CHAPTER III

MATERIALS AND METHODS

General apparatus and glassware

1. Analytical balance (5 digits), Model XP205DR, Max 81 g/ 220 g Mettler Toledo, Germany
2. Analytical balance (2 digits), Model BT420 S Sartorius AG, Germany
3. Amber vial, 2 mL with screw cap PTFE/silicone septa, Agilent technologies, Germany
4. Amber vial, 16 mL with screw cap silicone septa, Agilent technologies, Germany
5. Beakers 10, 50, 150, 250, 500 mL
6. Glass bottle 1000 mL
7. Glass syringe 10 mL, poper & sons, inc., Italy
8. Graduated cylinders 100, 250, 500 mL
9. Homogenizer
10. Hot air oven
11. Micro-pipettes 20-200 µL, 100-1000 µL and tips, BRAND, Germany
12. Milli-Q Plus185 ultrapure water system, Millipore, U.S.A.
13. Refrigerator, SANYO, Japan
14. Stainless beakers 500, 1000 mL
15. Syringe filters, PVDF, nylon 13 mm, 0.2 µm, Agela Technologies, U.S.A.
16. Thermometer
17. Ultrasonic bath, Model T 1060/H, Elma®, Germany
18. Vacuum pump, Model DDA-V130-BN, International controls corp.
19. Vial insert, 250 µL glass with polymer feet, Agilent, U.S.A.
20. Vial rack, for 2 mL with 50 positions
21. Volumetric flask: size 1000 mL
22. Volumetric flasks (amber): size 10, 25, 50 mL

23. Volumetric pipettes 0.5, 1, 2, 4, 5, 6 mL
24. Vortex mixer, Model genie-2™, Scientific Industries, U.S.A.

All experimental glasswares were washed with detergent, then allowed to dry at room temperature and rinsed with methanol before use.

Instrumentation and equipments

HPLC-MS

High performance liquid chromatography mass spectrometer (HPLC-MS) system: Agilent 1100 Series LC/MSD (VL model) consists of G1956A mass spectrometer detector, G1379A micro vacuum degasser, G1311A quaternary pump, G1313A autosampler and G1316A thermostat column compartment, Agilent Technologies, U.S.A.

HPLC column: Hypersil BDS C8, 150 mm x 3 mm I.D., 3 μm, Thermo scientific, U.S.A.

Guard column: Hypersil BDS C8, 10 mm x 3 mm I.D., 3 μm, Thermo scientific, U.S.A.

Reagents and chemicals

1. Reagents

1.1 0.1% v/v formic acid

A 0.1% v/v formic acid was prepared by pipette 1.0 mL of formic acid into 1,000 mL volumetric flask, then mixed and diluted to the mark with water. It was filtered through a 0.2 μm PVDF membrane filter then deaerated ultrasonically prior to use.

1.2 Water

Water was purified with a Milli-Q Plus 185 ultrapure water system (Millipore, U.S.A.).

2. Standards

2.1 Betamethasone, reference standard (USP, Rockville , U.S.A.)

2.2 Betamethasone 17-valerate, reference standard (USP, Rockville, U.S.A.)

2.3 Dexamethasone, reference standard (USP, Rockville , U.S.A.)

2.4 Hydrocortisone acetate, reference standard (USP, Rockville , U.S.A.)

2.5 Hydroquinone, reference standard (USP, Rockville , U.S.A.)

2.6 Prednisolone, reference standard (USP, Rockville , U.S.A.)

2.7 Triamcinolone acetonide, reference standard (USP, Rockville, U.S.A.)

2.8 Retinoic acid, reference standard (USP, Rockville , U.S.A.)

2.9 Betamethasone, (Dr. Ehrenstorfer GmbH, Augsberg, Germany)

2.10 Betamethasone 17-valerate, (Dr. Ehrenstorfer GmbH, Augsberg, Germany)

2.11 Dexamethasone, (Dr. Ehrenstorfer GmbH , Augsberg, Germany)

2.12 Hydrocortisone acetate, (Dr. Ehrenstorfer GmbH, Augsberg, Germany)

2.13 Hydroquinone, (Chem service, U.S.A.)

2.14 Prednisolone, (Dr. Ehrenstorfer GmbH, Augsberg, Germany)

2.15 Retinoic acid, (Dr. Ehrenstorfer GmbH , Augsberg, Germany)

2.16 Triamcinolone acetonide (Dr. Ehrenstorfer GmbH, Augsberg, Germany)

3. Chemicals

3.1 Acetonitrile , HPLC grade (Merck, Darmstadt Germany)

3.2 Formic acid 98-100%, AR grade (Merck, Germany)

3.3 Liquid nitrogen 99.999% (UHP) (Praxair, Thailand)

3.4 Methanol, AR grade for rinse glassware (Merck, Darmstadt Germany)

3.5 Methanol, HPLC grade for liquid chromatography (Merck, Darmstadt Germany)

Preparation of standard solutions

1. Stock standard solutions

For USP certificated, reference standard (dexamethasone and prednisolone) must be dry portion at 105 °C for 3 hours, and hydrocortisone acetate must be dry portion at 60 °C for 3 hours before using.

Separate stock solutions were prepared by dissolving an accurate weight of approximately 10 mg of each standard of pure compound in 10 mL volumetric flask with HPLC grade methanol to obtain concentrations of 1 mg/mL. The solutions were

sonicated for 1 minute and were then diluted to the mark with methanol. These stock standard solutions were kept in amber glass bottle with screw cap and were stored in the dark at -18 °C.

2. Individual intermediate standard solutions

2.1 Intermediate standard solution of hydroquinone

A 6.0 mL of stock standard solution at 1 mg/mL of hydroquinone was transferred into 10 mL volumetric flask and was diluted to volume with methanol. The individual intermediate standard solutions of hydroquinone had a concentration of 600 µg/mL.

2.2 Intermediate standard solution of retinoic acid

A 0.5 mL of stock standard solution at 1 mg/mL of retinoic acid was transferred into 25 mL volumetric flask and was diluted to volume with methanol. The individual intermediate standard solutions of retinoic acid had a concentration of 20 µg/mL.

2.3 Intermediate standard solution of betamethasone and dexamethasone

A 4.0 mL of stock standard solutions at 1 mg/mL of betamethasone and dexamethasone was transferred into a separate 25 mL volumetric flask and was diluted to volume with methanol. The individual intermediate standard solutions of betamethasone and dexamethasone had a concentration of 160 µg/mL.

2.4 Intermediate standard solution of betamethasone 17-valerate

A 1.0 mL of stock standard solution at 1 mg/mL of betamethasone 17-valerate was transferred into 25 mL volumetric flask and was diluted to volume with methanol. The individual intermediate standard solutions of betamethasone 17-valerate had a concentration of 40 µg/mL.

2.5 Intermediate standard solution of hydrocortisone acetate, prednisolone and triamcinolone acetonide

A 2.0 mL of stock standard solutions at 1 mg/mL of hydrocortisone acetate prednisolone and triamcinolone acetonide was transferred into a separate 25 mL volumetric flask and was diluted to volume with methanol. The Individual intermediate standard solutions of prednisolone, hydrocortisone acetate and triamcinolone acetonide had a concentration of 80 µg/mL.

These solutions were kept in amber glass bottle with screw cap and were stored in the dark at -18 °C.

3. Working standard mixture solutions

Calibration mixtures were prepared at four different concentration levels. The working standard solutions of the eight compounds were prepared on a daily from the individual intermediate standard solutions as followed;

For the working standard solutions, 50 µL of the individual intermediate standard solutions was diluted with methanol to 10 mL in volumetric flask to a final concentration of 3, 0.1, 0.8, 0.2, 0.8, 0.4, 0.4 and 0.4 µg/mL of hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide, respectively.

For the working standard solutions , 200 µL of the individual intermediate standard solutions was diluted with methanol to 10 mL in volumetric flask to a final concentration of 12, 0.4, 3.2, 0.8, 3.2, 1.6, 1.6 and 1.6 µg/mL of hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide, respectively.

For the working standard solutions , 400 µL of the individual intermediate standard solutions was diluted with methanol to 10 mL in volumetric flask to a final concentration of 24, 0.8, 6.4, 1.6, 6.4, 3.2, 3.2 and 3.2 µg/mL of hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide, respectively.

For the working standard solutions , 600 µL of the individual intermediate standard solutions was diluted with methanol to 10 mL in volumetric flask to a final concentration of 36, 1.2, 9.6, 2.4, 9.6, 4.8, 4.8 and 4.8 µg/mL of hydroquinone, retinoic acid, betamethasone, betamethasone 17-valerate, dexamethasone, hydrocortisone acetate, prednisolone and triamcinolone acetonide, respectively.

The standard mixture solutions were filtered through a 0.2 µm nylon membrane filter and were kept in amber glass bottle with screw cap. Then, these solutions were stored in the dark at -18 °C. They were used to prepare calibration curves. Calibration curves were obtained by plotting the responses of the analyte peaks against the analyte concentrations, with the results analyzed by linear regression.

Sample

Matrix blank is essentially matrices with no analyte. Then a process was prepared by omitting the ingredients such as hydroquinone, retinoic acid and corticosteroids in subsequent preparations. The second type is the sample; it is prepared for analyzing precision.

Skin creams contain a variety of ingredients. Some skin creams may contain small amounts of vitamins or other "nutrients". A basic and satisfactory skin cream can be prepared from stearic acid, lanolin, mineral oil, triethanolamine, and water [48].

1. Preparation of skin cream (matrix blank)

Preparation of skin cream as described in the procedure below. The ingredients and amounts shown in Table 4.

1.1 Phase 1 (cremophor A-6, cremophor A-25, finsolv TN, white oil 2076, GMS, WAX-C) mixed together in a stainless steel beaker and heated in a bath with temperature about 80 degrees celsius.

1.2 Phase 2 (water) was placed in another beaker of stainless steel and heated in a bath with temperature about 80 degrees celsius. After the water solution has reached a temperature from above, remove it from the source.

1.3 Phase 1 and phase 2 were mixed in the other beaker with temperature about 80 degrees celsius.

1.4 When a temperature of the solution dropped to 50 degrees celsius, we added phase 3 (alpha bisabolol, methyl paraben, propyl paraben, sodium sulfite) into the same beaker.

1.5 The mixture was mixed using homogenizer in cool bath with ice until it has a smooth cream.

Table 4 The components used to prepare the skin cream (matrix blank)

Phase	Component		% w/w	Function	Weight (g)
	Trade name	Chemical name			
1	cremophor A-6	ceteareth-6 and stearyl alcohol	1.5	emulsifier	3.06
	cremophor A-25	ceteareth-25	0.5	emulsifier	1.02
	finsolv TN	C12- C15 alcohol benzoate	3.0	emulsifier	5.98
	white oil 2076	-	5.0	ingredient	10.02
	GMS	glyceryl monostearate	3.0	emulsifier	5.98
	WAX-C	cetyl alcohol	1.5	viscosity	2.97
2	water	-	85.0	-	169.97
3	alpha-bisabolol	-	0.10	anti-irritant	0.20
	methylparaben	4-hydroxybenzoic acid methyl ester	0.125	preservative	0.25
	propylparaben	4-hydroxybenzoic acid propyl ester	0.125	preservative	0.25
	sodium sulfite	sodium sulfite	0.15	preservative	0.30

2. Preparation of the sample for analyzing precision

Preparation of the sample was prepared as well as the procedure of the skin cream (above), but the Phase 1 was spiked with hydroquinone (0.4509 g), retinoic acid (0.0292 g), betamethasone (0.1030 g), betamethasone 17-valerate (0.0488 g), dexamethasone (0.1040 g), hydrocortisone acetate (0.0963 g), prednisolone (0.0960 g) and triamcinolone acetonide (0.0932 g), respectively. Ingredients and amounts are shown in Table 5.

Table 5 The components used to prepare the sample for analyzing precision

Phase	Component		% w/w	Function	Weight
	Trade name	Chemical name			(g)
1	cremophor A-6	ceteareth-6 and stearyl alcohol	1.5	emulsifier	1.51
	cremophor A-25	ceteareth-25	0.5	emulsifier	0.50
	finsolv TN	C12- C15 alcohol benzoate	3.0	emulsifier	2.99
	white oil 2076	-	5.0	ingredient	4.98
	GMS	glyceryl monostearate	3.0	emulsifier	3.02
	WAX-C	cetyl alcohol	1.5	viscosity	1.52
		BM	0.10	analyte	0.1030
		BMV	0.05	analyte	0.0488
		DM	0.10	analyte	0.1040
		HCA	0.10	analyte	0.0963
		HQ	0.45	analyte	0.4509
		PRL	0.10	analyte	0.0960
		RA	0.03	analyte	0.0292
		TA	0.09	analyte	0.0932
2	water	-	83.98	-	85.05
3	alpha-bisabolol	-	0.10	anti-irritant	0.10
	methylparaben	4-hydroxybenzoic acid methyl ester	0.125	preservative	0.20
	propylparaben	4-hydroxybenzoic acid propyl ester	0.125	preservative	0.10
	sodium sulfite	sodium sulfite	0.15	preservative	0.15

HPLC-MS conditions

Analyzes were conducted with Agilent 1100 MSD equipped with a mass detector with electrospray ionization (ESI). For the screening step the chromatographic analysis was carried out on a Hypersil BDS C8 column (150 mm x 3.0 mm I.D., 3 μm, Thermo scientific, U.S.A.) with Hypersil BDS C8 guard column (10 mm x 3 mm I.D., 3 μm Thermo scientific, U.S.A.) using gradient condition (Table 6). The column temperature was maintained at 25 °C. The mobile phase A consisted of 0.1% v/v formic acid in water was filtered under vacuum through a 0.2 μm nylon member filter 47 mm and the mobile phase B consisted of acetonitrile. The mobile phase was prepared by mixing the mobile phase A with the mobile phase B in a required volume ratio, by programming the pump. The injection volume was 5 μL and the total runtime 80 minutes. During all experiments, mass spectra were obtained by scanning from range 50 to 550 *m/z* using both negative and positive ionization. In SIM mode, the different fragmentation voltages at 70, 80, 90, 100, 110, 120, 130, 140 and 150 V were applied in order to obtain the protonated molecular ion. The best acquisition parameters were selected.

Table 6 HPLC gradient elution program using 0.1% v/v formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow of 0.5-0.55 (mL/min)

Time (min)	% Mobile phase A*	% Mobile phase B**	Flow rate (mL/min)
0	90	10	0.50
3.5	90	10	0.50
4	76	24	0.50
27	76	24	0.50
31	60	40	0.50
40	60	40	0.50
54	40	60	0.50
57	10	90	0.55
58	90	10	0.50
80	90	10	0.50

Note: * 0.1% formic acid in water

* * Acetonitrile

Instrumental settings for the Agilent 1100 Series LC/MSD VL mass spectrometer operated with the ESI interface in positive ion mode with the condition provided in Table 7.

Table 7 Initial set up spray chamber conditions

Parameter	Condition
Mode	API-ES
Drying gas flow: nitrogen	12 L/min
Nebulizer pressure: nitrogen	40 psi
Drying gas temperature: nitrogen	350 °C
Capillary voltage	4000 volts
Quadupole temperature	100 °C
Optimized voltage of fragmentor	
betamethasone, dexamethasone, hydroquinone and prednisolone	100 V
betamethasone 17 valerate and retinoic acid	110 V
hydrocortisone acetate and triamcinolone acetonide	120 V

Method validation

Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for specific intended use are fulfilled [49]. In general, validation should check that the method performs adequately for the purpose throughout the range of analyte concentrations and test materials to which it is applied.

Non-standard method and laboratory-developed methods require validation. Method validation is an important requirement in the practice of chemical analysis. The ISO definition of method validation as the process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with what the application requires [50].The method was validated via the parameters included specificity/selectivity, linearity and range, recovery, precision, limit of detection (LOD), limit of quantitation (LOQ) and measurement uncertainty. There are three commonly encountered methods of employing these standards, namely the use of external standards, the use of standard additions and the use of internal standards. For this study, the use of external standards was selected.

System suitability tests are an integral part of liquid chromatographic method. The parameters necessary to be established for system suitability, e.g., resolution,

theoretical plate number and % RSD. The resolution is a critical value when working with complex samples. Therefore, it is an essential part of the system suitability measurement state before the quantitative sample. There are two peaks in same chromatographic (betamethasone and dexamethasone), a minimum resolution for the separation is set at 1.5. The theoretical plate number depends on eluting time but in general should be more than 1500 [51]. The system suitability will check by performing three replicate injection of the standard solution which contained 12 µg/mL HQ, 0.4 µg/mL RA, 3.2 µg/mL BM, 0.8 µg/mL MBV, 3.2 µg/mL DM and 1.6 µg/mL for HCA, PRL and TA, respectively. % RSD of peak area of three injections could below 5.

As noted above, in order to equilibrate the stationary phase surface modification, the mobile phase must be allowed to flow for at least 1 hour before injection.

1. Specificity/Selectivity

The selectivity of a method is the accuracy of its measurement in the presence of interferences. Methods that employ highly specific determinative procedures, such as chromatography/mass spectrometry, have the capability to be very selective [52]. It is necessary to establish that the signal produced at the measurement is only due to the analyte and not from the presence of something. In order to test the specificity of the method, standards, matrix blank (cream without any added analytes) and spiked matrix blank ware analyzed and checked for interfering compounds in the regions of interest. The retention times of the compounds in the matrix blank were compared with that of the compounds of interest.

2. Linearity and range

Linearity can be tested by examination of a plot of the produced by linear regression between response and concentration. Classical least squares regression is used to establish the equation of the relation between the instrumental response(y) and the concentration (x) which for a linear model is $y = ax + b$. In addition, the plot of residual values should be calculated by linear regression of the responses on the concentrations in an appropriate calibration set [53, 54].The procedure for the study of linearity and range can be described as follows:

2.1 System linearity

Calibration standards were prepared in methanol at 7 concentration levels as present in Table 8 and analyzed by HPLC-MS under optimized conditions (Table 6 and Table 7). Each concentration level was analyzed in 5 replicates using peak area. The intercepts, slopes and correlation coefficient (r) of calibration curves were presented. A plot of residuals also analyzed. Using residual plots, the x-axis is the concentration value of x, and the y-axis is the residual of x. In fact a random pattern of residue supports a linear model. Therefore, if the residuals randomly appear, it suggests that the model fits the data well.

Table 8 Concentrations of mixed standard solution (μg/mL) for linearity and range

Analyte	Conc. (μg/mL)						
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
HQ	1.50	3.00	6.01	12.01	24.02	36.04	60.06
RA	0.05	0.10	0.21	0.41	0.82	1.23	2.05
BM	0.41	0.81	1.63	3.25	6.51	9.76	16.27
BMV	0.11	0.21	0.42	0.84	1.68	2.53	4.21
DM	0.42	0.83	1.67	3.33	6.67	10.00	16.67
HCA	0.21	0.42	0.83	1.66	3.32	4.98	8.30
PRL	0.20	0.41	0.81	1.62	3.24	4.87	8.11
TA	0.20	0.41	0.82	1.63	3.26	4.89	8.15

2.2 Method linearity

The data from percent recovery has plotted between the percentage of added standard with percentage of spiked matrix blank. The correlation coefficient should be near 1.

3. Recovery

Analytical recovery is a bias usually associated with sample preparation, extraction of the analyte from a sample and other analytical procedures prior to determination [52]. It is often evaluated by spiking the matrix with know levels of

analyte standards at or near target values. It could include anything added to the sample in order to gauge the effect of the addition, therefore the recovery was corrected for losses during sample preparation. The expect recovery % values depend on the analyte concentration as shown in Table 9 [54].

Table 9 Acceptable recovery percentages at different concentrations as a function of the analyte concentrations

Analyte	Analyte ratio	Unit	Mean recovery (%)
100	1	100%	98-102
10	1.00E-01	10%	98-102
1	1.00E-02	1%	97-103
0.1	1.00E-03	0.10%	95-105
0.01	1.00E-04	100 ppm	90-107
0.001	1.00E-05	10 ppm	80-110
0.0001	1.00E-06	1 ppm	80-110
0.00001	1.00E-07	100 ppb	80-110
0.000001	1.00E-08	10 ppb	60-115
0.0000001	1.00E-09	1 ppb	40-120

Source: Taverniers, 2004

The study of recovery was estimated by added standard solutions to a test sample, in 1 g of matrix blank, into 50 mL volumetric flask with methanol at three different concentrations. Each concentration level was analyzed in five replicates with duplicate injection. The validation levels for hydroquinone were 0.22, 0.44 and 0.66% w/w; for retinoic acid were 0.0125, 0.025 and 0.0375% w/w; betamethasone and dexamethasone were 0.05, 0.10 and 0.15% w/w; for betamethasone 17 valerate were 0.02, 0.04 and 0.06% w/w; and for hydrocortisone acetate, prednisolone and triamcinolone acetonide were 0.045, 0.09 and 0.135% w/w. The concentrations of spiked matrix blank in 3 levels as present in Table 10 were evaluated. The four-levels, duplicate injection, of calibration curve were obtained by plotting the analyte peak

areas against the analyte concentrations in the calibrators using linear regression. Matrix blank and fifteen spiked matrix blank were analyzed within each run. The recovery was calculated by comparison of the peak areas of analytes from spiked matrix blank with those of injected standards. The recovery of the method was calculated and reported for each concentration in term of percent relative standard deviation (% RSD) and the mean percent recoveries.

Table 10 Concentrations of spiked matrix blank (% w/w) for recovery

Analyte	Amount (% w/w)		
	Level 1	Level 2	Level 3
HQ	0.22	0.44	0.66
RA	0.0125	0.025	0.0375
BM	0.05	0.10	0.15
BMV	0.02	0.04	0.06
DM	0.05	0.10	0.15
HCA, PRL, TA	0.045	0.09	0.135

4. Precision

The precision of a method is a measure of the closeness expected between independent replicate test results conducted under specified conditions. It is usually calculated and reported in terms of the standard deviation (SD), or relative standard deviation (RSD) of replicate results [52, 53]. The retention times of each analyte were compared in different days. Two measures of precision, termed repeatability and intermediate precision are selected. Calculated repeatability and intermediate precision values can be compared with those of existing methods. If there are no method with which to compare the precision parameters, theoretical relative reproducibility and repeatability standard deviations can be calculated from the Horwitz equation which is shown in Table 11 [54].

Table 11 Horwitz function as an empirical relationship between the precision of an analytical method and the concentration of the analyte regardless of the nature of the analyte, metrix and the method used.

Analyte	Analyte ratio	Unit	Horwitz % RSD
100	1	100%	2
10	1.00E-01	10%	2.8
1	1.00E-02	1%	4
0.1	1.00E-03	0.10%	5.7
0.01	1.00E-04	100 ppm	8
0.001	1.00E-05	10 ppm	11.3
0.0001	1.00E-06	1 ppm	16
0.00001	1.00E-07	100 ppb	22.6
0.000001	1.00E-08	10 ppb	32
0.0000001	1.00E-09	1 ppb	45.3

Source: Taverniers, 2004

4.1 Repeatability

Repeatability expresses the precision by the same analyst under the same operating conditions (same reagents, equipment, settings and laboratory) over a short interval of time [55]. The procedure for the study of repeatability was carried out in the same way as recovery. The extractions of sample were analyzed in five replicates with duplicate injection against a calibration curve within one day. If the concentrations of sample fall outside the calibration curve, these solutions were diluted with methanol. Repeatability was tested by analysis on a day. The precision of the method was calculated and reported in term of percent relative standard deviation.

4.2 Intermediate precision

Intermediate precision was tested by analysis the samples on five different days. Analysis of variance (ANOVA) was used to evaluate for intermediate precision.

5. Limit of detection (LOD)

The limit of detection is the lowest concentration of analyte that can be detected from base line noise [56]. The limit of detection was calculated as three times of signal-to-noise ratio (S/N=3) and was estimated by spiking standard solutions in the matrix blank. The method was determined by analyzing spiked matrix blank containing low concentration of analytes in 1 g of matrix blank into 50 mL volumetric flask by appropriate dilution with methanol. Ten replicates of spiked matrix blank were performed and were determined instrument detection limit (IDL).

6. Limit of quantitation (LOQ)

The limit of quantitation is the lowest concentration of analyte in the sample that may be determined with acceptable recovery and precision when the required procedure is applied [56]. The experimental procedure was similar to the LOD. This parameter was determined by analyzing spiked matrix blank that provided a peak height of signal-to-noise ratio equal to 10 (S/N=10). It was evaluated by spiking 1 g matrix blank with each standard analyte into 50 mL volumetric flask by appropriate dilution with methanol. Ten replicates of spiked matrix blank were performed.

7. Measurement uncertainty [57]

The International Organization of Standardization, ISO/IEC 17025, standard for the general requirements for competence of testing laboratories requires that testing laboratories shall have and shall apply procedures for estimating uncertainty of measurement. When estimating the uncertainty of measurement, all uncertainty components which are of importance in the given situation shall be taken into account using appropriate method for analysis. The parameter may be, for example, a standard deviation. A process of measurement uncertainty estimation identifies the key as follow:

7.1 Specify measurand

Write down a clear statement of what is being measured, including the relationship between the measurand and the input quantities (e.g. measured quantities, constants, calibration standard values etc.) upon which it depends. Where possible, include corrections for known systematic effects. The specification information should be given in the relevant Standard Operating Procedure (SOP) or other method description.

7.2 Identify uncertainty sources

List the possible sources of uncertainty. This will include sources that contribute to the uncertainty on the parameters in the relationship specified in Step 1, but may include other sources and must include sources arising from chemical assumptions.

7.3 Quantify uncertainty components

Measure or estimate the size of the uncertainty component associated with each potential source of uncertainty identified. It is often possible to estimate or determine a single contribution to uncertainty associated with a number of separate sources. It is also important to consider whether available data accounts sufficiently for all sources of uncertainty, and plan additional experiments and studies carefully to ensure that all sources of uncertainty are adequately account.

7.4 Calculate combined uncertainty

The information obtained in step 3 will consist of a number of quantified contributions to overall uncertainty, whether associated with individual sources or with the combined effects of several sources. The contributions have to be expressed as standard deviations, and combined according to the appropriate rules, to give a combined standard uncertainty. The appropriate coverage factor should be applied to give an expanded uncertainty.

The application of optimized condition of developed method in cosmetic product samples

Assays of samples of analytes in cosmetic products should be completed with a single determination without duplicate analysis if the assay method has acceptable variability. Ten samples of different brands were analyzed using the optimized HPLC-MS condition.