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“Simultaneous Determination of Seven Whitening Agents by Ion-Pair Reversed-Phase High Performance Liquid Chromatography”

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Most whitening ingredients are water-soluble and have high hydrophilicity. Due to these characteristics, it is not easy to separate and quantify them by reverse phase chromatography. Ion-pair reversed-phase chromatography offers another possibility for separation and quantification. By adding ion pair reagents to form lipophilic ion pair compounds with negatively charged compounds, better resolution and separation efficiency can be achieved.

In this study, An ion-air reversed-phase high performance liquid chromatography method with ultraviolet detector was developed for simultaneous determination of seven whitening agents. including ascorbic acid (AA), magnesium, ascorbyl phosphate (MAP), ascorbic acid 2-glucoside (AA2G) , 3-o-ethyl ascorbic acid , kojic acid (KA), arbutin (AR) and hydroquinone (HQ). the monitor wavelength is 264 nm. The mobile phase contains 0.05 M KH₂PO₄ and tetrabutylammonium hydroxide. Under optimum condition, seven agents were successfully separated. The calibration curve has good linearity in the concentration range of 5ug/mL to 100ug/mL, and the linear correlation coefficient is greater than 0.999. The recoveries of homemade 1% essence were evaluated. For the essence, the recoveries were between 93% and 106%, This method was also successfully used to analyze four commercially available whitening products. The deviation between the measured value and the labeled value was less than 5%, and the spike recoveries ranged from 95% to 106%. The above results show that this method can be used as a reliable method to analyze the whitening ingredients in products.

Keywords: ion-air reversed-phase chromatography, whitening agents, lipophilic compound

1. Introduction

Whitening agents are widely utilized in cosmetic formulations due to their ability to inhibit melanin synthesis and promote skin brightening. Most whitening ingredients, such as ascorbic acid derivatives and hydroquinone, are highly hydrophilic and water-soluble compounds (Kim, Uyama, & Kobayashi, 2008; Ando, Ryu, Hashimoto, & Ichihashi, 2010). Their polar nature, however, poses a challenge for effective separation and quantification using conventional reversed-phase high-performance liquid chromatography (RP-HPLC), which typically favors hydrophobic analytes (Dong, 2006).

Ion-pair reversed-phase chromatography (IPC) provides an alternative approach to overcome these limitations. By introducing ion-pairing reagents, such as quaternary ammonium salts, into the mobile phase, the hydrophilicity of polar analytes can be effectively masked, forming neutral or less polar complexes that enhance retention and resolution on reversed-phase columns (Snyder, Kirkland, & Dolan, 2012). This technique has been extensively applied in various fields. For instance, IPC is widely used to separate highly polar pharmaceutical compounds like amino acids and nucleotides (Honda et al., 1989), and to simultaneously analyze multiple organic acids in biological and food samples (Stella & Nti-Addae, 2007). In cosmetic science, IPC has been successfully employed to quantify active ingredients such as kojic acid and arbutin (de Orsi, Noto, & Rizza, 2009). Moreover, IPC plays a critical role in bioanalysis of charged biomolecules and environmental monitoring of anionic pollutants (Song & Xia, 2008; Kiss, 2001).

In this study, we aimed to develop and validate an ion-pair reversed-phase HPLC method coupled with ultraviolet (UV) detection for the simultaneous determination of seven whitening agents, including ascorbic acid (AA), magnesium ascorbyl phosphate (MAP), ascorbic acid 2-glucoside (AA2G), 3-O-ethyl ascorbic acid(3OEAA), kojic acid (KA), arbutin (AR), and hydroquinone (HQ) as shown in Fig 1. Optimization of chromatographic conditions was performed using potassium dihydrogen phosphate (KH_2PO_4) buffer and tetrabutylammonium hydroxide as the ion-pair reagent, with detection at 264 nm. The developed method was evaluated for linearity, accuracy, precision, and applicability to commercial whitening products, demonstrating its reliability and effectiveness for routine quality control of cosmetic formulations.

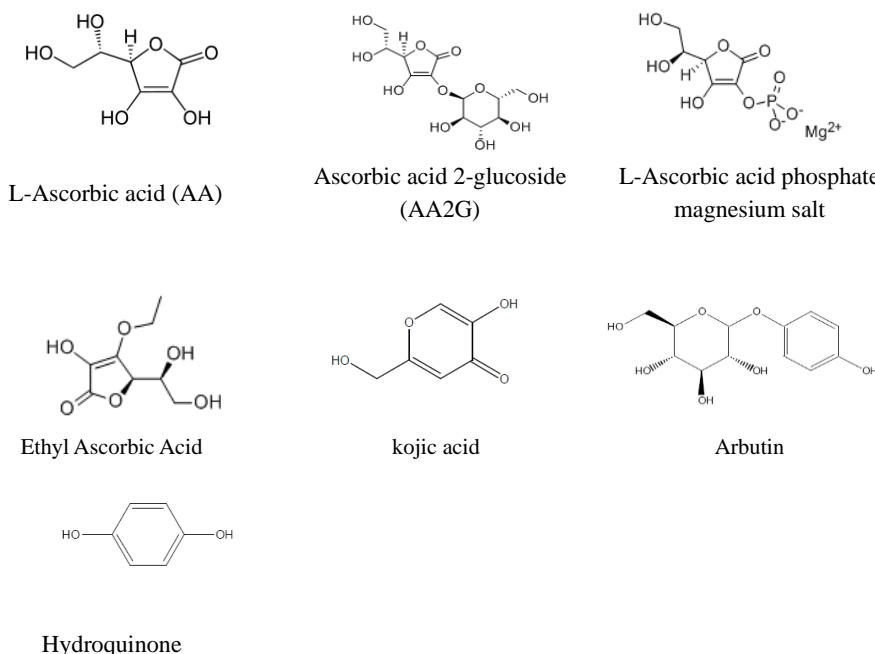


Figure 1: chemical structures of seven whitening reagents tested in this study

2. Materials and Methods

2.1 Materials

L-ascorbic acid was purchased from Sigma-Aldrich (USA). L-ascorbic acid phosphate magnesium salt n-hydrate was obtained from Wako Pure Chemical Industries (Japan). Ascorbic acid 2-glucoside was sourced from Hayashibara Co., Ltd. (Japan), and 3-O-ethyl ascorbic acid was supplied by Spec-Chem Industry (China). Kojic acid was purchased from Alfa Aesar (USA), while arbutin was obtained from Tokyo Chemical Industry (Japan). Hydroquinone was sourced from Sigma-Aldrich (USA).

Sodium hydroxide was purchased from United Chemical Reagent Company (Taiwan). Potassium dihydrogen phosphate was obtained from Kojima Chemical (Japan), and dipotassium hydrogen phosphate was purchased from Merck (Germany). Phosphoric acid was sourced from J.T. Baker (USA). Tetrabutylammonium hydroxide solution (TBAH, 40% w/w in water) was purchased from Alfa Aesar (USA). HPLC-grade methanol was obtained from J.T. Baker (USA).

2.2 Instruments

High-performance liquid chromatography (HPLC) analyses were carried out using a Hitachi L-7100 HPLC system equipped with a Hitachi L-7100 pump and a Hitachi L-7420 UV-Vis detector. Separation was performed on an Agilent HC-C18 column (4.6 × 250 mm, 5 µm particle size). The wavelength for detection is set at 264 nm. The HPLC system was operated under controlled laboratory conditions at 25°C.

2.3 Selection of Ion-Pair Reagent Concentration

Stock solutions of seven whitening agents were prepared at 1000 µg/mL in deionized water. A working solution of 50 µg/mL was obtained by mixing equal volumes of each stock and diluting with deionized water.

HPLC analysis was performed using an isocratic mobile phase of 0.05 M KH_2PO_4 (pH 2.5) containing TBAH and methanol (95:5, v/v), at a flow rate of 1.0 mL/min. Detection was carried out at 264 nm with an injection volume of 20 µL.

TBAH concentrations of 2, 5, 10, and 15 mM were tested based on the method of Miaw-Ling Chang et al. (Chang, M.-L et al 2003). Retention times and resolution were evaluated to determine the optimal TBAH concentration.

2.4 Effect of Organic Modifier Ratio and Buffer pH on Separation

Stock solutions of seven whitening agents were prepared at 1000 µg/mL in deionized water, and mixed equally to obtain a 50 µg/mL working solution.

A mobile phase of 0.05 M KH_2PO_4 buffer (pH 2.5) containing 2 mM TBAH was prepared and mixed with different proportions of methanol (2%, 3%, 4%, 5%, and 6%, v/v). Experiments were performed under the chromatographic conditions described in Section 2.3 to evaluate changes in retention time and resolution, and to determine the optimal methanol proportion.

Additionally, different buffer pH values were tested. Buffer solutions were prepared as follows: pH 2.5 using 0.05 M KH_2PO_4 and phosphoric acid; pH 5.0 using 0.1 M citric acid and sodium hydroxide; and pH 7.0 using 0.005 M K_2HPO_4 and 0.005 M KH_2PO_4 with sodium hydroxide adjustment. All buffers contained 2 mM TBAH and 4% methanol (v/v). Retention time variations were monitored to select the optimal buffer pH for analysis.

2.5 Preparation of Standard Solutions and Calibration Curves

L-ascorbic acid, magnesium ascorbyl phosphate, ascorbic acid 2-glucoside, 3-O-ethyl ascorbic acid, arbutin, hydroquinone, and kojic acid were accurately weighed and each dissolved in deionized water to prepare 10,000 µg/mL stock solutions. These stock solutions were further diluted with deionized water to obtain standard solutions at concentrations of 5, 10, 20, 50, and 100 µg/mL for calibration curve construction.

2.6 Sample Preparation and Spike Recovery Test

(a) Sample Preparation

Self-prepared emulsions containing different whitening agents and commercial products were evaluated. A 0.1 g portion of each sample was weighed into a 50 mL volumetric flask, diluted to volume with deionized water, sonicated, and filtered through a 0.45 µm nylon membrane before HPLC analysis.

(b) Spike Recovery Test

For recovery studies, 0.1 g of each sample was placed into volumetric flasks of different volumes, spiked with standard solutions at various concentrations, diluted to volume with deionized water, sonicated, and filtered through a 0.45 µm nylon membrane prior to HPLC injection.

2.7 Precision and Accuracy

To evaluate the intra-day precision and accuracy of the developed method, stock solutions of L-ascorbic acid, magnesium ascorbyl phosphate, ascorbic acid 2-glucoside, 3-O-ethyl ascorbic acid, arbutin, hydroquinone, and kojic acid were prepared at 1000 µg/mL in deionized water. These were diluted to concentrations of 5, 10, 20, 50, and 100 µg/mL. Each concentration level was analyzed by HPLC/UV detection in triplicate ($n = 3$).

2.8 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The determination of LOD and LOQ followed the guidelines established by the Environmental Analysis Laboratory . Stock solutions of the seven whitening agents were prepared at 1000 µg/mL, then diluted to 100 µg/mL as intermediate solutions. Calibration curves were established across the range of 0.5 to 20 µg/mL. Samples at 0.5, 1, and 2 µg/mL were each injected seven times, and the standard deviation (SD) of the peak areas was calculated. The LOD was determined by applying the SD to the calibration curve equation, while the LOQ was estimated as approximately 3.3 times the LOD.

3. Results and Discussions

3.1 Effect of TBAH Concentration on the Chromatographic Separation of Whitening Agents

Seven whitening agents at 50 µg/mL were analyzed using TBAH at concentrations of 2, 5, 10, and 15 mM. As shown in Figure 2, 15 mM TBAH significantly extended the retention time of magnesium ascorbyl phosphate to 30 minutes, while 2 mM TBAH greatly shortened the retention time.

At 2 mM TBAH, the resolution between ascorbic acid and ascorbic acid 2-glucoside was 2.71, and between kojic acid and arbutin was 6.07, both indicating complete separation. Moreover, complete separation was achieved within 20 minutes. Considering analysis time, resolution, and solvent consumption, 2 mM TBAH was selected as the optimal ion-pair reagent concentration.

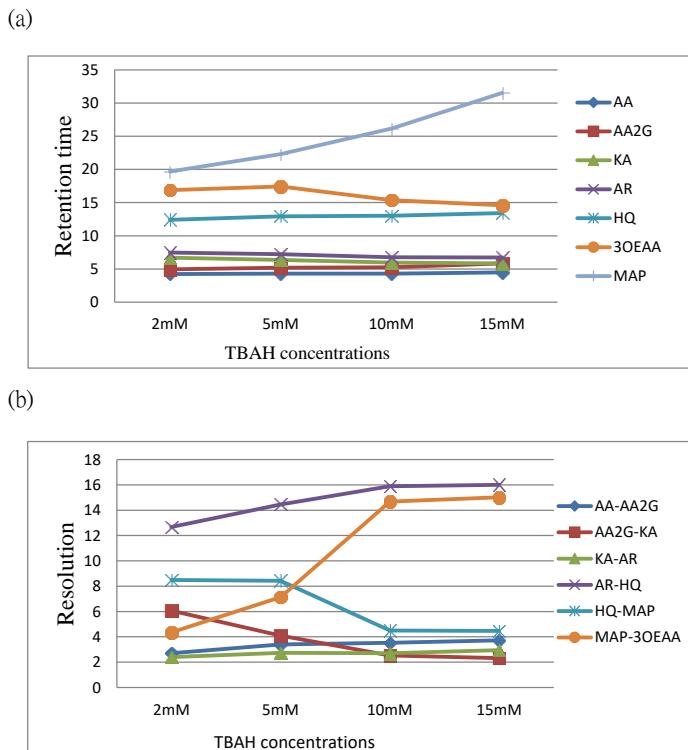


Figure 2. The effect of Different ion-pair reagent concentrations on (a) retention times and (b) resolutions.

3.2 Effect of Organic Modifier Ratio on the Chromatographic Separation of Whitening Agents

A standard solution of seven whitening agents at 50 µg/mL was analyzed with varying proportions of methanol as the organic modifier. As shown in Figure 3, increasing the methanol content to 6% enhanced the elution strength but also reduced the retention times, resulting in poor separation. With 5% methanol, all seven agents were separated; however, the resolution between ascorbic acid and ascorbic acid 2-glucoside (2.71) was slightly lower compared to that achieved with 4% methanol (2.83). Considering both retention time and resolution, adding 4% methanol to the mobile phase was determined to be the most appropriate condition.

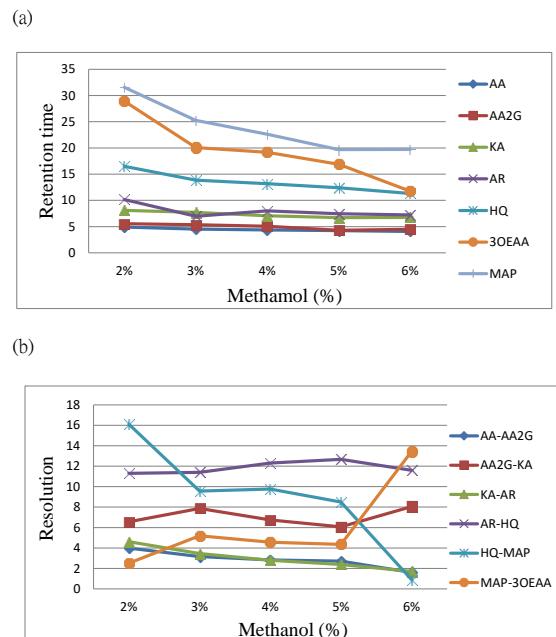


Figure 3. The effect of Organic Modifier Ratio on (a) retention times and (b) resolutions.

3.3 Effect of Buffer pH on the Chromatographic Separation of Whiting Agents

The pH of the mobile phase buffer can influence the ionization of analytes, thereby affecting chromatographic separation. In this study, a standard solution of seven whitening agents at 100 µg/mL was analyzed using buffer solutions at pH 2.5, 5.0, and 7.0. As shown in Fig. 4, the retention time of magnesium ascorbyl phosphate increased significantly with higher pH values.

Among the seven agents, achieving good resolution between kojic acid and arbutin was the most challenging. The resolution between ascorbic acid and ascorbic acid 2-glucoside was highest at pH 2.5, reaching 2.83, compared to lower resolutions at pH 5 and pH 7. Additionally, magnesium ascorbyl phosphate exhibited the shortest retention time at pH 2.5, and complete separation of all analytes was achieved under this condition. Therefore, the mobile phase buffer pH was adjusted to 2.5 for subsequent analyses.

Based on the optimized parameters, the mobile phase was composed of 0.05 M KH₂PO₄ (pH 2.5) with 2 mM TBAH and methanol in a 96:4 (v/v) ratio. Detection was performed at 264 nm with an injection volume of 20 µL, and the column was maintained at room temperature. The optimized chromatogram is shown in Figure 4.

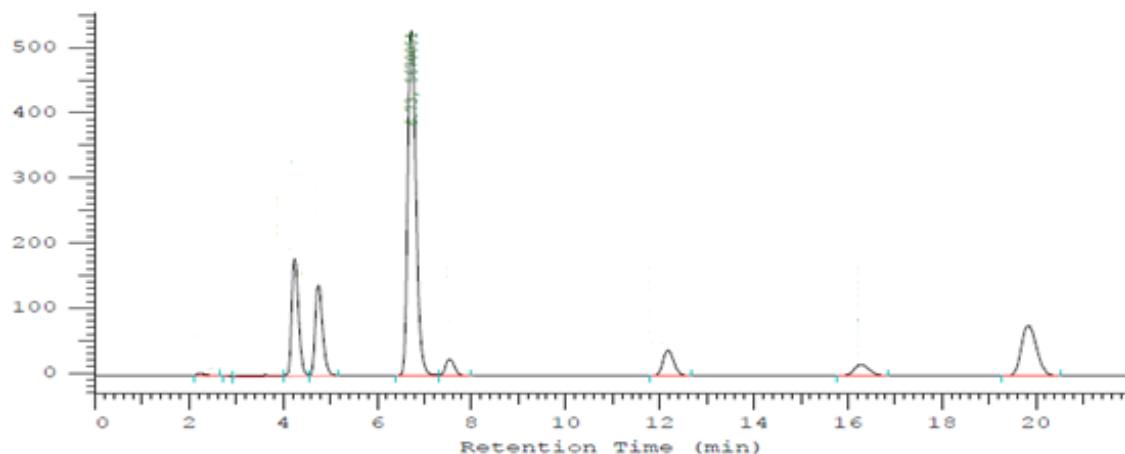


Figure 4 the optimum chromatography condition for seven whiting reagents. Separation condition: 0.050 M KH₂PO₄ (pH2.5), 0.2 mM TBAH, Peaks identification: AA(4.24 min); AA2G(4.94 min); KA(6.70 min); AR (7.46 min); HQ(12.41 min); MAP(16.89 min), 3OEAA(19.68 min).

3.5 Calibration Curve Establishment and Detection Limit

Mixed standard solutions of seven whitening agents at various concentrations were analyzed under optimized HPLC conditions. As shown in Table 1, all analytes exhibited excellent linearity within the concentration range of 5–100 µg/mL, with correlation coefficients (R^2) greater than 0.9993.

For the determination of detection limits, standard solutions at 0.5, 1, and 2 µg/mL were each injected seven times. The standard deviation (SD) of peak areas was calculated and

applied to the calibration curve to determine the limit of detection (LOD), while the limit of quantification (LOQ) was estimated as 3.3 times the LOD.

The LODs for the seven whitening agents were determined as follows: 0.03 µg/mL for ascorbic acid, 0.05 µg/mL for ascorbic acid 2-glucoside, 0.02 µg/mL for kojic acid, 0.16 µg/mL for arbutin, 0.19 µg/mL for hydroquinone, 0.40 µg/mL for magnesium ascorbyl phosphate, and 0.07 µg/mL for 3-O-ethyl ascorbic acid, as summarized in Table 1.

Table 1 the calibration curve, correlation coefficient LOD and LOQ of seven compounds

Compounds	Calibration curve	Correlation Coefficient	LOD ug/mL	LOQ ug/mL
AA	y= 2352.4x +28832	0.9996	0.03	0.09
AA2G	y=15464x +69022	0.9993	0.05	0.16
KA	y =2591.4x +1628.8	0.9995	0.02	0.06
AR	y=8448.5x +68507	0.9998	0.16	0.52
HQ	y=2291.4x -4817.6	0.9997	0.19	0.62
MAP	y=2591.4 x +1628.8	0.9995	0.4	1.32
3OEAA	y=27925x +95503	0.9993	0.07	0.23

* n=7 for LOD and LOQ

3.6 Spiking Recovery Determination of Whitening Agents in home-made Samples

The ion-pair chromatographic method established in this study was applied to self-prepared samples to evaluate reproducibility. The results of content determination and spike recovery are summarized in Table 2. The method demonstrated good recovery for samples containing ascorbic acid, ascorbic acid 2-glucoside, magnesium ascorbyl phosphate, 3-O-ethyl ascorbic acid, kojic acid, arbutin, and hydroquinone.

For both essence formulations, the recovery rates ranged from 97.49% to 112.56%, indicating that the proposed method is capable of accurately extracting and quantifying whitening agents from different cosmetic formulations.

The recovery rate was calculated using the following formula:

$$\text{Recovery (\%)} = \left(\frac{C_{\text{found}} - C_{\text{original}}}{C_{\text{spiked}}} \right) \times 100$$

Where:

- C_{found} is the concentration measured in the spiked sample,
- C_{original} is the concentration measured in the unspiked sample,
- C_{spiked} is the theoretical concentration of the added standard.

Table 2 spiking recoveries of 7 compounds in home-made samples

Compounds	Addition (%)	Measures (%)	Spiking Recovery(%)	
AA	0.5	0.52±0.07	101.08	
AA2G	0.5	0.53±0.08	102.09	
KA	0.1	0.13±0.005	112.15	
AR	0.5	0.49±0.01	100.73	
HQ	0.5	0.52±0.03	112.56	
MAP	0.5	0.47±0.03	97.49	
3OEEA	0.5	0.53±0.007	104.72	n=3

3.7 Intra-Day and Inter-Day Precision Analysis

The intra-day repeatability and inter-day reproducibility of the method were evaluated by assessing the precision of repeated injections. For intra-day testing, three consecutive injections were performed within a single day. For inter-day testing, the same procedure was conducted over three consecutive days.

Standard solutions of the seven whitening agents at concentrations of 10 and 100 µg/mL were used for the precision studies. The coefficient of variation (CV%) was calculated by dividing the standard deviation (SD) of the peak areas by the mean peak area and multiplying by 100.

As shown in Table 3, the intra-day CV% values for the seven analytes ranged from 0.16% to 3.04%, indicating good precision and reliability (CV% < 5%). For inter-day analysis, CV% values ranged from 0.04% to 10.32%, which were relatively higher compared to intra-day results, reflecting slightly lower precision and accuracy across different days.

Table 3 Intra-Day and Inter-Day Precision Analysis

Compounds	Intra-day CV(%)				Inter-day CV(%)			
	10µg/ml	20µg/ml	50µg/ml	100µg/ml	10µg/ml	20µg/ml	50µg/ml	100µg/ml
AA	3.04	0.23	1.13	0.43	1.08	0.75	0.77	1.11
AA2G	1.91	0.59	1.82	0.70	2.68	1.87	0.005	0.96
KA	1.68	0.76	0.34	0.10	1.48	2.15	0.65	1.14
AR	2.59	1.30	3.19	1.51	2.51	7.63	0.53	0.04
HQ	1.28	0.31	0.27	0.11	1.43	1.39	1.15	1.29
MAP	0.80	1.64	0.52	0.71	4.29	2.60	4.42	1.87
3OEEA	0.53	0.16	1.16	0.19	2.70	2.68	1.15	0.78

n=3

3.8 Analysis of Commercial Whitening Products

The validated ion-pair HPLC method was applied to the analysis of commercial whitening products. The selected samples included various formulations such as facial masks, essences, serum emulsions, and lotions. Products were chosen based on labeling claims that emphasized whitening or spot-lightening effects and indicated the presence of at least one of the seven whitening agents targeted in this study. All experiments were performed in triplicate.

The results, summarized in Table 4, showed that for Sample 1, although the label indicated the presence of both arbutin and magnesium ascorbyl phosphate, only arbutin (3.3%) was detected, while magnesium ascorbyl phosphate was not found. This may be due to the absence of the ingredient or degradation during manufacturing or storage. In Sample 3 (labeled arbutin 6%) and Sample 4 (labeled ascorbic acid 5%), the detected concentrations were consistent with the labeled claims. The recovery rates were $98.24 \pm 1.09\%$ and $99.20 \pm 2.98\%$, respectively.

Moreover, hydroquinone, which was not listed on the packaging, was not detected in any products containing arbutin. All measured concentrations were within the regulatory limits for labeled ingredients.

Table 4 Analysis of Commercial Whitening Products

No.	Labeled compound	Labeled content(%)	Measured content (%)	Spiking Recoveries(%)
01	Arbutin	---	3.33 ± 0.04	97.67 ± 1.89
	MAP	---	ND	104.45 ± 4.10
02	Ascorbic acid 2-glucoside	---	0.01 ± 0.001	103.08 ± 5.02
03	Arbutin	6%	6.05 ± 0.04	98.24 ± 1.09
	3-O-ethyl ascorbic acid	---	0.53 ± 0.004	104.78 ± 5.74
04	Ascorbic acid	5%	5.08 ± 0.13	99.20 ± 2.98

4. Conclusion

A robust and reliable ion-pair reversed-phase HPLC method with UV detection was successfully developed and validated for the simultaneous quantification of seven hydrophilic whitening agents, including ascorbic acid, ascorbic acid 2-glucoside, magnesium ascorbyl phosphate, 3-O-ethyl ascorbic acid, kojic acid, arbutin, and hydroquinone. Through systematic optimization of the ion-pair reagent concentration, organic modifier ratio, and buffer pH, the method achieved complete separation within 20 minutes under isocratic conditions. The method exhibited excellent linearity ($R^2 > 0.9993$), high precision (intra-day CV% < 5%), and satisfactory recovery rates across both self-prepared and commercial cosmetic formulations. The detection limits ranged from 0.02 to 0.40 µg/mL, demonstrating the method's high sensitivity and suitability for trace analysis.

Application to commercial products confirmed the method's practical utility, accurately identifying and quantifying labeled whitening agents while ensuring compliance with regulatory requirements. Importantly, no unauthorized hydroquinone residues were detected.

Overall, the established method provides a powerful analytical tool for routine quality control and regulatory inspection of cosmetic products containing multiple hydrophilic whitening agents. Its rapid analysis time, high sensitivity, and broad applicability make it a valuable contribution to the field of cosmetic product testing and quality assurance.

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