International Journal of Applied Science and Engineering
2011. 9, 4: 287-299

Simultaneous Determination of Five Whitening Agents by Ion-Pair Reversed-Phase High Performance Liquid Chromatography

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Abstract: A gradient ion-air reversed-phase high performance liquid chromatography method was developed for simultaneous determination of 5 whitening agents (ascorbic acid (AA), magnesium ascorbyl phosphate (MAP), kojic acid (KA), arbutin (ART) and hydroquinone (HQ)). The gradient elution was performed using Inertsil ODS-3V column (250 × 4.6 mm, 5 μm). The analytes were detected by ultraviolet light absorption at the wavelength of 270 nm, the flow rate was set at 1.0 mL/min, and the sample injection volume was 10 μL. The buffer solution contains 50 mM sodium phosphate monobasic dihydrate and 2 mM n-hexadecyltrimethyl ammonium bromide at pH 4.5. The precision of the proposed method, expressed as relative standard deviation (RSD) (%), was determined by the analysis of each compound. The RSD range was from 0.23 to 0.55%. The calibration curves were found to be linear in 8.12-40.6 μg/mL (KA), 12.44-62.20 μg/mL (ART), 8.28-41.40 μg/mL (HQ), 8.16-40.80 μg/mL (MAP) and 4.25- 1.80 μg/mL (AA). The correlation coefficient of linear regression analysis (R^2) was within the range of 0.9968 - 0.9997. The analytical results were satisfied for these 5 whitening agents. The recoveries ranging from 96.97% to 101.22% for lotion, and from 97.17 to 100.80% for cream, were obtained at both low and high concentrations. The proposed method is suitable for routine analysis of the 5 whitening agents in commercial cosmetic products, or examined the allowable amount of whitening agents for the Department of Health.

Keywords: Ion-pair reversed-phase high performance liquid chromatography whitening agent; ascorbic acid; magnesium ascorbyl phosphate; kojic acid; arbutin; hydroquinone

1. Introduction

In recent years, the break in the ozonosphere makes the ultraviolet rays stronger than ever. Excessive exposure to ultraviolet rays leads to the development of skin spots, which is a common problem for more people. Therefore, skin-whitening cosmetics are very popular. Skin-whitening products are used to lighten the skin tone. Ascorbic acid (AA) and magnesium ascorbyl phosphate (MAP, a derivative of AA) inhibit the production of

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Accepted for Publication: October 24, 2011

melanin, and are used as skin whiteners and radical scavengers in cosmetic products [1-4]. Kojic acid (KA) is a tyrosinase inhibitor produced by various fungal species such as *Aspergillus, Acetobacter,* and *Penicillium* [5-6]. It is used in cosmetic preparations to achieve a skin-lightening effect by inhibiting melanin formation; it works by chelating the copper ions in tyrosinase [7-9]. Its presence in cosmetic products also enhances shelf life of the product through its preservative actions against both chemical and microbial degradation [10]. Arbutin (ART), a hydroquinone glycoside, is also widely used to lighten the skin [11-12], and its active compound is hydroquinone (HQ), a metabolite of ART [13]. Both ART and HQ inhibit the conversion of tyrosine to melanin by inhibiting tyrosinase activity [14-15]. KA, MAP, ascorbyl glucoside, and ART were all permitted as whitening ingredients for cosmetics by the Department of Health, Executive Yuan, R.O.C. in 2000 [16], and their permissible amounts are 3, 2, 2 and 7%, respectively [16]. The Department of Health announced that HQ should be used only as a drug because of its side effects, which include dermatitis, erythema, burning, and hyperpigmentation [17]. The HQ released by ART can be regarded as an impurity, and the maximum permissible amount is 20 μg/mL [16]. Ascorbic glucoside decomposition can produce AA. In Taiwan, no limit was set on the concentration of AA in cosmetic products by the government. In general, the preferred analysis methods employ high-performance liquid chromatography (HPLC). According to the literature, the levels of the above-mentioned 5 whitening agents can be determined by reversed-phase high performance liquid chromatography [18-27]. The purpose of this study was to develop a rapid and simple quantitative method for the simultaneous determination of the above-mentioned 5 whitening agents in cosmetic products. This work is of great importance for public safety. Several parameters were investigated, including the acetonitrile ratio in the mobile phases, different ion-pair reagents, the concentration of ion-pair reagents, and the pH of the mobile phase. Furthermore, this method was applied to the analysis of commercial whitening cosmetic products. The chromatographic method we developed is suitable for routine analysis of the 5 whitening agents in commercial cosmetic products.

2. Materials and methods

2.1. Reagents and Standards

KA, AA, ART, MAP and HQ were purchased from Alfa Co. (American), Alfa Co. (American), Wako (Japanese), Lipo. Co. (American), and Sigma (St. Louis, MO, USA), respectively. Commercial whitening cosmetics, from various manufacturers, were purchased from retail stores and a local pharmacy. N-hexadecyltrimethylammonium bromide (CTAB) was purchased from Sigma (St. Louis, MO, USA). Tetrabutylammonium bromide (TBA), were purchased from TCI Co. (Tokyo, Japan).

All the mobile phase and the solution for injection were degassed in an ultrasonic bath and were filtered through a 0.45 μm nylon membrane before used.

2.2. Apparatus and Chromatographic Conditions

HPLC analysis were preformed on Agilent system equipped with a 1100 quaternary pump, 1100 autosampler, 1100 diode array detector and LAN Interface. The absorption measurements were carried out on a Hitachi U-2001 UV-VIS spectrophotometer. The colum used was Inertsil ODS-3V 250 mm × 4.6 mm (5 μm particle size), Inertsil ODS-3 250 mm × 3.0 mm (5μm particle size).

The mobile phase of gradient elution composed of the acetonitrile : buffer mixture (1: 99, v/v) from 0 to 20 min, acetonitrile : buffer mixture (70 : 30, v/v) from 21 to 40 min and acetonitrile : buffer mixture (1: 99, v/v) from 41 min; post time was 10 min. The flow rate was 1.0 mL/min, sample injection volume was 10 μL, and the detector wavelength was set at 270 nm.

2.3. Calibration Curve

Standard stock solutions were prepared by accurately weighing the individual agents, and then dissolving them in methanol, except MAP was dissolved in a mixture of methanol and deionized water in the ratio of 1 : 1 (v/v). Five working standard solutions were prepared from their standard stock solutions by appropriate dilution.

2.4. Recovery Study

A sample lotion and cream without any whitening agents were used as blanks in the accuracy study. To prepare the sample cream, 0.5 g was weighed into a 100-mL volumetric flask and diluted to volume with methanol; 5 mL of the resulting solution was transferred into a 25-mL volumetric flask, again diluted to volume with methanol, and then, filtered through a Millipore (0.45 μm) nylon filter. The accuracy of the proposed method was evaluated by comparing the results to the values obtained by adding known concentrations of the 5 whitening agents to the blank lotion and cream. Each concentration was analyzed in triplicate. Recovery was calculated as the relationship between the experimental concentration (*C*exp) and the theoretical concentration (*C*teo), expressed as percentage ((*C*exp/*C*teo) × 100).

2.5. Precision

To evaluate the intra-day precision and inter-day precision of the method, results were expressed as the relative standard deviation (RSD) of peak area measurements obtained under the same conditions. The intra-day precision measurements were performed on the same day (n = 3), and the inter-day precision measurements were determined on 3 consecutive days.

2.6. Application to Real Cosmetic Products

To analyze the MAP content of whitening cosmetic lotion, 1 g was weighed into a 50-mL volumetric flask and diluted to volume with a 1 : 1 mixture of methanol : deionized water; then the flask was immersed in an ultrasonic bath for 1 min, and 3 mL of the resulting solution was transferred into a 25-mL volumetric flask, diluted to volume with methanol, and then filtered through a Millipore (0.45 μm) nylon filter.

3. Results and Discussion

3.1. Determination of Optimization Wavelength for UV Detector

To test the specific absorption peak wavelength of the 5 compounds using an ultraviolet light spectrometer, the maximum absorption peaks were listed in Table 1. Wavelengths of 220, 240, 254, 270 and 290 nm were selected. AA (24 μg/mL), KA (20 μg/mL), ART (47 μg/mL), HQ (21 μg/mL), and MAP (30 μg/mL) were assayed. The results showed that the detection range of wavelengths between 240 and 270 nm is appropriate for these 5 whitening components (Table 2). The choice of the most suitable wavelength was based on the absorption of the whitening ingredients under 5 different wavelengths. At 270 nm, AA and KA have the strongest absorption, ART and MAP have medium-intensity absorption, and HQ has the weakest absorption. However, when combined with absorption at the other 4 groups of wavelengths, its integral area is not very different. After assessment, we chose 270 nm as the wavelength to be studied for analysis method.

Table 1. The maximum absorption peak

Compound	The maximum absorption peak（nm）
Ascorbic acid	264
Kojic acid	218、270
Arbutin	222、284
Hydroquinone	222、290
Mg ascorbyl phosphate	256

Table 2. Different wavelengths and the peak integral area

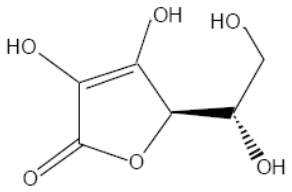
wavelength	Area (mAU)				
	Ascorbic acid	Kojic acid	Arbutin	Hydroquinone	Mg ascorbyl phosphate
220	-	20817.3	12580.0	-	62.5
240	609.2	710.1	167.8	316.4	667.9
254	1002.3	921.0	93.9	219.8	956.4
270	1156.8	1276.9	238.7	155.7	750.0
290	296.8	514.2	317.5	513.3	71.2

3.2. Chromatographic Conditions

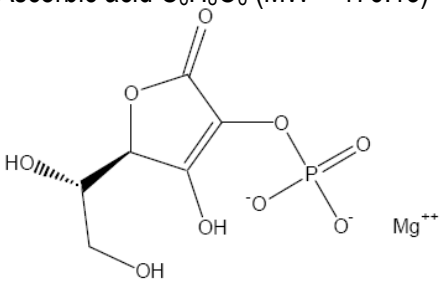
The preliminary experiments were performed using the experimental conditions described by Shih [24]. The eluent was 0.5 mM tetrabutylammonium bromide (TBA) and 0.05 M phosphate buffer (pH 5), containing 5% methanol; UV detection was performed at 254 nm and the flow rate was 1.0 mL/min. The experimental results indicated that the retention time of AA was too short (3 min), and that KA and ART were eluted at the same time (5.3 min). Therefore, the above conditions need to be improved. Initially, various mobile phases and columns were tested in an attempt to obtain the best resolution of the 5

whitening agents, and an Inertsil ODS-3V column (250 × 4.6 mm, 5 μm) was found to be the most appropriate, allowing adequate separation of the 5 whitening agents. A mobile phase consisting of a buffer solution containing 50 mM sodium phosphate monobasic dihydrate, 2.0 mM n-hexadecyltrimethyl ammonium bromide (CTAB) at pH 4.5 and acetonitrile was selected in this study to obtain the optimum analytical conditions. Gradient elution was performed with a mobile phase composed of the acetonitrile: buffer mixture (1 : 99, v/v) from 0 to 20 min, acetonitrile : buffer mixture (70 : 30, v/v) from 21 to 40 min and acetonitrile : buffer mixture (1 : 99, v/v) from 41 min; post time was 10 min. The flow rate was 1.0 mL/min, sample injection volume was 10 μL, and the detector wavelength was set at 270 nm.

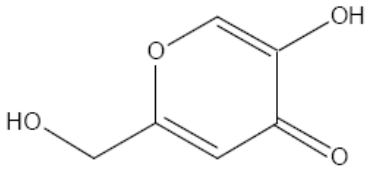
The ion-pair reagent (CTAB) was used to enhance the separation of the analytes. The positive charge ion (CTAB) forms an uncharged ion pair with the analyte ion of negative charge (AA, MAP, KA, ART, and HQ) in the mobile phase. This ion pair then partitions into the nonpolar stationary phase, giving differential retention of solutes based on the affinity of the ion pair for the two phases. In addition, it was important to identify suitable separation conditions for the simultaneous analysis of all 5 analytes in cosmetic products.



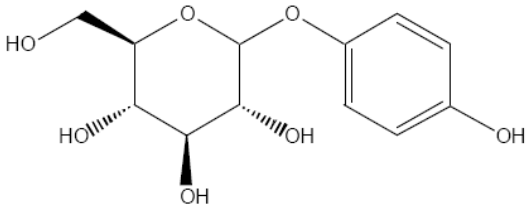
(1) Ascorbic acid $C_6H_8O_6$ (MW = 176.13)



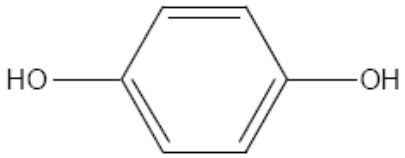
(2) Mg ascorbyl phosphate: $C_6H_7MgO_9P$ (MW=278)



(3) Kojic acid：C6H6O4 (MW = 142.11)

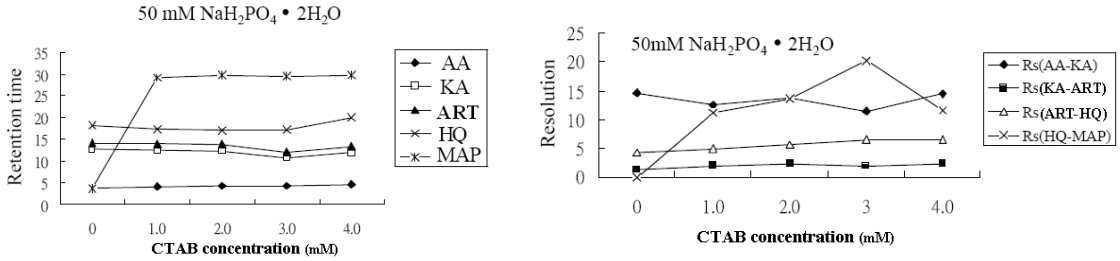


(4) Arbutin $C_{12}H_{16}O_7$ (MW = 272.25)



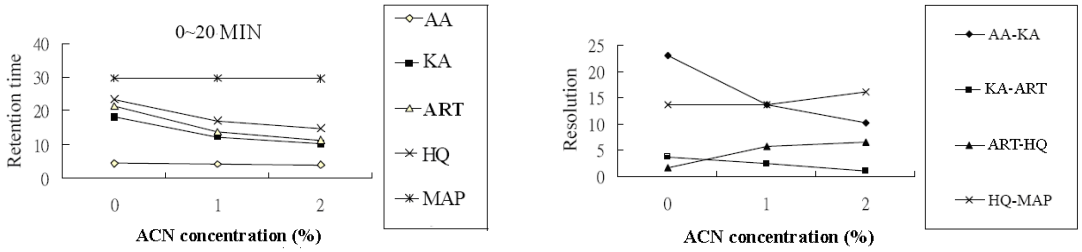
(5) Hydroquinone $C_6H_6O_2$ (MW = 110.11)

Figure 1. Chemical structures of 5 whitening agents



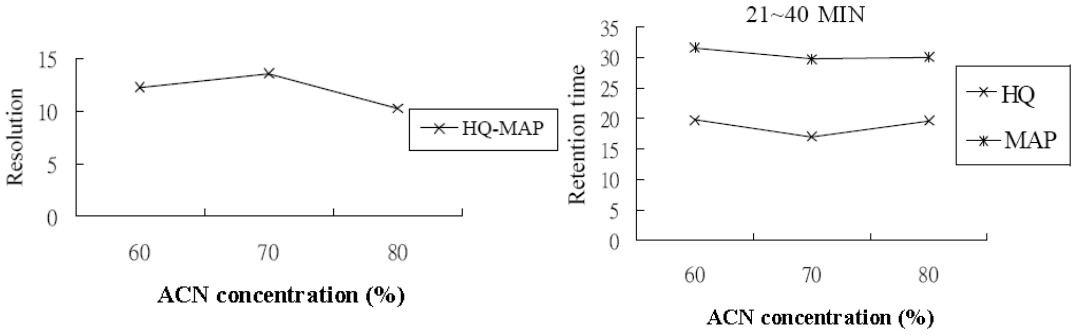
[A] [B]

Figure 2. Effect of n-hexadecyltrimethyl ammonium bromide concentration (0 to 4 mM) on the values of retention time (A) and resolution (B). Gradient elution with mobile phase composed : acetonitrile: buffer mixture (1 : 99 v/v) at 0 to 20 min, acetonitrile: buffer mixture (70 : 30 v/v) at 21 to 40 min and acetonitrile : buffer mixture (1 : 99 v/v) at 41 min; post time was 10 min. The flow rate was set at 1.0 mL/min, sample injection volume was 10 µL, and the detector wavelength was set at 270 nm.



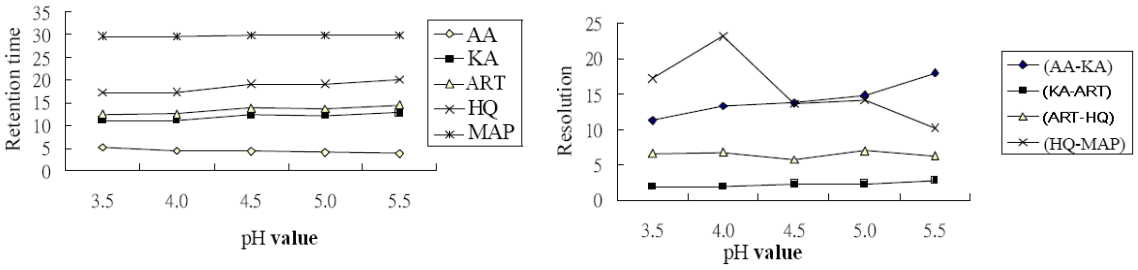
[A] [B]

Figure 3. Effect of acetonitrile concentrations on the values of retention time (A) and resolution (B). Gradient elution with mobile phase composed : acetonitrile : buffer mixture (0 : 100, 1 : 99 and 2 : 98 v/v) at 0 to 20 min, acetonitrile : buffer mixture (70 : 30 v/v) at 21 to 40 min and acetonitrile:buffer mixture (1 : 99 v/v) at 41 min; post time was 10 min. The flow rate was set at 1.0 mL/min, sample injection volume was 10 µL, and the detector wavelength was set at 270 nm.



[A] [B]

Figure 4. Effect of acetonitrile concentrations on the values of retention time (A) and resolution (B). Gradient elution with mobile phase composed : acetonitrile : buffer mixture (1: 99, v/v) at 0 to 20 min, acetonitrile : buffer mixture (60 : 40, 70 : 30, 80 : 20 v/v) at 21 to 40 min and acetonitrile:buffer mixture (1 : 99 v/v) at 41 min; post time was 10 minutes. The flow rate was set at 1.0 mL/min, sample injection volume was 10 µL, and the detector wavelength was set at 270 nm.



[A] [B]

Figure 5. Effect of pH values of the buffer solution (3.5-5.5) on the values of retention time (A) and resolution(B). Gradient elution with mobile phase composed : acetonitrile : buffer mixture (1 : 99 v/v) at 0 to 20 min, acetonitrile : buffer mixture (70 : 30 v/v) at 21 to 40 min and acetonitrile : buffer mixture (1 : 99 v/v) at 41 min; post time was 10 min. The flow rate was set at 1.0 mL /min, sample injection volume was 10 µL, and the detector wavelength was set at 270 nm

In order to obtain the optimum analytical conditions, key chromatographic parameters such as the concentration of CTAB, percentage of acetonitrile, and pH of the buffer solution were optimized first. The following different chromatographic conditions were assessed:

(a) Mobile phases of different buffer/ acetonitrile ratios
(b) different pH (3.5 to 5.5)
(c) different ion-pair reagent (CTAB) concentration (0 to 4.0 mM).

The results of these tests allowed a suitable mobile phase to be chosen to provide good resolution, and the results obtained are illustrated in Figure 2-5.

(I) *Ion-pair Reagent Concentration*

Two ion-pair reagents were tested: TBA and CTAB. After a series of pretests, we found that TBA could not effectively separate KA and ART but CTAB could provide the best separation of the 5 whitening agents; it was, therefore, selected for
further method development and optimization. Five different CTAB concentrations were prepared. As shown in Figure 2, when a CTAB concentration of 3 mM was used, the resolution between the peaks of KA and ART was very small (1.88). The resolution between AA and KA, ART and HQ, and HQ and MAP, however, was greater than 5.

Therefore, resolution between the peaks of KA and ART is the key to selecting an appropriate ion-pair reagent concentration, and the concentration of the ion-pair reagent selected was 2 mM.

(II) *The Percentage of Mobile Phase Solvent*

In this study, we used gradient elution with mobile phases of different ratios of acetonitrile : buffer mixture in the experiment. The following mobile phase was used and the injection was performed in triplicate.

For the first time period of 0 - 20 min, 3 different volume ratios of acetonitrile were used, i.e., 0 to 2% (Figure 3), and for the second period of 21-40 min, 3 different volume ratios of acetonitrile, i.e., 60 to 80%, were used (Figure 4). From Figure 3, it can be observed that in the 21-40 min period, when the acetonitrile volume was 0%, the resolution for each of the whitening agent was more than 3, and the resolution between the adjacent peaks of ART and HQ was 1.71. When the volume of acetonitrile was increased to 2%, the reso-

lution between the adjacent peaks of KA and ART was 1.05. Thus, the resolution worsened with the increasing acetonitrile volume; therefore, we chose to use 1% acetonitrile for the 0-20 min period. Figure 4 shows that in the 21-40 min period, when the elution volume was 60% acetonitrile, MAP had a longer retention time; on the other hand, when the elution volume was increased to 80% acetonitrile, the resolution between the adjacent peaks of HQ and MAP was relatively small (10.3); therefore, during the 21-40 min period, we chose to use 70% acetonitrile.

(III) *The Buffer Solution pH Value*

The effects of different pH values (3.5, 4.0, 4.5, 5.0, and 5.5) of the buffer solution were studied under the conditions described, and the results indicated that the resolution of KA and ART is the worst. At a pH of 4.5-5.5, the resolution was greater than 2.0, with the best resolution at pH 5.5 (2.7). However, the retention time of HQ was longer at higher pH; therefore, a buffer pH value of 4.5 was chosen. Overall, the optimized buffer mixture solution contained 50 mM sodium phosphate monobasic dihydrate and 2 mM CTAB at pH 4.5. The gradient elution of the mobile phase consisted of acetonitrile-buffer mixture (1:99) from 0 to 20 min, acetonitrile-buffer mixture (70:30) from 21 to 40 min, and acetonitrile-buffer mixture (1:99) from 41 min; the post time was 10 min, the flow rate was set at 1.0 mL/min, the detection wavelength was 270 nm, and the sample injection volume was 10 μL. The HPLC analysis method was as developed in this study. Mixtures of the standard solutions of the 5 whitening agents were analyzed by this method. The HPLC chromatograms are shown in Figure 6.

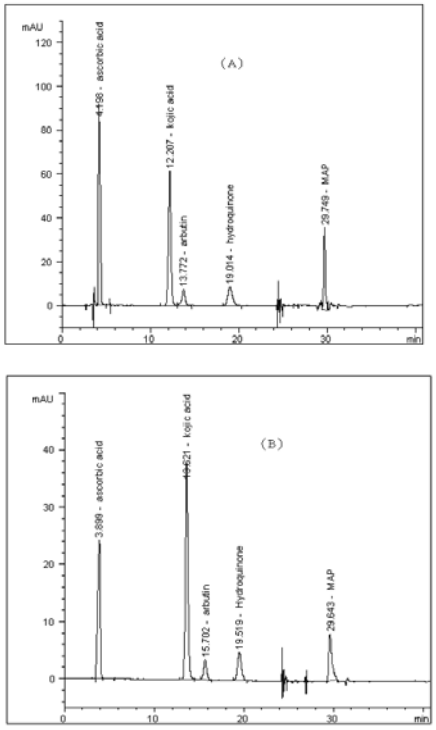


Figure 6. HPLC chromatogram of five whitening agents. (A) HPLC-UV detector and (B) HPLC -PDA detector. The gradient elution of mobile phase was acetonitrile : buffer mixture (1 : 99 v/v) at 0 to 20 min, acetonitrile : buffer mixture (70 : 30 v/v) at 21 to 40 min and acetonitrile : buffer mixture (1 : 99 v/v) at 41 min (buffer solution pH 4.5), post time was 10 min, The flow rate was set at 1.0 mL/min, sample injection volume was set at10 μL and the detector wavelength was set at 270 nm.

3.3. Method Validation

(I) *Linearity*

In this paper, 5 concentration levels were used to study the linear dynamic range of the method. Five different concentrations of standard working solutions (Table 3) prepared from the stock solutions were analyzed. Calibration curve, linear regression equations, and correlation coefficients (R^2) were obtained by

plotting the individual peak area (y) versus its concentration (x). The calibration curves were found to be linear in the concentration range. The correlation coefficient of linear regression analysis (R^2) was within the range of 0.9968 to 0.9997 (Table 3).

(II) *Accuracy*

A sample lotion and cream containing no whitening agents were used as blanks in the recovery study. Recovery of the 5 whitening components was obtained at both low and high concentrations. Varying amounts of the 5 whitening agents were added to the sample lotion and cream, and they were then assayed using the procedure described. The concentrations are given, and the results are summarized in Table 4 and 5. Recoveries (n = 3) ranging from 96.97 to 101.22% for lotion, and from 97.17 to 100.80% for cream, were obtained at both low and high concentrations. The coefficients of variance calculated from the 3 replicates were all less than 2.43%.

Table 3. Calibration curves of 5 whitening agents

Compound	Concentration (μg/mL)	Regression equation	R^2
Ascorbic acid	4.25,8.32,12.84, 17.12,21.80	y=43.234x−123.41	0.997
Mg ascorbyl phosphate	8.16,16.32,24.48, 32.68,40.80	y=25.107x−123.89	0.999
Kojic acid	8.12,16.24,24.36, 32.48,40.60	y=68.718x−30.917	0.996
Arbutin	12.44,24.88,37.32, 49.76,62.20	y=4.1875x+9.29	0.998
Hydroquinone	8.28,16.56,24.84, 33.12,41.40	y=10.108x−4.018	0.998

Table 4. Recovery of whitening compounds in cosmetic lotion formulations

Compound	Amount added (μg/mL)	Amount detected (μg/mL)	Recovery (%)	RSD (%)
Ascorbic acid	8.32	8.21	98.68	2.43
	21.80	21.14	96.97	1.54
Mg ascorbyl phosphate	16.32	16.28	99.75	0.77
	40.80	41.30	101.22	1.81
Kojic acid	16.24	16.25	100.08	1.48
	40.60	40.79	100.47	0.96
Arbutin	37.32	37.37	100.13	0.52
	62.20	61.97	99.64	0.74
Hydroquinone	24.84	24.63	99.17	1.41
	41.40	40.88	98.74	0.70

Each value is the mean of triplicates.

Table 5. Recovery of whitening compounds in cosmetic cream formulations

Compound	Amount added (μg/mL)	Amount detected (μg/mL)	Recovery (%)	RSD (%)
Ascorbic acid	8.32	8.20	98.52	2.69
	21.80	21.46	98.44	3.51
Mg ascorbyl phosphate	16.32	16.13	98.86	3.71
	40.80	40.66	99.67	3.02
Kojic acid	16.24	16.16	99.51	1.43
	40.60	40.92	100.80	0.94
Arbutin	37.32	36.26	97.17	0.94
	62.20	61.89	99.50	1.65
Hydroquinone	24.84	24.48	98.54	0.93
	41.40	40.30	97.34	1.64

Table 6. Intra-day precision of the proposed HPLC method.

Compound	Concentration (μg/mL)	peak area (Mean) (mAU)	SD	RSD (%)
Ascorbic acid	12.84	402.17	1.136	0.28
Mg ascorbyl phosphate	24.48	483.75	2.428	0.50
Kojic acid	24.36	1615.03	4.044	0.25
Arbutin	37.32	162.77	0.379	0.23
Hydroquinone	24.84	237.06	0.880	0.37

Each value is the mean of triplicates.

Table 7. Inter-day precision of the proposed HPLC method (Ascorbic acid is not stable so do not do this experiment)

Compound	Concentration (μg/mL)	peak area (Mean) (mAU)	SD	RSD (%)
Mg ascorbyl phosphate	24.48	484.03	2.65	0.55
Kojic acid	24.36	1620.12	5.59	0.34
Arbutin	37.32	162.69	0.44	0.27
Hydroquinone	24.84	236.87	0.69	0.29

Each value is the mean of three repetitive analyses of each componed. performed on three consecutive days

(III) *Precision*

The precision of the proposed method, expressed as relative standard deviation (RSD) (%), was determined by analysis of each compound. The intra-day precision was determined from the results of 3 repeat analyses of each compound performed on the same day. The inter-day precision was determined from

the results of 3 repeat analyses of each compound performed on 3 consecutive days. The results of intra-day precision and inter-day precision are shown in Table 6 and 7, respectively. The precision of the method was calculated as the RSD of assays containing the 5 whitening agents. RSD was found to be from 0.23 to 0.55%. The results were excellent for all the 5 whitening agents.

3.4. Application

Four commercial whitening cosmetic products were tested: 3 cosmetic lotions (cosmetic number 1, cosmetic number 2, and cosmetic number 3) and 1 whitening mask (cosmetic number 4). These cosmetics were analyzed using the procedure described in this study, and the results are shown in Table 8. Each value is the mean of triplicates.

Quantitatively measured values for MAP (labeled as 1.5%) in the lotions ranged from 1.35 to 1.47%, indicating little difference in comparison to the labeled concentration. MAP contents in the lotion were within 90 to 98% of the labeled amount. Quantitatively measured values for ART in the whitening mask, however (labeled as 2.0%), were 2.26%; thus, the contents were 113% of the labeled amount. The results indicated that the concentration of ART exceeded the allowed amount (2%) [16].

Table 8. Assay results for the HPLC analysis of MAP in three commercial cosmetic products

Sample	Whitening agents	Label claim (MAP%, w/w)	Found (MAP%, w/w)	% of label
Cosmetic number 1	MAP	1.5	1.36	90.7
Cosmetic number 2	MAP	1.5	1.35	90.0
Cosmetic number 3	MAP	1.5	1.47	98
Cosmetic number 4	ART	2	2.26	113

4. Conclusion

In this report, we had developed a feasible gradient ion-pair high performance chromatographic method for the simultaneous determination of 5 whitening agents, and the chromatographic method we developed is suitable for routine analysis of the 5 whitening agents in commercial cosmetic products, or examined the allowable amount of whitening agents for the Department of Health.

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