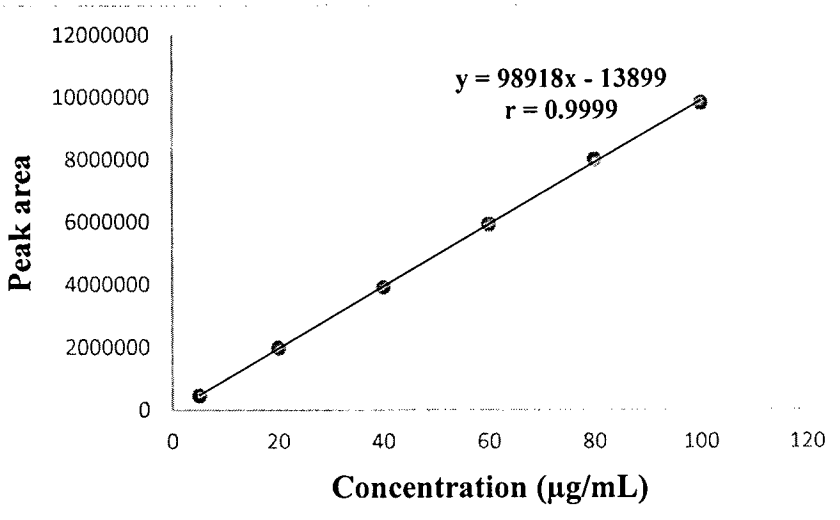


Figure 36 Calibration curve for BEMT analysed on reverse phase gradient HPLC using system 5 as mobile phase.

3.2 Accuracy

The accuracy of the method can be expressed in many ways. One of the most common ways is to calculate the percentage recovery. The accuracy is assessed by a triplicate of three different concentrations at the 10, 50 and 90 μg/mL (Table 22). The average of % recovery of all UV filters was in the acceptable range of 95-105% (The European Cosmetics Association, 2011) with RSD values less than 2.5%. Pocklington (1990) suggested that for a complex sample like cosmetics, the RSD value should not exceed 2.5%. Therefore, these results showed that the UV filters analysis could be accurate without prejudice under using the gradient HPLC method developed.

3.3 Precision

The repeatability and intermediate precision were performed by intra-day and inter-day in three different concentrations at 10, 50 and 90 μg/mL (Table 23). The variation of peak area of was expressed by coefficient of variation (CV). The %CV calculated from calculation of all UV filters was in the range of 0.18-1.94. Thus, the results showed that the gradient HPLC method developed was precisely for analyze all of UV filters. The variations within a laboratory and within different days was in acceptable range (CV < 5%) (Ermer and Miller, 2005).

3.4 Specificity

For the specificity, the three of sunscreen formulation bases which do not contain UV filters including S1, S2 and in-house base formulation were tested to observe the interference such as excipients and degradations in the sample matrix. The results showed the stable baseline which has no interferences in sample matrices of the base formulations (Figure 37-39).

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

In addition, LOD and LOQ were determined by the serial dilutions of UV filters stock solution to find the signal to noise ratio of 3:1 and 10:1 for LOD and LOQ, respectively. The observation found the lowest concentration range 5 μg/mL of standard curve was the minimum amount of analytes for all filters that could be quantitatively determined which the signal/noise equal to 10:1. Then, the 5 μg/mL was further diluted to various lower concentrations. The signal per noise ratio of 3:1 could be measured at approximately 0.05 μg/mL of all UV filters.

Table 21 Linearity of calibration curve of ten UV filters covering the range of concentration 5-100 µg/mL

UV filters	Linear equation	Correlation coefficient (r)	Slope	Intercept	Residual sum of squares
BZ-3	y = 56950x + 10832	0.9999	56950	10832	5.37×10^{10}
OCR	y = 30645x + 13736	0.9997	30645	13736	2.43×10^{10}
BMDBM	y = 67935x + 31582	0.9999	67935	31582	7.49×10^{10}
EHMC	y = 56660x + 56348	0.9998	56660	56348	6.69×10^{10}
ED-PABA	y = 50266x + 78673	0.9995	50266	78673	8.22×10^{10}
EHS	y = 8059.6x + 2832.9	0.9999	8059.6	2832.9	1.11×10^{9}
HMS	y = 6448.9x + 2151.1	0.9999	6448.9	2151.1	9.30×10^{8}
EHT	y = 35500x + 109611	0.9993	35500	109611	5.16×10^{10}
MBBT	y = 35167x + 41945	0.9995	35167	41945	2.63×10^{10}
BEMT	y = 98918x - 13899	0.9999	98918	-13899	1.87×10^{11}

Table 22 Accuracy of the quality control samples expressed as percentage recovery

UV filters	%Recovery (%RSD)		
	10 µg/mL	50 µg/mL	90 µg/mL
BZ-3	100.29 (1.84)	99.50 (0.43)	101.12 (1.76)
OCR	100.49 (2.03)	99.66 (0.50)	100.98 (1.79)
BMDBM	96.86 (1.69)	99.93 (0.54)	101.23 (1.69)
EHMC	97.17 (2.50)	100.62 (0.42)	99.69 (0.46)
ED-PABA	96.03 (2.25)	101.64 (0.48)	100.98 (1.64)
EHS	100.27 (1.45)	100.22 (0.46)	101.14 (1.77)
HMS	99.09 (1.80)	100.28 (0.50)	101.32 (1.78)
EHT	96.11 (0.61)	103.77 (1.18)	101.04 (1.79)
MBBT	96.54 (1.52)	102.04 (0.49)	101.57 (0.28)
BEMT	100.70 (1.90)	100.39 (0.48)	100.40 (0.19)

Table 23 The % Coefficient of variation of intra- and inter-day precision at the concentration of 10, 50 and 90 µg/mL

UV filters	%CV					
	10 µg/mL		50 µg/mL		90 µg/mL	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
BZ-3	1.83	1.70	0.43	1.56	1.81	1.54
OCR	1.91	1.92	0.54	1.64	1.72	1.56
BMDBM	1.65	1.17	0.57	1.60	1.63	1.34
EHMC	1.94	1.91	0.43	1.61	0.49	0.70
ED-PABA	0.86	1.93	0.49	1.59	1.62	1.44
EHS	1.33	1.54	0.45	1.51	1.71	1.58
HMS	1.03	1.93	0.51	1.54	1.70	1.56
EHT	0.52	1.94	1.20	1.87	1.74	1.49
MBBT	1.43	1.80	0.51	1.54	0.24	0.51
BEMT	0.71	1.02	0.48	1.41	0.18	1.30

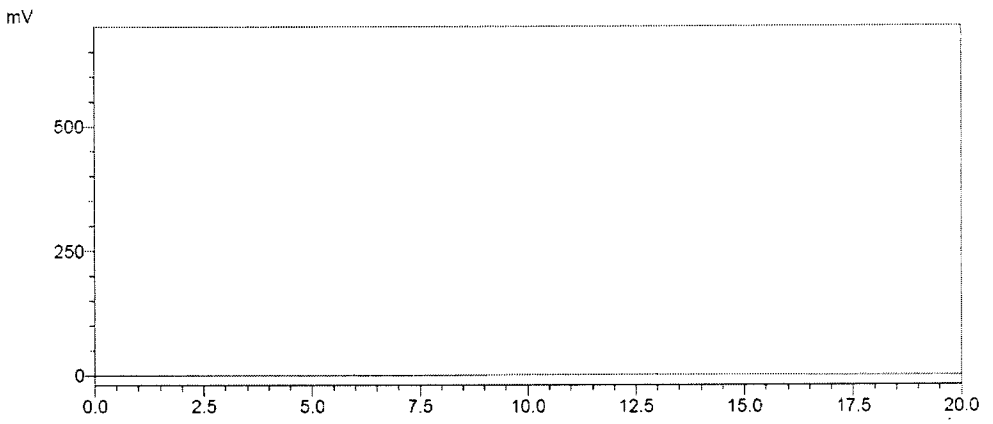


Figure 37 Chromatogram of base matrix of standard S1 sunscreen formulation dissolving in a mixture of acetonitrile and ethyl acetate (1:1)

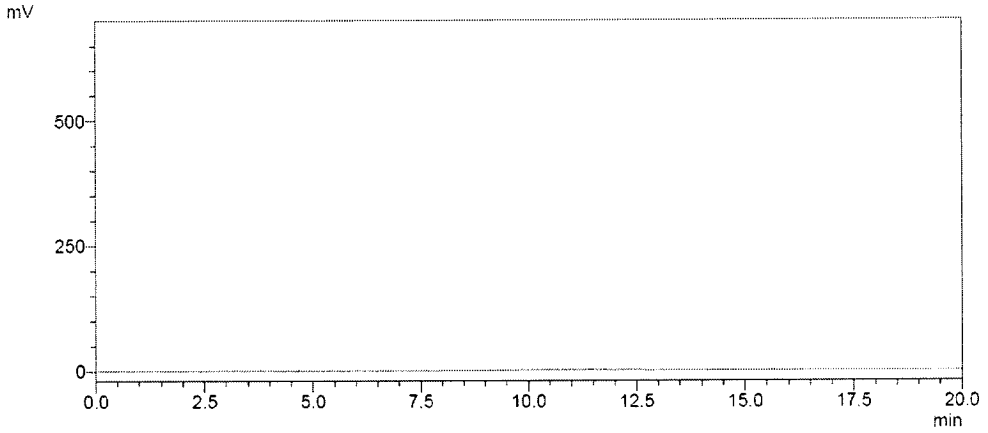


Figure 38 Chromatogram of base matrix of standard S2 sunscreen formulation dissolving in a mixture of acetonitrile and ethyl acetate (1:1)

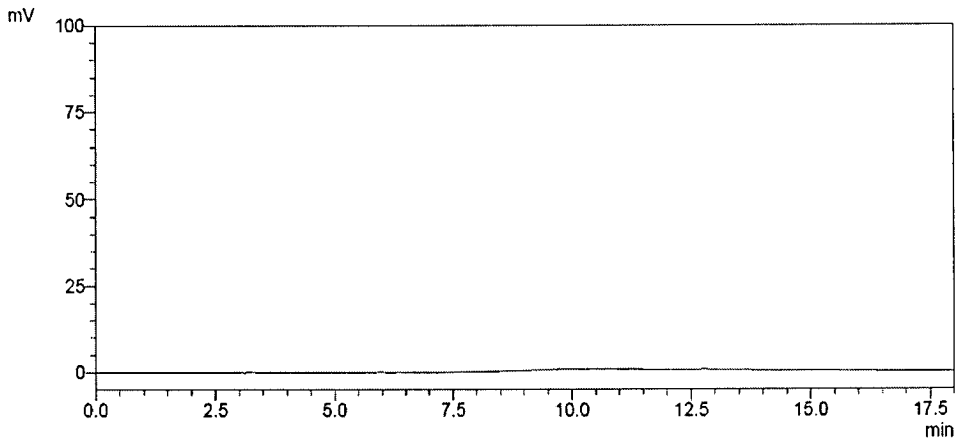


Figure 39 Chromatogram of base matrix of in-house sunscreen formulation dissolving in a mixture of acetonitrile and ethyl acetate (1:1)

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

The developed method was applied to analyze a various combination of UV filters in standard sunscreens, formulated sunscreens and commercial sunscreens. The samples was prepared and determined with three determinations (n=3).

Standard sunscreens

Initially, the standard sunscreen was examined to confirm the applicability of the gradient HPLC method developed. The reference formulations of S1 (SPF 5.1 ± 0.38) and S2 (SPF 16.6 ± 1.22) from US FDA and COLIPA were chosen to proof this

method. The results for quantitative analysis of S1 and S2 are tabulated in Table 24 and the chromatograms are shown in Figure 40 and 41. It was observed that, the chromatogram of standard S1 and S2 sunscreen formulation presented UV filters peak on the chromatogram correlated with the type of UV filters containing in standard sunscreen formulation. In addition, the percent found of UV filters were correlated with the % added in formulations. In this step, it was clearly shown that this method could correctly analyze the percentage of UV filters presented in standard sunscreen formulations.

Table 24 Analysis of standard formulations

Formulation	SPF (SD)	UV filters identified	%Add in formulation	% Found (RSD)
S1	4.27 (0.21)	HMS	8.00	7.90 (0.81)
S2	12.08 (1.05)	BZ-3	3.00	3.00 (0.54)
		ED-PABA	7.00	7.23 (0.15)

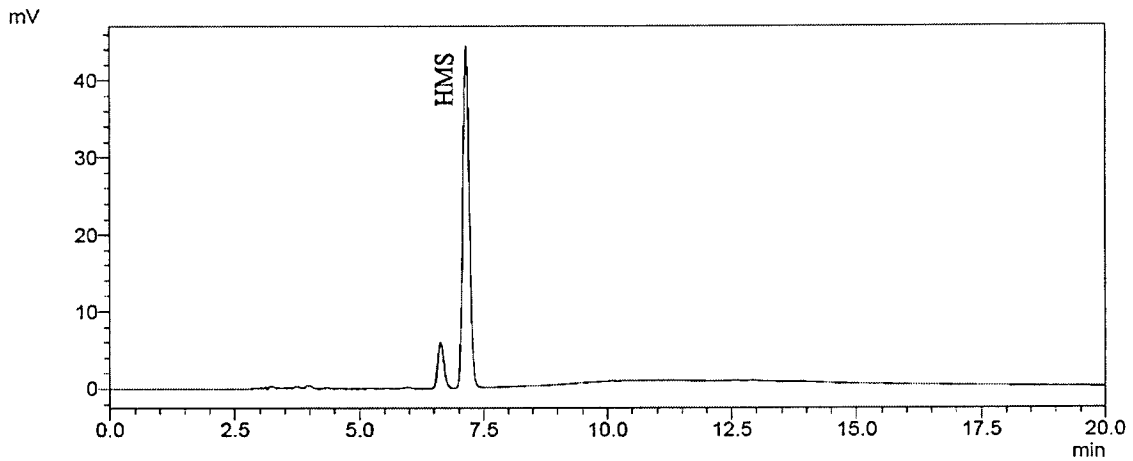


Figure 40 HPLC Chromatograms for standard S1 analysis.

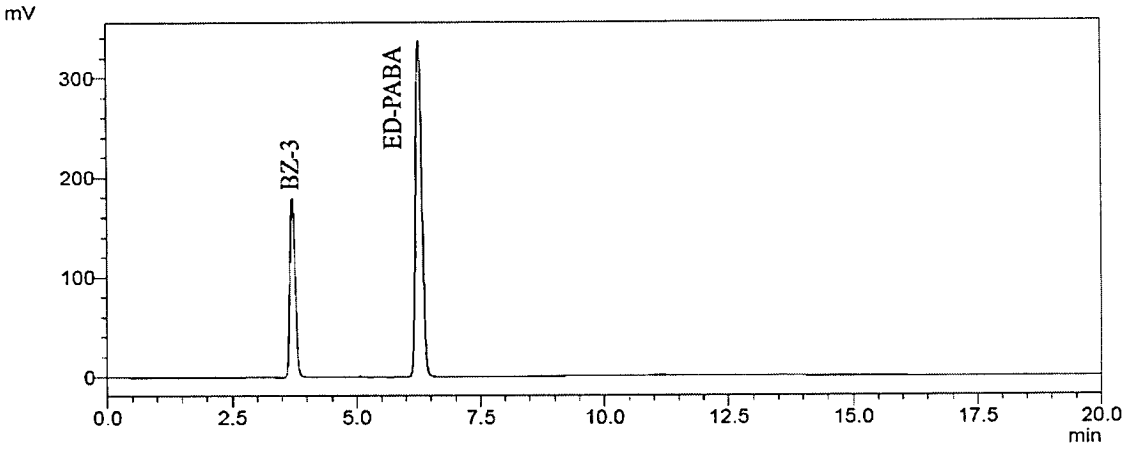


Figure 41 HPLC Chromatograms for standard S2 analysis.

Formulated sunscreens

Second step, applicability of the gradient HPLC method developed was tested using in-house sunscreen formulations. The sunscreen formulations prepared consisted of single and combination UV filters formulations. The formulations contained single UV filters were prepared at middle and high concentration levels. On the other hands, the formulations with combination of UV filters were prepared using various types and concentrations of UV filters which covered all ten UV filters analysis. The results showed that the amount of UV filters obtained from the HPLC analysis was correlated to the types of UV filters and percent added in the formulations (Table 25). Thus, the results could confirmed that the method was sufficient for the quantitation of single UV filters and combination of UV filters in formulation. The deviation of the results was shown to be within the acceptable range (RSD < 5%) (Ermer and Miller, 2005).

Table 25 Analysis of in-house formulated sunscreen formulations

Formulation	UV filters identified	%Add in formulation	% Found (RSD)
A1	BZ-3	5.00	4.81 (0.56)
A2	BZ-3	10.00	9.98 (1.59)
A3	OCR	5.00	5.44 (1.08)
A4	OCR	10.00	10.68 (0.76)
A5	BMDBM	2.50	2.44 (0.46)
A6	BMDBM	5.00	5.27 (0.28)
A7	EHMC	5.00	5.42 (0.24)
A8	EHMC	10.00	10.23 (0.32)
A9	ED-PABA	5.00	4.51 (0.22)
A10	ED-PABA	10.00	9.10 (0.66)
A11	EHS	2.50	2.39 (0.81)
A12	EHS	5.00	5.10 (1.42)
A13	HMS	5.00	5.04 (0.44)
A14	HMS	10.00	10.69 (0.32)
A15	EHT	2.50	2.40 (0.50)
A16	EHT	5.00	5.46 (0.51)
A17	MBBT	5.00	4.52 (0.22)
A18	MBBT	10.00	9.02 (1.07)
A19	BEMT	5.00	5.13 (0.26)
A20	BEMT	10.00	9.08 (0.61)
A21	BZ-3	4.00	3.89 (0.35)
	OCR	5.00	5.03 (0.37)
	EHMC	7.00	7.30 (0.44)
A22	BZ-3	5.00	5.04 (0.93)
	EHMC	7.50	7.61 (0.66)
	EHS	5.00	4.86 (0.55)

Table 25 (Cont.)

Formulation	UV filters identified	%Add in formulation	% Found (RSD)
A23	BMDBM	1.00	0.95 (1.25)
	EHMC	7.50	7.42 (2.09)
	BEMT	3.00	2.91 (2.10)
A24	BMDBM	2.00	2.08 (0.82)
	EHMC	7.50	7.81 (0.81)
	BEMT	5.00	5.21 (0.85)
A25	BZ-3	2.00	2.09 (0.22)
	EHMC	7.50	7.84 (0.19)
	EHS	5.00	5.29 (0.29)
	HMS	5.00	5.49 (0.25)
A26	BMDBM	5.00	4.95 (2.82)
	EHMC	7.50	7.43 (1.29)
	EHS	5.00	4.83 (0. 61)
	MBBT	10.00	10.24 (2.50)
A27	BZ-3	2.00	2.07 (0.59)
	EHMC	1.80	1.86 (0.30)
	BMDBM	2.00	2.01 (0.35)
	EHS	5.00	5.45 (0.10)
	HMS	9.50	9.98 (0.16)

Commercial sunscreens

Commercial sunscreen products (13 samples) were obtained as a gift from manufacturers as well as were purchased from local hypermarkets. The information of commercial sunscreens used in this study indicating ingredients and manufacturing date is shown in Appendix D. Eleven samples have SPF between 30 to 50^{+} which are complied with new regulation of Thai FDA (became effective in September 2013) which limited the maximum SPF at 50+ (Table 26). However, there were two products have SPF of 60 and 110. Protection grade of UVA (PA) for all commercial sunscreen

products obtained were +++ and ++++. Numbers of UV filters found in these products were between 1 to 5 different UV filters per product. Type of the UV filters found in these products covered all ten UV filters which were aimed to develop an analytical method in this thesis.

In the analysis of commercial sunscreens, the amount of each UV filters was unknown. Thus, the analysis was aimed to find out whether the amount of detected UV filters was complied with Thai FDA or not? In addition, the type of UV filter as stated on the products' label was also observed. It was found that the type of UV filters detected using the gradient HPLC method developed was conform With the UV filters listed on the product labels. In addition, the amount of UV filters detected in all products was within the permitted authorized levels regulated by Thai FDA (Table 26). Moreover, the interferences by any others materials in the base formulations such as emulsifier (e.g. glyceryl monostearate), oil (e.g. isopropyl palmitate, jojoba oil), polymer (e.g. acrylates/C10-30 alkyl acrylate crosspolymer) preservative (e.g. methyl paraben, propyl paraben, phenoxyethanol), additive (e.g. vitamin C, vitamin E, Niacinamide) were not observed. On the top of that, physical UV filters includes titanium dioxide and zinc oxide presenting in the commercial sunscreen products were also not interfered the analysis.

Table 26 Analysis of commercial sunscreens

Formulation	Protection efficacy on labeling	UV filters identified	% Found (RSD)	% Allowed in Thailand
C1	SPF 40, PA+++	EHMC	3.40 (2.22)	10
C2	SPF 40, PA+++	EHMC	4.89 (0.34)	10
C3	SPF 60, PA++++	OCR	9.92 (0.98)	10
		BMDBM	3.00 (0.40)	5
C4	SPF 30, PA+++	EHMC	8.25 (1.33)	10
		MBBT	7.31 (0.81)	10

Table 26 (Cont.)

Formulation	Protection efficacy on labeling	UV filters identified	% Found (RSD)	% Allowed in Thailand
C5	SPF 110, PA+++	BZ-3	3.43 (0.21)	10
		OCR	7.99 (0.16)	10
		BMDBM	2.50 (0.22)	5
C6	SPF 50, PA+++	BZ-3	3.13 (2.04)	10
		EHMC	7.63 (2.02)	10
		EHS	4.47 (2.20)	5
C7	SPF 50+, UVB &	OCR	8.82 (0.26)	10
	UVA protection	BMDBM	4.53 (0.78)	5
		BEMT	2.90 (0.73)	10
C8	SPF 40, UVB &	BZ-3	4.60 (2.29)	10
	UVA protection	EHMC	6.63 (2.38)	10
		EHS	4.70 (2.33)	5
		HMS	6.89 (2.15)	10
C9	SPF 50, PA+++	OCR	2.71 (1.85)	10
		BMDBM	3.82 (1.85)	5
		EHMC	6.43 (1.85)	10
		HMS	6.55 (1.86)	10
C10	SPF 50, PA+++	OCR	4.04 (0.30)	10
		BMDBM	4.20 (0.37)	5
		EHMC	0.96 (0.85)	10
		MBBT	3.08 (1.91)	10
C11	SPF 30, PA++++	BMDBM	4.57 (0.41)	5
		EHMC	8.62 (0.30)	10
		EHT	1.96 (2.12)	5
		BEMT	2.39 (0.13)	10

Table 26 (Cont.)

Formulation	Protection efficacy on labeling	UV filters identified	% Found (RSD)	% Allowed in Thailand
C12	SPF 50+, UVB &	OCR	8.42 (2.83)	10
	UVA protection	BMDBM	4.25 (3.54)	5
		EHS	4.15 (3.90)	5
		HMS	4.03 (3.02)	10
		BEMT	2.77 (3.45)	10
C13	SPF 50, UVB &	OCR	8.77 (1.05)	10
	UVA protection	BMDBM	4.56 (0.98)	5
		EHS	5.25 (3.88)	5
		HMS	8.43 (1.11)	10
		BEMT	1.94 (3.18)	10