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High-performance liquid chromatographic determination of arbutin in skin-whitening creams and medicinal plant extracts

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Synopsis

A high-performance liquid chromatographic method was developed for quantitative analysis of arbutin. The arbutin was separated on an ODS Hypersil® C_{18} column with a mobile phase of water:methanol:0.1 M hydrochloric acid (89:10:1, v/v/v). The level of arbutin was measured by means of UV detection at 222 nm. The optimum conditions for arbutin quantitative analysis were investigated. The calibration curve was found to be linear up to 1,000 $\mu g/ml^{-1}$ of arbutin concentration, and the working calibration curve for arbutin determination over the range 0.5–30.0 $\mu g/ml^{-1}$ of arbutin (r^2 = 0.9999) was established. The relative standard deviations for intraday and interday were found to be 0.98% and 1.15%, respectively. A detection limit (3σ) and quantitation limit (10σ) of 0.02 $\mu g/ml^{-1}$ and 0.2 $\mu g/ml^{-1}$, respectively, and a mean percentage recovery of the spiked arbutin of 99.88 ± 1.12% were obtained. The proposed method has been applied to the determination of arbutin in commercial skin-whitening creams (Arbuwhite® cream, Super Whitening® cream, and Shiseido® cream) with average contents of 7.60, 5.30, and 57.90 mg/g^{-1}, respectively. It was also applied to the determination of arbutin in medicinal plant extracts from *Betula alnoides* Buch. Ham., *Clerodendrum petasites* S. Moore, *Curculigo latifolia* Dryand. Var. latifolia, and *Hesperethusa crenulata* (Roxb.) Roem, levels of which were found to be 3.50, 1.50, 1.10, and 0.12 $\mu g/g^{-1}$, respectively (no article reported in the literature about arbutin analysis). The proposed HPLC method is rapid, simple, and selective for routine analysis.

INTRODUCTION

Skin-whitening products have become increasingly in demand in the past few years. The main purpose for skin-lightening products is to lighten the skin as well as to even out skin tone or to treat pigmentation disorders such as freckles, melasma, pregnancy marks, and age spots (1). The most successful recent and natural skin-whitening agents are arbutin, vitamin C, kojic acid, licorice extract, burnet root extract, scutellaria extract,

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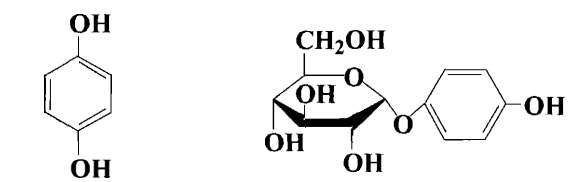
and mulberry. These agents are all tyrosinase inhibitors, which inactivate tyrosinase (the enzyme responsible for skin pigmentation) by chelating with its vital copper ion and suppressing tautomerization from dopachrome to DHICA. Normal skin color is formed by melanin, a natural pigment that also determines hair and eye color. In the skin, the enzyme tyrosinase biochemically converts the amino acid tyrosine into melanin. Hyperpigmentation occurs when too much melanin is produced and forms deposits in the skin. Hyperpigmentation is not a medically harmful condition. However, it is always advisable to have new brown spots checked by a dermatologist to make sure they are not skin cancers (2). Arbutin is a naturally occurring glycoside of hydroquinone (Figure 1).

Arbutin is found in the bark and leaves of various plants, usually occurring together with methylarbutin. Naturally occurring arbutin was first characterized by Kawalier (3), who obtained it from bearberry leaves. It is also found in the leaves of blueberry, cranberry, and pear. Synthetic arbutin was first reported by Mannich (4), and later by others. Commercial arbutin is almost always synthetic in origin. Because of its antibacterial properties, arbutin is a constituent of the traditional medicine *uva ursi,* and it is widely used in a variety of formulations (5). The ability of arbutin to inhibit human melanin synthesis has given rise to its wide use in many cosmetic formulations (6). Arbutin protects the skin against damage caused by free radicals. It is a skin-whitening agent that is very popular in Japan and Asian countries for skin depigmentation. Arbutin inhibits the formation of melanin pigment by inhibiting tyrosinase activity (7). Back in the 18th century arbutin was first used in medical areas as an anti-inflammatory and antibacterial agent. It was used particularly for cystitis, urethritis, and pyelitis. These uses have been applied until today, when natural medicine uses only natural ingredients to treat any disease. It may be used to repress the virulence of bacterial pathogens and to repress contaminating bacteria. It is also used for treating allergic inflammation of the skin. It can be used to whiten the skin, to prevent liver spots and freckles, to treat sunburn marks, and to regulate melanogenesis (8).

Arbutin is a very safe skin-whitening agent for external use, which does not have toxicity, a stimulating effect, an unpleasant odor, or side effects such as hydroquinone (Figure 1a). Hydrophilic arbutin can be incorporated in lipophilic media by encapsulation. Arbutin has three main properties: a whitening effect, an anti-aging effect, and a UVB/UVC filter (9). Arbutin is determined by many methods: the spectrophotometric method (10–12), capillary zone electrophoresis (13), and thin-layer chromatography-densitometry (14). The proposed HPLC method for determining arbutin in skin-whitening cosmetic products and some medicinal plants is more sensitive, precise, and less time-consuming than the previous HPLC methods described in the literature (15–



Hydroquinone (a) Arbutin (b)

Figure 1. Chemical structures of hydroquinone (a) and arbutin (b).

18). Furthermore, there is no data in the literature about the isolation and quantitative analysis of arbutin in *Betula alnoides* Buch. Ham., *Clerodendrum petasites* S. Moore, *Curculigo latifolia* Dryand. Var. latifolia, and *Hesperethusa crenulata* (Roxb.) Roem. Therefore, it is interesting to investigate the arbutin content in these medicinal plants, because naturally occurring arbutin is very safe skin-whitening agent.

EXPERIMENTAL

APPARATUS

HPLC analyses were carried out with Hewlett Packard Model 1100 liquid chromatograph with autosampler, thermostatic column compartment, online degasser, and a UV-visible detector model G 1313 A. The column used was ODS Hypersil® C_{18} (125 mm × 4 mm, 5.0 µm [Chromtech, Stockholm, Sweden]) with a Lichrosphere® 100 RP-18 (4 mm × 4 mm, 5.0 µm) guard column. The mobile phase was a mixture containing varying ratios of methanol, water, and 0.1 M hydrochloric acid, vacuum-filtered through 0.45-µm nylon membranes (Germany) before use. The following instruments were also used: a simultaneous spectrophotometer (Spekol 1200), a pH-meter (Model pH 900, Precisa, Switzerland), a water bath and shaker (Model SB-200-10, Thailand), an ultrasonicator (Model 889, Cole Parmer, USA), and a rotary evaporator (EYELA N-N series).

REAGENTS

The following standard reagents were used: arbutin HPLC grade 98% (Sigma, St Louis, MO) and resorcinol 98% (Fluka). The following reagents were used: hydrochloric acid (AR) (Farmitalia Carlo Erba, Italy), glacial acetic acid (AR) (Farmitalia Carlo Erba), acetonitrile (HPLC grade) (Lab-Scan Analytical Sciences, Ireland), ethyl acetate (AR) (BDH laboratory supplies, England), methanol (HPLC grade) (Lab-Scan Analytical Sciences), and ether (AR) (Lab-Scan Analytical Sciences). De-ionized distilled water was used throughout.

SAMPLES

Skin whitening products. Arbuwhite® cream (Nature Best Health Product Co., Ltd., Thailand), Super Whitening® cream (Aunyamanee Herbs, Thailand), and Shiseido® cream (Shiseido Co., Ltd., Tokyo, Japan) were used.

Plant material and location. The bark of *Betula alnoides* was collected at Bah Bae Village, Mae Dtang District, Chiang Mai Province, Thailand, in October 2003 and was identified by W. Thongchai 1. Voucher specimens have been deposited at CMU Herbarium, Chiang Mai University, Chiang Mai, Thailand. The roots of *Clerodendrum petasites* S. Moore were collected from Chiang Dow, Chaing Mai, Thailand. The tubers of *Curculigo latifolia* Dryand. Var. latifolia were collected from Papae, Maetang, Chiang Mai, Thailand. The trunk of *Hesperethusa crenulata* (Roxb.) Roem. was collected from Mae Sai, Chiang Rai, Thailand.

PROCEDURES

Sample preparation (skin-whitening cream). About 0.5 g of each whitening cream was accurately weighed and transferred into three separate 25-ml volumetric flasks and dissolved in methanol. To each flask 50 $\mu g/ml^{-1}$ of resorcinol was added as an internal standard. The solution was sonicated vigorously for 30 min, centrifuged at 4000 rpm for 30 min, and filtered on a Millipore membrane (0.45 μm) to obtain a transparent solution. The supernatant liquid was used for chromatographic analysis.

Extraction of medicinal plants. The dried medicinal plants were powdered. Then 6 kg of the powder was extracted with two successive portions of 5.0 l of de-ionized water and methanol. They were shaken in a wrist-action shaker for five hours and filtered. Then the solvent of the filtrate could be removed either by using a spray-dried technique (temperature 100°C and flow rate 1.0 ml/min^{-1}) to give a brown powder, or by using a rotatory evaporator to give a dark brown crude residue.

Preparation of standard solutions. A 1,000 $\mu g/ml^{-1}$ stock solution of arbutin standard was prepared in methanol. A series of each standard solution containing 0.5, 1.0, 3.0, 5.0, 10.0, and 30.0 $\mu g/ml^{-1}$ was prepared from the stock standard solution.

Preparation of sample solutions. Three sets of medicinal crude extracts (5 g) and cosmetic samples (0.5 g) of each set were extracted under reflux with 100 ml of 75% methanol for 30 min and filtered. The filtrate was evaporated to about 12 ml and transferred into a 250-ml separator followed by addition of 50 ml of water. The mixture was then extracted with ether (2 × 30 ml). The combined aqueous layer was extracted with ethyl acetate (3 × 50 ml). The combined ethyl acetate extract was then evaporated to dryness and dissolved in 10 ml of methanol.

Preliminary investigation. A preliminary investigation was carried out to separate some chemical constituents by TLC. The crude extract was extracted with 75% methanol under reflux for 30 min and then filtered. The filtrate was evaporated to about 12 ml and transferred to a 250-ml separating funnel together with 50 ml of water. This solution was extracted three times with 50 ml of ethyl acetate, and the combined ethyl acetate extracts were evaporated to dryness and the residues dissolved in 10% methanol. The sample solution and the standard solutions were separated on a silica gel GF_{254} (20 × 20 cm) glass plate, using ethyl acetate:methanol (9:1) as a developing solvent. The crude extract gave five well-defined spots. The R_f value of each spot was exactly the same as that obtained from each spot of standard.

Optimization of experimental conditions for RP-HPLC. RP-HPLC was performed under isocratic conditions. All experimental conditions were optimized by means of a univariate method as follows:

Analytical wavelength. Optimum absorbance of each standard solution was determined by injection of the same amount of mixed standard solutions (5.0 $\mu g/ml^{-1}$) at different wavelengths from 200 nm to 400 nm. The mobile phase was a mixture consisting of water:methanol (80:20 v/v) with a flow rate of 1.0 ml/min^{-1}. As the optimum to obtain the best sensitivity, λ_{max} was chosen.

Mobile phase. Various solvent systems were tested as the mobile phase for the separation of arbutin in the samples, e.g., water:acetonitrile:0.1 M hydrochloric acid (94:5:1, v/v/v), water:methanol:0.1 M hydrochloric acid (89:10:1, v/v/v), and methanol:100 mM phosphate buffer, pH 2.1 (10:90 v/v).

Mobile phase flow rate. The optimum flow rate of the mobile phase should provide good separation, high sensitivity, and short analysis time. In this work, after the optimal wavelength was selected, optimization of the flow rate was carried out by injecting the same concentration of mixed standard solutions at varying flow rates from 0.5 ml/min^{-1} to 1.0 ml/min^{-1}.

Recommended RP-HPLC procedure. A sample and/or standard solution containing arbutin was separated on a reverse-phase ODS Hypersil® C_{18} column (125 mm × 4 mm, 5.0 μm) and detected at 222 nm. An aliquot of 100 μl of a series of arbutin standard solutions and 100 μl of sample extract was injected into the LC system and eluted with the mobile phase, water:methanol:0.1 M hydrochloric acid (89:10:1, v/v/v) (flow rate = 1.0 ml/min^{-1}). The area of the arbutin peak was measured. Arbutin concentration in the plant extract was determined by reference to the calibration curve prepared under identical experimental conditions.

RESULTS AND DISCUSSION

A high-performance liquid chromatographic method for the determination of arbutin in skin-whitening creams and medicinal plant extracts containing arbutin was developed. The experimental conditions were investigated and the proposed method was also validated.

OPTIMIZATION OF RP-HPLC CONDITIONS

The optimal conditions of HPLC for determining arbutin were carried out under isocratic conditions. Various mobile phase systems with different compositions were investigated. First, the optimal wavelength for the detection of arbutin and other compounds, as mentioned earlier, was investigated, and the UV spectrum of each standard compound showed the absorption maxima at 222 nm. A wavelength of 222 nm showed the highest sensitivity for arbutin. Second, among the mobile phases studied, a mixture consisting of water:methanol:0.1 M hydrochloric acid (89:10:1, v/v/v) was used as the mobile phase, and it was found that this mobile phase was the most suitable because it resulted in good retention times, resolution, and satisfactory peak profiles (Figure 2). Finally, the optimum flow rate was 1.0 ml/min^{-1}, as it gave a good resolution, high sensitivity, a short analysis time, etc. In the RP-HPLC analysis, arbutin and resorcinol (internal standard) showed single symmetrical peaks (retention time 5.7 min and 10.7 min), respectively.

METHOD VALIDATION

The proposed method was validated according to U.S. Pharmacopoeia; USP (19).

Sensitivity. The sensitivity of the assay was determined in terms of the detection limit (LOD) and the quantitation limit (LOQ). Detection limits and quantitation limits were estimated for each of the examined compounds. The values were calculated from the standard deviation (S.D.) of response and the slope of the curve (S) by means of the equations: LOD = 3.3 (S.D./S) and LOQ = 10 (S.D./S). Low LOD and LOQ values were

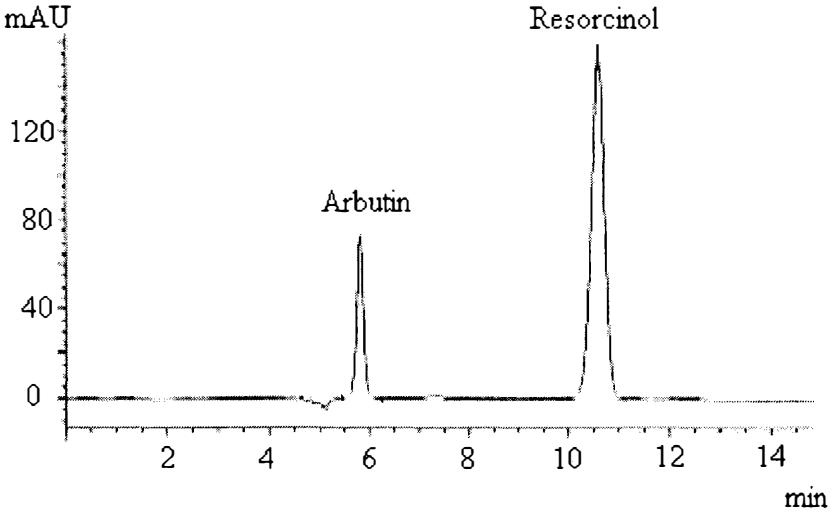


Figure 2. HPLC chromatogram of the arbutin standard (5 $\mu g/ml^{-1}$) and the resorcinol internal standard (5 $\mu g/ml^{-1}$).

obtained as shown in Table I, which indicates the good sensitivity of the proposed LC method.

Linearity. To determine linearity, five different concentrations of the arbutin standard were used in a working range of 0.5–30.0 $\mu g/ml^{-1}$. The linear regression equations and the correlation coefficient (r^2) values for arbutin and the internal standard are given in Table I. The r^2 values show good linearity in the examined concentration range.

PRECISION AND ACCURACY

The intraday reproducibility study was performed during a period of three days. The results obtained showed that the arbutin peak area variabilities for standard solutions were within 0.00–0.02% R.S.D. For interday reproducibility, five replicate injections of various concentrations of arbutin were made within a day. The results obtained showed that the arbutin peak area variabilities for the standard solutions were within 0.00–0.02% R.S.D. The results are shown in Table II. The R.S.D. values demonstrated good precision.

Table I
Linear Regression Analysis (n = 3) and Limits of Detection (S/N = 3) and Quantitation (S/N = 10)

Standard	Linearity range ($\mu g/ml^{-1}$)	Equation Y = SX + C	r^2 Mean (±S.D.)	LOD ($\mu g/ml^{-1}$)	LOQ ($\mu g/ml^{-1}$)
Arbutin	0.5–30	Y = 132.84 (±0.36)X − 9.32 (±1.23)	0.9999 ($\pm 5.77 \times 10^{-5}$)	5.07×10^{-3}	1.01×10^{-2}
Resorcinol*	0.5–30	Y = 257.03 (±0.91)X + 7.34 (±0.04)	0.9999 ($\pm 1.49 \times 10^{-8}$)	5.04×10^{-3}	1.00×10^{-2}

* Internal standard.

Table II
Intraday and Interday Precision Analysis of Arbutin Standard Solutions

Concentration added ($\mu g/ml^{-1}$)	Within-day variability (n = 5) (concentration found)		Between-day variability (n = 5) (concentration found)	
	Mean ± S.D. ($\mu g/ml^{-1}$)	%R.S.D.	Mean ± S.D. ($\mu g/ml^{-1}$)	%R.S.D.
1.00	0.99 ± 0.02	0.02	1.01 ± 0.02	0.02
5.00	5.00 ± 0.02	0.00	5.00 ± 0.02	0.00
10.00	10.09 ± 0.07	0.01	10.06 ± 0.11	0.01
15.00	15.02 ± 0.08	0.00	15.02 ± 0.08	0.00
20.00	19.88 ± 0.28	0.01	19.88 ± 0.28	0.01

The accuracy of the proposed method was determined by analyzing sample extract solutions spiked with three different concentrations of standard arbutin solution (3.0, 5.0, and 10.0 $\mu g/ml^{-1}$, respectively), using the proposed LC method. The recoveries of the arbutin standard solutions were also analyzed. The results are presented in Table III. The excellent recoveries of the standard addition amounts suggest that the method is accurate.

APPLICATION TO COSMETIC AND MEDICINAL PLANTS

The proposed RP-HPLC method was applied to the determination of arbutin in whitening creams and medicinal plant extracts after TLC extraction. The samples were separated on a silica gel GF_{254} (20 cm × 20 cm) glass plate using ethyl acetate:methanol:water (85:17:13, v/v/v) as mobile phase. After visualization, each sample showed a blue spot with an R_f value of 0.36, which corresponded to that of the standard. Arbutin in each sample was then determined by the proposed RP-HPLC method (Figure 3). A comparative determination of arbutin in the original skin-whitening cream samples was also carried out using the UV spectrophotometric method. The results are shown in Table IV. It is indicated that the amounts of arbutin found by both methods are quite identical. Excellent correlation between the two methods was obtained. The performance of the method was assessed by calculation of the Student *t*-test compared with the UV spectrophotometric method. It was evident that the *t*-value for arbutin determination, compared to the results obtained by the UV spectrophotometric method, was 1.38, which did not exceed the theoretical value (2.44) at the 95% confidence limit for the six degrees of freedom. These results indicated that the 95% confidence limit between the proposed HPLC method and the UV spectrophotometric method for the arbutin assay does not differ significantly. Four medicinal plant samples were collected: *Betula alnoides* Buch. Ham, *Clerodendrum petasites* S. Moore., *Curculigo latifolia* Dryand. Var. latifolia,

Table III
Accuracy of the Proposed LC Method (n = 7)

Standard	Concentration added ($\mu g/ml^{-1}$)	Concentration found: mean ± S.D. ($\mu g/ml^{-1}$)	Recovery (%)	Relative error (%)
Arbutin	3.00	2.96 ± 0.006	98.71 ± 0.22	0.04
Arbutin	5.00	5.03 ± 0.003	100.64 ± 0.06	-0.03
Arbutin	10.00	10.15 ± 0.009	101.53 ± 0.09	-0.15

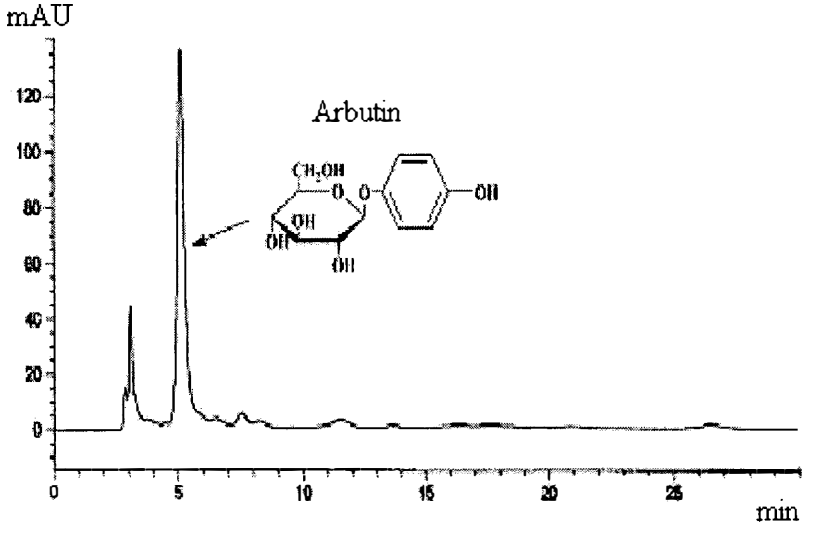


Figure 3. HPLC chromatogram of a sample containing arbutin from skin-whitening cream.

Table IV
Comparison of Two Methods for Measuring Arbutin in Samples of Commercial Whitening Cream

Cosmetic sample (Arbuwhite® cream)	Amounts of arbutin found (g%)		Calculated *t*-value[b]
	RP-HPLC method[a]	Spectrophotometric method[a] (20)	
	0.75	0.77	
	0.75	0.78	
	0.77	0.80	
	0.76	0.78	1.38
	0.76	0.79	
	0.75	0.78	
	0.77	0.80	
Average	0.76	0.79	

[a] Each value is the average of 7 determinations.
[b] Calculated *t*-value for p = 005 and six degrees of freedom is 2.44.

and *Hesperethusa crenulata* (Roxb.) M. Roem. The samples were extracted with water and methanol. Then the amounts of arbutin in the samples were determined by the RP-HPLC method. The amounts of arbutin found in the aqueous extract were 3.50, 1.50, 1.10, and 0.12 $\mu g/g^{-1}$, respectively. The amounts of arbutin found in the methanol extract were 0.77, 0.0002, and 0.0012 $\mu g/g^{-1}$, respectively. The method was successfully applied to the determination of arbutin in three commercial skin-whitening creams. The amounts of arbutin found in the samples (Arbuwhite® cream, Super Whitening® cream, and Shiseido® cream) were 0.76, 0.58, and 5.79 mg/g^{-1}, respectively.

CONCLUSION

The reversed-phase high-performance liquid chromatographic procedure has been developed for determining arbutin in commercial skin-whitening creams and some me-

dicinal plant extracts, respectively. The method was also validated for limit of detection, limit of quantitation, repeatability, reproducibility, and accuracy. The optimum conditions and analytical characteristics for RP-HPLC determination of arbutin exhibited good resolution, short analysis time, and rather high sensitivity. In the proposed method for determining arbutin in skin-whitening creams, the working calibration curves over the ranges of 0.5–30.0 $\mu g/ml^{-1}$ were established. The method was successfully applied to the determination of arbutin in three commercial skin-whitening creams. The content of arbutin found in the samples (Arbuwhite® cream, Super Whitening® cream, and Shiseido® cream) were 0.76, 0.58, and 5.79 mg/g^{-1}, respectively. The method was also applied to the determination of arbutin in some medicinal plant extracts. The amounts of arbutin in *Betula alnoides* Buch. Ham., *Clerodendrum petasites* S. Moore., *Curculigo latifolia* Dryand. Var. latifolia, and *Hesperethusa crenulata* (Roxb.). Roem in the aqueous extracts were 3.50, 1.50, 1.10, and 0.12 $\mu g/g^{-1}$, respectively. Arbutin from these medicinal plants can be used for the production of skin-whitening cosmetics. The benefits of the proposed method are simplicity, convenience, rapidity, sensitivity, good precision, and accuracy. The method is suitable for routine analysis of arbutin in commercial cosmetics and raw plant materials.

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