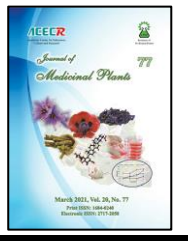
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Research Article

Optimization of cleaning and analytical method for determination of arbutin, hydroquinone and kojic acid in cosmetic products

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ABSTRACT

Background: Hydroquinone, arbutin and kojic acid are herbal-based skin whitening compounds that are used in many cosmetics. Today, the lack of side effects of arbutin and kojic acid has made them a valuable alternative to hydroquinone. Due to the toxic effects of whitening compounds, it is necessary to control the amount of use of these in the whitening products so that their concentration is within the permissible range. **Objective:** Therefore, the purpose of this study was to identify and quantify these three compounds in anti-lacquer products. In this research, the best separation of analytes has been tried by changing the HPLC mobile phase. **Methods:** Different samples were taken from the supply level. After extraction and preparation of samples, the solution was injected into HPLC-UV. In order to optimize the method, three different mobile phases were used to identify the compounds. By comparing the obtained peaks with the standard peaks, the Whitening compounds were identified and quantified. **Results:** The results showed that hydroquinone and kojic acid were applied in three products and arbutin in one product and the concentration of all the compounds used were within the standard range. Four out of the eight companies surveyed did not correctly identify the compounds used in their products. **Conclusion:** Reverse Phase HPLC-UV is also one of the best methods for determination and quantification the whitening compounds and water composition: acetonitrile with a ratio of 80:20 v/v is the most appropriate mobile phase in this method.

Abbreviations: HPLC, High Performance Liquid Chromatography; IMS, Ion Mobility Spectrometry; TLC, Thin Layer Chromatography; LC/MS, Liquid Chromatography/Mass Spectrometry

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1. Introduction

The most successful recent and natural skin whitening agents are arbutin, vitamin C, kojic acid, licorice extract, burnet root extract, scutellaria extract and mulberry [1]. Whitening agents are of major importance because of the negative effect of exposure to UV radiation on the skin [2]. Skin whitening products have become increasingly in demand in the past few years.

The main purpose for skin lightening products is lighten the skin as well as to even out skin tone or to treat pigmentation disorders such as freckles, melisma, pregnancy marks, and age spots [1]. These hyperpigmentation disorders can be treated using skin lighteners which inhibit biosynthesis of melanin via different mechanism [3]. Some of the whitening agents such as hydroquinone have side effects. Hydroquinone is potentially carcinogenic and is known to be a skin and respiratory irritant. Because of its carcinogenic properties, it has been banned in some countries like Kenya and Indonesia because of the fear of a cancer risk and only allowed to be used as a drug [3]. The lack of significant adverse effects of arbutin and its derivatives makes them a valuable alternative to hydroquinone [4]. However, the excessive use of arbutin and kojic acid would have complications. The allowable levels of skin lighteners in cosmetic products are 2 % for kojic acid, 7 % for arbutin [5, 6]. Over the counter hydroquinone products can contain 0.5 % to 2 %. Sometimes higher concentrations of 4 % and above are available from dermatologist in some countries for the gradual lightening of hyper-pigmented skin in conditions such as melasma, freckles and senile lentigenines as well as chloasma [3]. Whitening agents can be identified and determined using several analytical methods such as gas chromatography-mass spectrometry, kinetic spectrophotometry, flow injection analysis, HPLC-UV, TLC densitometry, capillary electrochromatography and differential pulse voltammetry. The most common approach for analysis of whitening agents in skin whitening products is HPLC. After HPLC separation, the detection of analytes is performed mostly by UV [7-9]. In recent studies the LC/MS was used as identification and determination of arbutin in nautral sorces [10]. Also using of nanomaterials for extraction of arbutin and hydroquinone before LC/MS analysis was reported [11].

Hydroquinone (1,4-benzenediol and also 1,4-dihydroxybenzene) [12] occurs naturally as a conjugate with beta-D-glucopyranoside in leaves, barks and fruits of several plants such as cranberry, cowberry, bearberry and blueberry [3].

Arbutin (4-hydroxyphenol β-D-glucopyranoside) [13] is glycoside derivative of hydroquinone [7]. It is the most popular and safest skin lightening agent [3] and exists naturally in plants and high quantities of it were found in plant families such as: Lamiaceae, Ericaceae, Saxifagaceae and Rosaceae [13].

Kojic acid obtains through isolation from the mycelia of *Aspergillus oryzae* grown on steamed rice. The term koji means steamed rice in Japanese [14]. It can also be derived from mushroom [3]. The structures of these components are shown in Fig. 1.

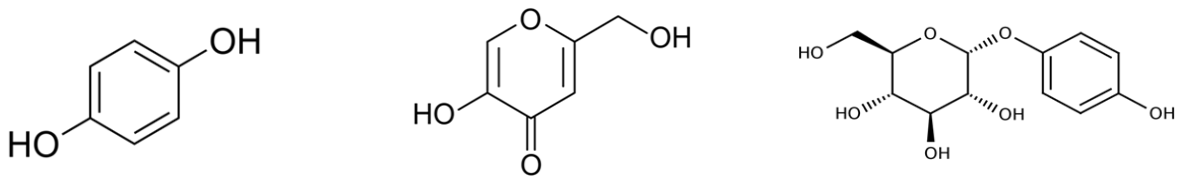
In this study the amount of kojic acid, arbutin and hydroquinone in some whitening products are determined by HPLC after preconcentration by liquid-liquid extraction.

2. Materials and Methods

2.1. Chemicals and skin whitening products

Hydroquinone, arbutin and kojic acid were purchased from Sigma-Aldrich. Acetonitrile, water and methanol were HPLC grade (Merck). Skin whitening products S_1 to S_8 were purchased from local stores. Details of each product are shown in Table 1.

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Hydroquinone (HQ) **Arbutin (AR)** **Kojic acid (KA)**

Fig. 1. Hydroquinone (HQ), arbutin (AR) and kojic acid (KA) structural formula

Table 1. The details of skin whitening products

Sample	Product type	Expiration date	HQ	KA	AR	Others
S_1	Penn soap	2020.08	-	-	*	*
S_2	Cream	2020.09	-	-	-	*
S_3	Gel	2021.02	-	-	-	*
S_4	Cream	2021.11	-	*	-	*
S_5	Cream	2020.10	*	-	-	*
S_6	Cream	2021.05	-	*	-	*
S_7	Cream	2020.11	-	-	*	*
S_8	Penn soap	2019.08	*	-	*	*

2.2. Extraction procedure

The extraction solvent in this study was water and acetonitrile (80:20 v/v). The internal standard method was used to eliminate the effect of product's matrix and improve the precision of quantitative analysis. For this purpose 50 ppm resorcinol was added to all products as an internal standard. The internal standard should behave as the analyte but should provide a signal that can be distinguished from that of the analyte.

About 1 g of each product was accurately weighted into a 15 ml centrifuge tube and dissolved in the extraction solvent. After mixing thoroughly with a vortex mixer for five minutes, it was sonicated 30 minutes, and centrifuged 10 minutes at 6000 rpm. The supernatant was filtered through a 0.45 µm HPLC syringe filter prior to HPLC analysis. The samples were injected through a 50 µl injection loop [14].

2.3. Instrumentation and chromatographic conditions

The HPLC method was performed on a liquid chromatograph with a KNAUER K-1001 pump. A UV visible detector KNAUER K-2501 and an injector fitted with a 50 µl loop were used in this research. The HPLC was carried out at a flow rate of 0.7 ml/min using a mobile phase constituted of water and acetonitrile (80:20 v/v) and detection was made at 280 nm. A Eurospher100-5 C_{18} column (250 × 4.6 mm) was used.

3. Results

3.1. Optimization of extraction conditions

Extraction was optimized by changing some factors.

a) Extraction solvent

In this method they have three extraction solvents had been used:

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1. A mixture of water and ethanol in the ratio of 45:55 (H_2O: MeOH) v/v.

2. A mixture of acetonitrile and methanol in the ratio of 80:20 (ACN: MeOH) v/v.

3. A mixture of water and acetonitrile in the ratio of 80:20 (H_2O: ACN) v/v.

The best extraction occurred using the third extraction solvent.

Based on the obtained results, maximum extraction recovery obtained by mixture of water and acetonitrile in the ratio of 80:20 (H_2O: ACN) v/v (Fig. 2a).

Since the tree target analyte have pKa > 9 so the acidic extraction solvent can improve extraction. The pH range of 3-9 were set for the mixture of acetonitrile and water and the pH 3 was the better pH for extraction. The pH blow 3 was not tested because may damage to the HPLC column.

b) Salting out

Salting out is a purification method that utilizes the reduced solubility of certain molecules in a solution of very high ionic strength. Salting out was used to investigate the extraction rate of the compounds from the sample. For this purpose, the extraction recovery at 1 %, 5 % and no added sodium chloride salt were investigated. The studies showed that salting out did not have a significant effect on the separation rate of compounds from the sample matrix (Fig. 2b).

c) Extraction time

For obtaining the best time to maximaze extraction the extraction times of 1, 2, 5 and 10 min were applied. The best extraction recovery obtained in 5 minutes at 6000 rpm. After 5 minute the extraction recovery didn't change significantly (Fig. 2c).

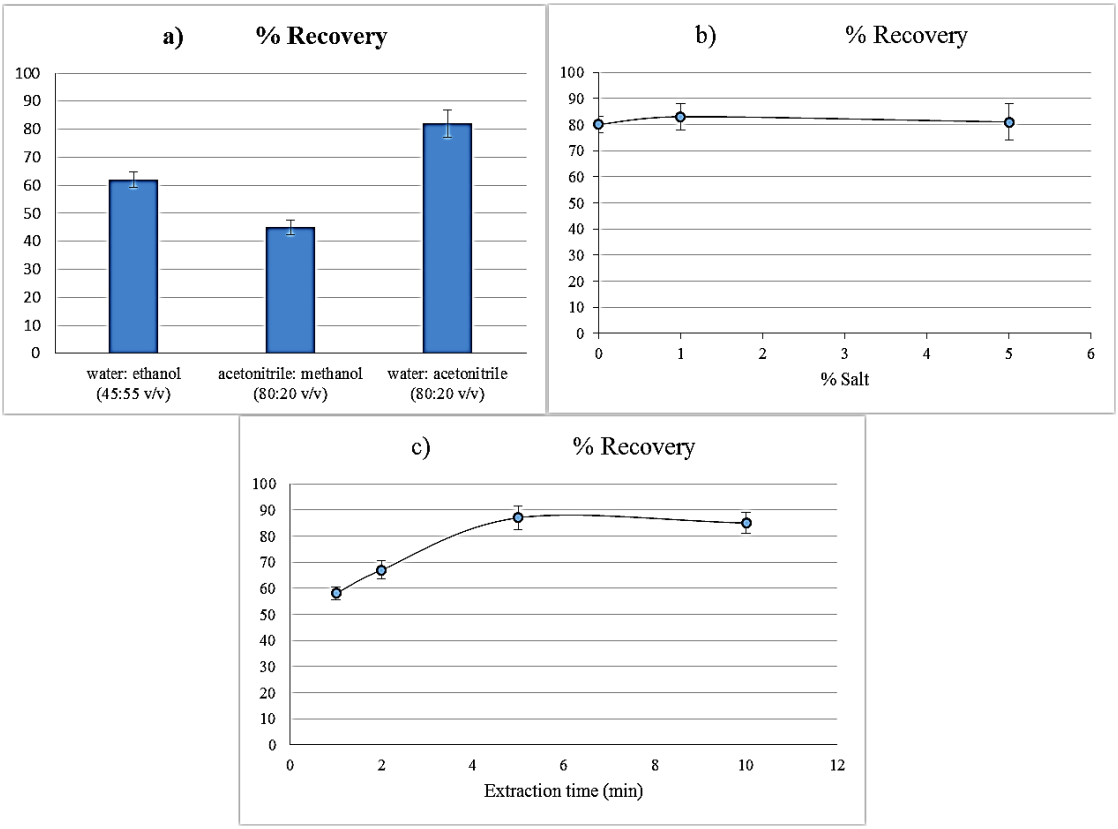


Fig. 2. The optimization charts for a) solvent extraction, b) salting out effect and c) extraction time

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3.2. Optimization of chromatographic conditions

The flow rate was tested from 1.2 ml/min to 0.5 ml/min. The best chromatograms were obtained at a flow rate of 0.7 ml/min.

In order to study the effect of different mobile phases on the separation of lightening agents, three mobile phases had been used:

1. A mixture of 0.05 M KH_2PO_4 buffer with methanol in the ratio of 99:1 v/v.
2. A mixture of acetonitrile: KH_2PO_4 in the ratio of 70:30 v/v.
3. A mixture of water: acetonitrile in the ratio of 80:20 v/v.

The best chromatograms were obtained using water and acetonitrile as a mobile phase.

3.3 Method validation

In optimized conditions with the best solvent, standard solutions were prepared in different concentrations to obtain the calibration curves and method's properties. The plots were linear in the range of 0.096-300 ppm for arbutin and 0.032-100 ppm for hydroquinone and kojic acid. A good linearity when the value of R^2 obtained was 0.99. The repeatability of method is below 5 % due to RSD values. Limit of detection (LOD) and limit of quantification of these compounds were estimated from the calibration curves. LOD values were found to be 0.0288 ppm for arbutin and 0.0096 ppm for hydroquinone and kojic acid. LOQ values were 0.096 ppm for arbutin and 0.032 ppm for hydroquinone and kojic acid. The values obtained which indicated high sensitivity of the proposed HPLC method. The results and chromatogram obtained are shown in Table 2 and Fig. 2 respectively.

Table 2. Result of method optimization

Whitening agents	Calibration equation	LR (ppm)	LOQ (ppm)	LOD (ppm)	RSD% (n = 3)		R^2
					Inter-day	Intra-day	
Hydroquinone (HQ)	A=100706C+63981	0.032-100	0.032	0.0096	3.18	4.25	0.9999
Kojic acid (KA)	A=115747C+170702	0.032-100	0.032	0.0096	1.29	2.54	0.9986
Arbutin (AR)	A=60598C-339378	0.096-300	0.096	0.0288	1.86	3.17	0.9909

3.4. Analysis of skin whitening products

As shown in Table 3, eight different formulations that claimed to contain at least one skin whitening agent were analyzed in this study.

The chromatograms of some of the products are shown in Fig. 3.

Sample S_1 and S_8 were two penn soap with labels claiming they contained arbutin while hydroquinone was identified about 0.025 % and 0.13 % in both of them, respectively. Product S_3 did not contain the desired lightening agents. About 1.5 % and 0.004 % of kojic acid were identified in product S_4 and S_6, respectively. Product S_2 contained about 0.027 % kojic acid, while claiming it was not used in this product. Product S_5 was a cream with about 1.6 % hydroquinone and product S_7 contained about 1.9 % of arbutin.

The results of the study show that hydroquinone and kojic acid are used in three products and arbutin is used in one product and all the compounds are used in the standard range of concentration while Three out of five companies did not list the compounds used in their products correctly.

In comparison with reported methods in Table 4, our chromatographic protocol described above, showed higher sensitivity and precision where the limit of detection and limit of quantification were less than other methods.

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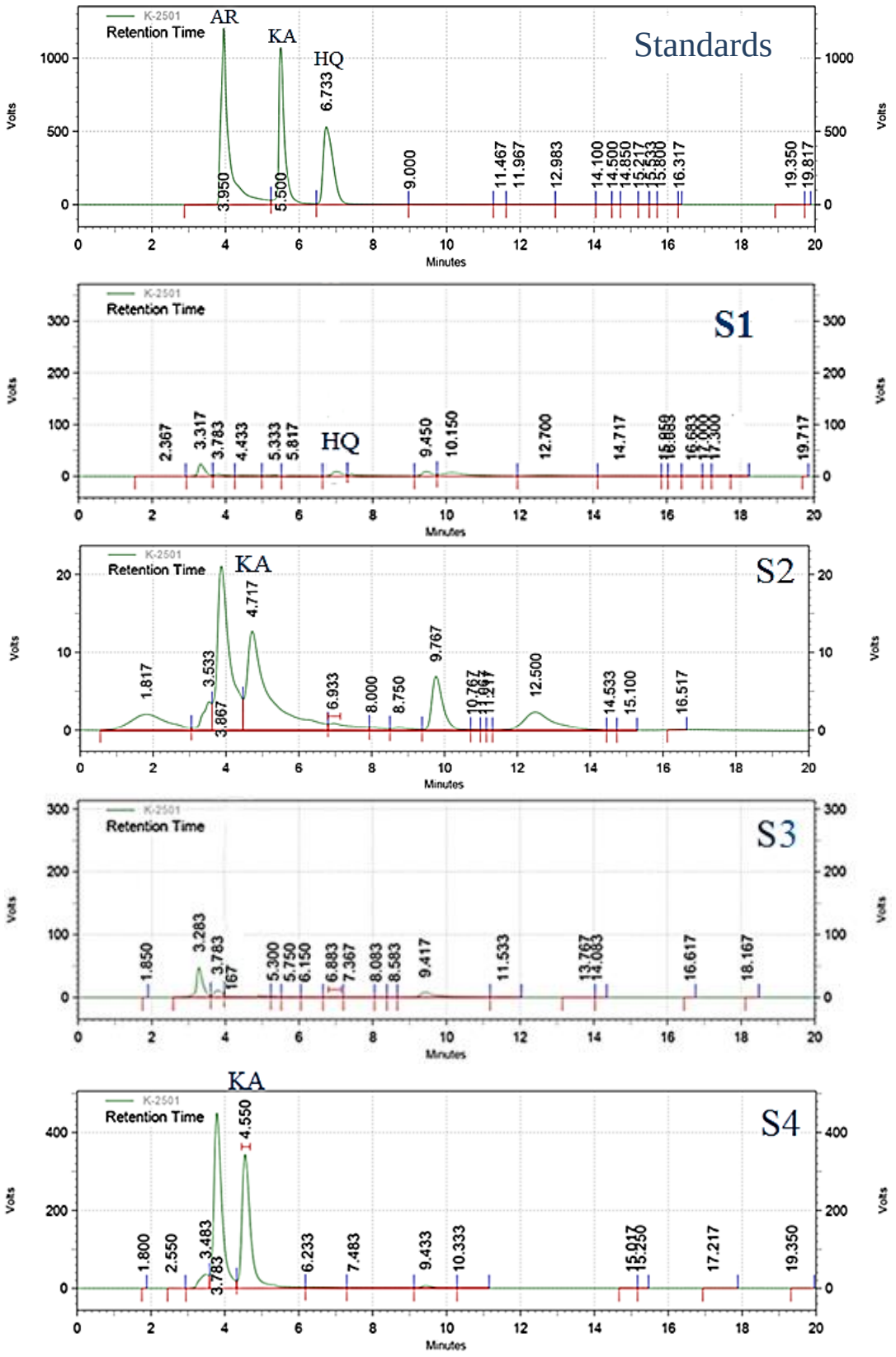


Fig. 3. The Chromatograms of standards and samples (S_1-S_8)

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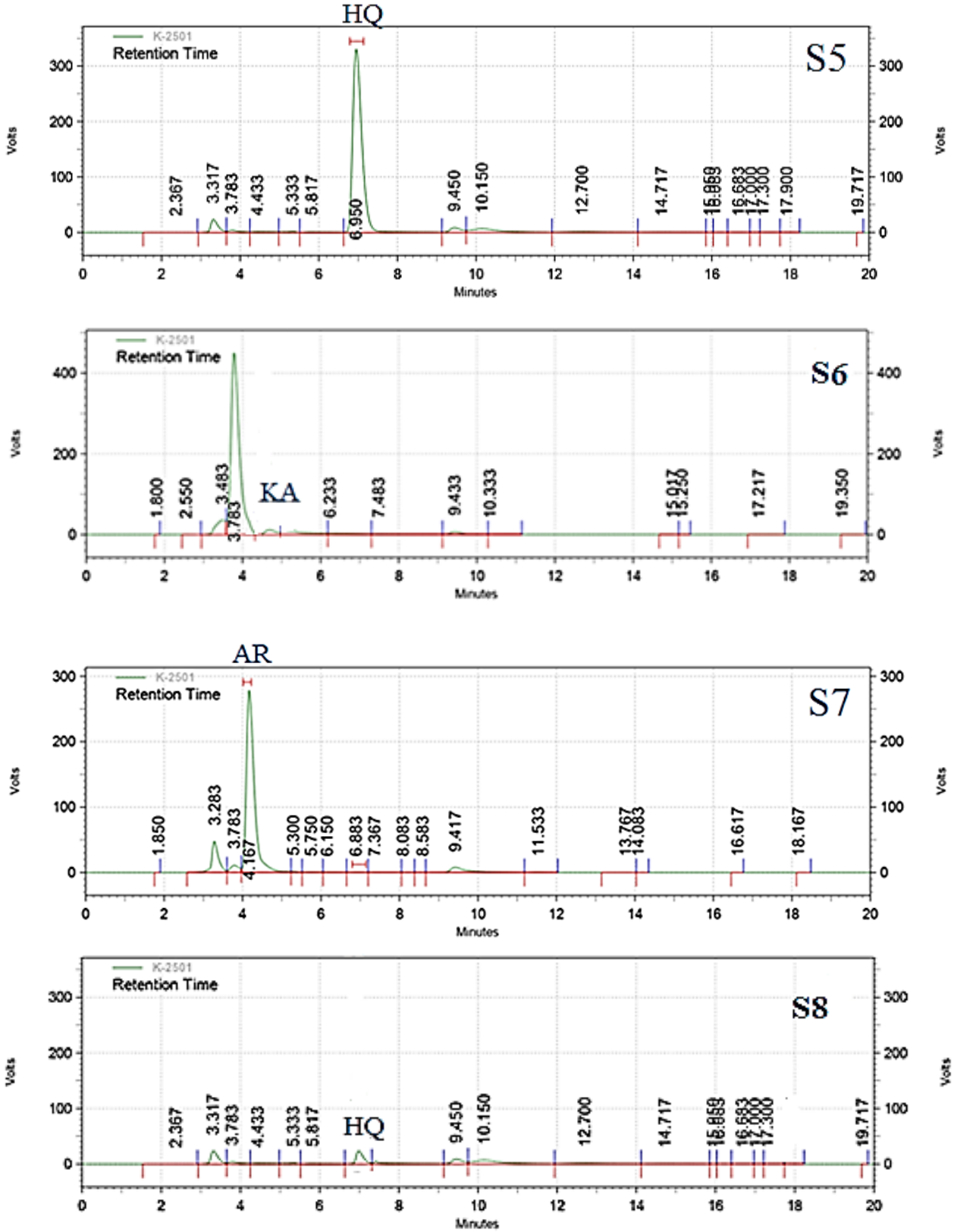


Fig. 3. The Chromatograms of standards and samples (S_1-S_8) (Continued)

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Table 3. Concentration of each whitening agents in the desired samples

Sample	Skin whitening agent		
	Hydroquinone (mg/g)	**Kojic acid (mg/g)**	**Arbutin (mg/g)**
S_1	0.251	-	-
S_2	-	0.275	-
S_3	-	-	-
S_4	-	15.306	-
S_5	15.843	-	-
S_6	-	0.0468	-
S_7	-	-	19.413
S_8	1.345	-	-

Table 4. Methods comparison

Analysis method	Extraction solvent	Analyte	LR (ppm)	LOD (ppm)	LOQ (ppm)	Year
HPLC	Methanol :water v/v 20:80	Hydroquinone	6-30	0.08	0.26	2005
UVDS	Sulfuric acid 0.1 N	Hydroquinone	10-26	0.14	0.46	2005
HPLC	Methanol	Arbutin	0.5-30	0.00507	0.001	2007
Flow Injection	Calcium carbonate, Lead acetate, Sodium phosphate	Arbutin	1-30	0.04	0.13	2009
RP-HPLC	Mixture of 20 M NaH_2PO_4 with 10 % methanol	Hydroquinone	-	0.1	0.3	2015
RP-HPLC	Mixture of 20 M NaH_2PO_4 with 10 % methanol	Kojic acid	-	0.05	0.2	2015
Proposed method	Water: acetonitrile 80:20 v/v	Hydroquinone	0.032-100	0.0096	0.032	2020
		Kojic acid	0.032-100	0.0096	0.032	
		Arbutin	0.096-300	0.0288	0.096	

4. Discussion

In comparison with reported methods in Table 4, our chromatographic protocol described above, showed higher sensitivity and precision where the limit of detection and limit of quantification were less than other methods. Wide linear dynamic range compared to other method shows the ability of this method to determine the both trace and major value of the target analytes accurately. P. Lopez Garcıa *et al*. in 2005 validated a HPLC and a UV derivative spectrophotometric methods for determination of hydroquinone in gel and cream preparations [15]. In this study HPLC method was about 20 times more sensitive than UVDS method and its HPLC results is comparable with our study. In other

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study, arbutin was determined by HPLC in skin-whitening creams and medicinal plant extracts. A detection and quantitation limit of 0.005 ppm and 0.001 ppm, respectively. This reported method was optimized based on analysis of only arbutin otherwise the proposed method can determine three whitening agent simultaneously [16]. In 2015 the method was reported by Y. Hong Wamg for quantitative determination of α-arbutin, β-arbutin, kojic acid, nicotinamide, hydroquinone, resorcinol, 4-methoxyphenol, 4-ethoxyphenol, and ascorbic acid from skin whitening products by HPLC [7]. In comparison with our study the obtained sensitivity was lower.

5. Conclusion

The proposed method was showed that the reverse phase HPLC-UV after proper extraction method is one of the best methods of determination and quantification of the number of lightening agents.

This method was so sensitive with low detection and quantification limit. In order to optimize the method, three different mobile phases were used to identify the compounds. Also, with the help of internal standard method the number of analytes obtained with high accuracy and repeatability.

Author contributions

N. E. carried out the experiments and collected available literatures and prepared the first version of the manuscript with the help of F. T. and H. R. F. T. also analyzed the statistical data and investigated the accuracy of the tests. HR. A-A, Sh. R. and M. T. replicated all the evaluations.

Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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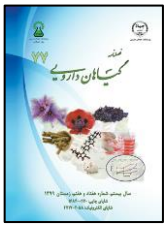
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مقاله تحقیقاتی

بهینه‌سازی روش استخراج و اندازه‌گیری معتبر شده برای تعیین مقدار آربوتین، هیدروکینون و کوجیک اسید در محصولات آرایشی و بهداشتی

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اطلاعات مقاله

گل‌واژگان:
ترکیبات روشن‌کننده
هیدروکینون
آربوتین
کوجیک اسید
HPLC-UV

چکیده

مقدمه: هیدروکینون، آربوتین و کوجیک اسید ترکیبات روشن‌کننده‌ای با منشا گیاهی هستند که در بسیاری از فرآورده‌های آرایشی مورد استفاده قرار می‌گیرند. امروزه، فقدان عوارض جانبی آربوتین و کوجیک اسید، آنها را به جایگزین ارزشمندی برای هیدروکینون تبدیل کرده است. **هدف:** با توجه به اثرات سمی ترکیبات روشن کننده، لازم است که میزان استفاده از این ترکیبات در محصولات روشن‌کننده به صورت کمی کنترل شود تا غلظت آنها در محدوده‌ی مجاز قرار داشته باشند. از این رو هدف از این تحقیق، شناسایی و تعیین مقدار این سه ترکیب در فرآورده‌های ضدلک است. **روش بررسی:** نمونه‌های مختلف، از سطح عرضه تهیه شد. پس از استخراج و آماده‌سازی نمونه‌ها، محلول حاصله به دستگاه HPLC-UV تزریق شد. به منظور بهینه‌سازی روش، از سه فاز متحرک مختلف به منظور شناسایی ترکیبات استفاده شد. با مقایسه پیک‌های حاصله با پیک‌های استاندارد، ترکیبات روشن‌کننده شناسایی و تعیین مقدار گردیدند. **نتایج:** نتایج حاصل از بررسی‌ها بیانگر آن است که هیدروکینون و کوجیک اسید در سه محصول و آربوتین در یک محصول به کار گرفته شده است و غلظت تمامی ترکیبات استفاده شده، در محدوده‌ی استاندارد قرار دارند. این در حالی است که چهار شرکت از هشت شرکت مورد بررسی، ترکیبات مورد استفاده در محصولات خود را به درستی بیان نکرده‌اند. **نتیجه‌گیری:** HPLC-UV فاز معکوس، یکی از بهترین روش‌های شناسایی و تعیین مقدار ترکیبات روشن‌کننده و ترکیب آب: استونیتریل با نسبت ۸۰:۲۰ مناسب‌ترین فاز متحرک در این روش است.

مخففها: HPLC، کروماتوگرافی مایع با کارایی بالا؛ IMS، طیف‌سنج تحرک یونی؛ LC/MS، کروماتوگرافی مایع متصل به طیف‌سنج جرمی، TLC، کروماتوگرافی لایه نازک

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