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ปีการศึกษา 2552

METHOD DEVELOPMENT FOR DETERMINATION OF ALPHA- AND BETA-ARBUTINS IN COSMETICS BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry

Department of Chemistry
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Thesis Title	Method Development for Determination of Alpha- and Beta-arbutins in Cosmetics by Ultra Performance Liquid Chromatography
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มัณฑินี มาลา : การพัฒนาวิธีตรวจวัดแอลฟาและบีตาอาร์บูทินในเครื่องสำอางโดยอัลตราเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี (METHOD DEVELOPMENT FOR DETERMINATION OF ALPHA- AND BETA-ARBUTINS IN COSMETICS BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. ปกรณ์ วรานุศุภากุล 78 หน้า.

เครื่องสำอางที่ช่วยให้ผิวขาวกำลังเป็นที่นิยมในปัจจุบัน ไฮโดรควิโนนเคยให้ใช้ในเครื่องสำอางเพื่อช่วยให้ผิวขาวได้ในอดีต แต่เนื่องจากว่าเมื่อใช้ไปนานๆจะเกิดผลเสียรุนแรงต่อผิวหนัง จึงถูกจัดเป็นสารห้ามใช้ในเครื่องสำอาง ดังนั้นจึงมีสารที่ช่วยให้ผิวขาวชนิดอื่นๆที่นำมาใช้แทนไฮโดรควิโนน เช่นอาร์บูทิน บีตาอาร์บูทินซึ่งเป็นอนุพันธ์ของไฮโดรควิโนน และได้จากธรรมชาติ ขณะที่อัลฟาอาร์บูทินได้จากการสังเคราะห์ อัลฟาอาร์บูทินมีความเสถียรและมีประสิทธิภาพในการออกฤทธิ์ได้ดีกว่าบีตาอาร์บูทินแต่ราคาแพงกว่ามาก ดังนั้นในการคุ้มครองผู้บริโภค จึงมีความจำเป็นต้องพัฒนาวิธีการตรวจวัดเพื่อหาปริมาณบีตา-และอัลฟาอาร์บูทินให้ได้ในคราวเดียวกัน เพื่อให้ผู้บริโภคได้ใช้ผลิตภัณฑ์ที่มีสารสำคัญตรงตามฉลากระบุ สำหรับวิธีนี้เป็นการพัฒนาวิธีการตรวจวัดหาปริมาณบีตา-และอัลฟาอาร์บูทินในผลิตภัณฑ์เครื่องสำอางโดยใช้เครื่องมือสมัยใหม่ที่เรียกว่าอัลตร้าเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี (UPLC™) เครื่องตรวจวัดยูวีที่ความยาวคลื่น 283 นาโนเมตร โดยการศึกษาจากคอลัมน์หลายประเภท ใช้เฟสเคลื่อนที่ในระบบเกรเดียนที่ประกอบด้วย 0.1% อะซิตริกแอซิดกับเมทานอล เวลาที่ใช้วิเคราะห์น้อยกว่า 6 นาที โดยมีช่วงของความเป็นเส้นตรงของสารทั้งสองชนิดที่ 2-30 ไมโครกรัมต่อมิลลิลิตร เปอร์เซ็นของการคืนกลับจากการเติมสารมาตรฐานลงในตัวอย่างเครื่องสำอางที่มีเนื้อเมทริกซ์ต่างๆ (ครีม, โลชั่น และเจล) อยู่ระหว่าง 98-102 % สำหรับวิธีนี้ทำให้ได้ค่าการแยกของสารที่ดีและมีความรวดเร็วกว่าวิธีที่ใช้เครื่องไฮเพอร์ฟอร์มานซ์ลิควิดโครมาโทกราฟี

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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MUNTINEE MALA : METHOD DEVELOPMENT FOR DETERMINATON OF ALPHA- AND BETA-ARBUTINS IN COSMETICS BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY. THESIS ADVISOR : ASST. PROF. PAKORN VARANUSUPAKUL, Ph.D., 78 pp.

Skin whitening products are now among the most desire cosmetic for beauty-care customers. In the past, hydroquinone was used as a whitening agent but it was banned due to its severe effects to human skin. Since then, there have been other alternative whitening agents available in the market that included arbutins. Beta-arbutin has been widely used since it is a natural product whereas alpha-arbutin is synthetic. Both are skin whitening active but alpha-arbutin offers higher stability and more efficiency, acts faster than beta-arbutin, so it is more expensive. For health consumer protection, it is necessary to develop a reliable method to assure that the cosmetic product contain such arbutin as it is claimed. This work aims to develop a method for simultaneous determination of alpha- and beta-arbutin in cosmetic products by using UPLC™. The method was developed on Waters ACQUITY UPLC™ system with UV/VIS spectrometric detection at 283 nm. Several columns were investigated and gradient elution profile of 0.1% acetic acid and methanol as a mobile phase were optimized. The analysis time was less than 6 min. The linear working ranges of both arbutins were 2-30 µg/mL (R^2=0.9999). The %recoveries of spiked synthetic cosmetics in various matrices (cream, lotion and gel) were between 98-102% with %RSD less than 0.6. Developed method offered better resolution, speed and sensitivity than a typical HPLC analysis method.

Department : Chemistry Student's Signature

Field of Study : Chemistry Advisor's Signature Pakorn Varanusupakul

Academic Year : 2009

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LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
UPLC	Ultra performance liquid chromatography
RPD	Relative percent different
RSD	Relative standard deviation
PDA	Photo diode array
N	Theoretical plate
R_S	Resolution
t_R	Retention time
k´	Retention factor
LOD	Limit of detection
HSS	High strength silica
BEH	Bridged ethyl hybrid
R^2	Coefficient of determination
r	Correlation coefficient
PVDF	Polyvinylidene fluoride

CHAPTER I

INTRODUCTION

The skin is responsible for protecting human body from the external environment such as pollutions, diseases and especially UV radiation by creating a melanin to absorb the UV radiation. Melanocytes are cells located at the bottom layer of the skin epidermis that are stimulated by enzyme tyrosinase to produce the melanin [1]. Too much production of melanin can cause abnormality of the pigment on the skin. Sunlight is the main cause for appearance of brown-spots such as freckle and melasma which are not desirable to some people [2]. Therefore, whitening cosmetic products have become more popular these days because of their abilities to repair such undesirable marks on the skin. There are many types of whitening agents available, such as hydroquinone, arbutin, kojic acid, kojic dipalmitate, licolice and vitamin C, which function based on the inhibition of the enzyme tyrosinase [3] subsequently, the production of melanin would be lessen and the freckle or the melasma would be subsided.

Before the year of 1996, hydroquinone had been a specially controlled substance [4]. The maximum does of 2.0% w/w was allowed for topical application in skin whitening products. Since then, it has been banned due to its effect on the skin such as irritation, mild itching, reddening of the skin and permanent skin damage if continually used for a long period of time. Moreover, hydroquinone is easily oxidized so that its potency could be lost [5]. Alternatively, there are other whitening agents available already mention above. Arbutin is one of the most popular whitening agents available in the market. Beta-arbutin is a natural product that is extracted from the bearberry plant [6]. Despite beta-arbutin is a derivative of hydroquinone, it does not cause skin irritation and other side effects. It is less reactive to the oxidation reaction compared to hydroquinone. In addition, it is relatively inexpensive. However, it is not

stable at the very acidic condition (about pH 4.5) and may cause color change in the final product if stored for a long period of time.

In the year of 2002, a synthetic alpha-arbutin was introduced. It offers higher stability and more efficiency than beta-arbutin [7]. Alpha-arbutin acts faster than beta-arbutin. It is stable at the more acidic condition (about 3.5). Furthermore, its color and odor is not changed during storage. Therefore, it is about 8 times more expensive than beta-arbutin. For this reason, there may be some manufacturers taking advantage by misleadingly advertising that there products contain alpha-arbutin to increase their sale price. For the quality control purpose, it is necessary to develop a reliable method for the simultaneous determination of alpha- and beta-arbutin.

Literature reviews

Several methods have been reported for determination of beta-arbutin in whitening cosmetic products and all of them were based on High Performance Liquid Chromatography (HPLC) methodology. M.-O. Masse. *et al*. [8] presented the HPLC analysis to confirm the preliminary identification of kojic acid and arbutin in skin whitening cosmetics by Tin Layer Chromatography (TLC). The HPLC conditions consisted of using the Lichrosorb 10 Diol column (chrompack; 10 μm, 250x4.6mm) and ACN : 0.05 M KH_2PO_4 (70:30), as a mobile phase and flow rate of 1.0 mL/min, UV detector at 270 and 286 nm for kojic acid and arbutin, repectively. The analysis times of kojic acid and arbutin were less than 5 min (t_R of kojic acid = 3.4 min, arbutin = 3.9 min). N. Kittipongpatana *et al*. [9] simultaneously separated arbutin and hydroquinone by HPLC using a reversed phase Apollo C18 column (4.6x150 mm). The mobile phase was methanol : water (10:90) at a flow rate of 0.9 mL/min. A UV detector was used at 280 nm. The analysis times within 10 min (t_R of arbutin = 3.9 min, hydroquinone = 5.8 min). M.L. Chang and C.M. Chang [10] determined four hydrophilic whitening agents (glycolic acid, ascorbic acid, arbutin and magnesium ascorbyl phosphate) using ion-pair agent as a mobile phase (0.005 M KH_2PO_4 buffer solution, tetrabutylammonium hydroxide (TBAH), methanol and phosphoric acid). The analysis times were within 15 min where arbutin was eluted at about 5 min. W. Thongchai *et al*. [11] developed a method for

quantitative determination of arbutin in commercial cosmetic samples. Using a reverse phase to examine but has not reported the retention time of arbutin. C.H. Lin *et al*. [12] developed an on-line HPLC method coupled with microdialysis for determination of arbutin from cream, lotion and essence cosmetic samples. The analysis time was 30 min per sample. The retention time of arbutin was about 3.05 min. Finally, micelle electrokinetic capillary electrophoresis (MEKC) with uncoated fused-silica capillary for simultaneous analysis of arbutin, kojic acid and hydroquinone was proposed by Y.H. Lin *et al*. [13]. The method was tested for several matrices such as cream, gel and ointment. The analysis time for separation of three whitening agents was within 6 min, which was faster than the HPLC method (18 min).

Ultra Performance Liquid Chromatography (UPLC™) is a new liquid chromatography technology that developed from the conventional HPLC to achieve better resolution, speed and sensitivity than HPLC system. None of the method has been reported for determination of both arbutins. There were several methods describing the performance of UPLC™ system compared with the conventional HPLC system. Z. Han *et al*. [14] separated and quantified four alkaloid by UPLC™-MS/MS using ACQUITY® BEH C18, 1.7 μm, 2.1x50 mm column and 10 mM ammonium acetate pH3 and ACN as a mobile phase with gradient condition. The analysis time of UPLC™ system was within 9 min whereas that of HPLC system was about 38 min. E.M. Paliakov *et al*. [15] determined the oil soluble vitamins and coenzyme Q10 in human serum by UPLC™ system using ACQUITY UPLC® BEH Shield RP18 column and a mixture of (MPA) (MPB) (90:10), MPA = ACN : water (90:10), MPB = methanol:2-propanol (70:30) as a mobile phase. The UPLC™ method could reduce the analysis time for about 2-3 times when compared with the HPLC method.

This work aims to develop a method for simultaneous determination of beta and alpha-arbutin in skin whitening cosmetic products by using UPLC™ system. It was developed from the typical HPLC but it would be more efficiency. The separation efficiency performance is based on the use of the column that contains small size particles (1.7 to 1.8 μm) of which the number of theoretical plate (N) are much improved according to the Van Deemter equation, resulting to high resolution power with fast flow

rate. The UPLC™ system comes with the high pressure pump (15,000 psi pressure limit) and used about 10 times less system volume than the HPLC system. UPLC™ offers better separation, rapid analysis time and high sample throughput, which are suitable for routine analysis in laboratory quality control.

CHAPTER II

THEORY

2.1 The whitening agents

The objective of the whitening cosmetic products is to reduce dark spots on the skin. Melasma or freckle may be caused by the high volume generation of melanin, especially when the skin is stimulated by the UV radiation. The mechanism of the production of melanin is shown in Figure 2.1

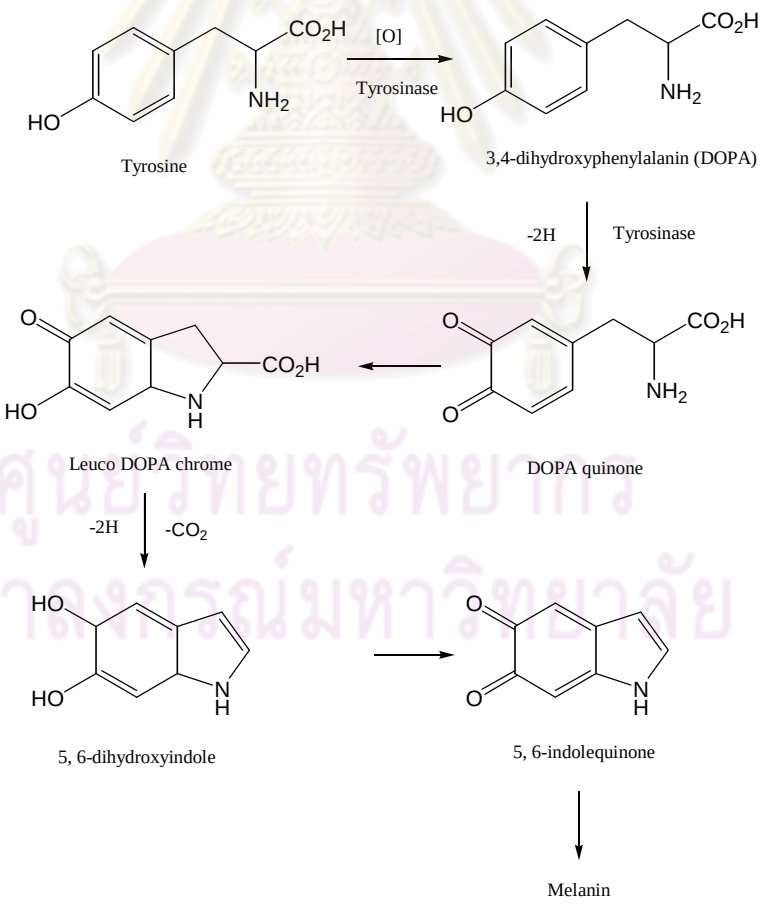


Figure 2.1 The mechanism of the pigmentation [16].

Figure 2.1 describes a melanin production. First tyrosinase is oxidized at the ortho position of aromatic ring by enzyme tyrosinase yielding 3,4-dihydroxyphenylalanin (DOPA) and further dehydrogenated to dopaquinone followed by cyclization of dopaquinone to leucodopachrome, rearranged to 5,6-dihydroxyindole by CO_2 and H_2 loosens, then to 5,6-indolequinone and finally, to melanin. The oxidation step by enzyme tyrosinase is where the whitening agents will play an important role as enzyme tyrosinase inhibitor. Examples of the whitening agents used in cosmetics are given.

2.1.1 Hydroquinone

The chemical structure of hydroquinone is shown in Figure 2.2

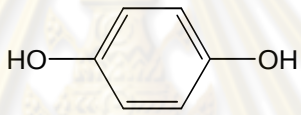


Figure 2.2 The chemical structure of hydroquinone.

Hydroquinone or so called 1,4-dihydroxybenzene or benzene-1,4-diol, is an aromatic organic compound having a molecular weight of 110.1 g/mol. Its appearance is a white crystal, easily oxidized in air and light that turns into a brown color. It can be decomposed in basic conditions. It has a good solubility in alcohol and water. In a medical purpose, hydroquinone is used as a topical application in skin whitening to reduce the color of skin. Hydroquinone does not bleach the skin but stifles the creation of melanin by effectively inhibiting enzyme tyrosinase. Since it works directly on the skin, it may cause skin irritation.

2.1.2 Kojic acid

The chemical structure of kojic acid is shown in Figure 2.3

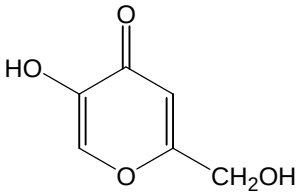


Figure 2.3 The chemical structure of kojic acid.

Kojic acid is a natural product from fungal metabolites. Its chemical name is 5-hydroxyl-2-hydroxyethyl-1,4-pyrone, having a molecular weight of 142.1 g/mol. It appears in white needle crystal, which is not stable to light, heat and oxidation reaction. Like hydroquinone, it inhibits enzyme tyrosinase. It is relatively inexpensive. Now, it has been banned in some countries in Asia.

2.1.3 Ascorbic acid

The chemical structure of ascorbic acid is shown in Figure 2.4

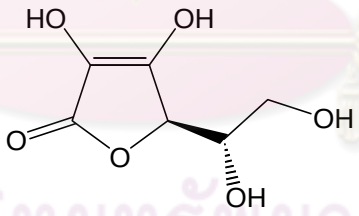


Figure 2.4 The chemical structure of ascorbic acid.

Ascorbic acid is also known as vitamin C. Its molecular weight is 176.12 g/mol. Its appearance is a white or light yellow solid that is easily dissolve in water. It is used to interrupt the production of melanin by inhibiting enzyme tyrosinase as well.

2.1.4 Arbutin

Arbutin is a derivative of hydroquinone that has two configuration forms;

forms; i.e., alpha-arbutin and beta-arbutin. Beta-arbutin that was known as arbutin in the past, is a natural product while alpha-arbutin is a synthetic one. Both arbutins are enzyme tyrosinase inhibitors. Figure 2.5 shows the chemical structure of beta-arbutin and Figure 2.6 shows the chemical structure of alpha-arbutin.

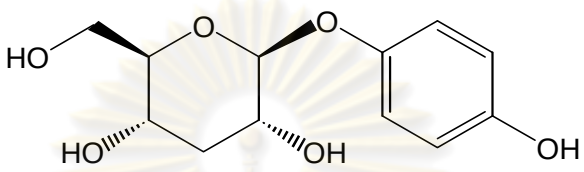


Figure 2.5 The chemical structure of beta-arbutin.

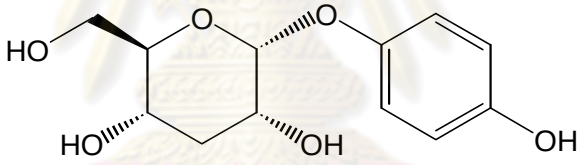


Figure 2.6 The chemical structure of alpha-arbutin.

Beta-arbutin is a phenolic glucoside which is found in many plants. Its chemical name is 4-hydroxyphenyl-β-D-glucopyranoside. The molecular weight is 272.25 g/mol. Beta-arbutin is more stable and there is no irritation and side effect. Alpha-arbutin or 4-Hydroxyphenyl-α-D-glucopyranoside is a synthetic compound. Its appearance is white to off-white powder. It provides higher ability in inhibition of melanin than beta-arbutin. It also promotes the skin lightening and minimizes brown spots so faster than beta-arbutin.

2.2 Theory of Liquid Chromatography by Ultra Pressure (Ultra Performance Liquid Chromatography, UPLC™)

The UPLC™ is the new technology of high performance liquid chromatography that has been developed to increase separation efficiency such as speed, sensitivity and resolution, The UPLC™ feature is shown in Figure 2.7.

Figure 2.7 The feature of Ultra Performance Liquid Chromatography (UPLC™, Waters, USA) [17].

2.2.1 The equipment of the UPLC™ system

2.2.1.1 Binary Solvent Manager (BSM)

It is equivalent to pump in conventional HPLC system but it has been developed for high pressure (15,000 psi pressure limit) having two inlet check valves (primary and accumulator), four selected solvents (A1 or A2, B1 or B2) and six channel solvent degassers. The total system volume is less than 100 μL. The time for system equilibration is short when compared with conventional HPLC system. It has

been designed for binary gradient, where the gradient curve profiles and the resulting chromatograms are shown in Figure 2.8 and 2.9, respectively.

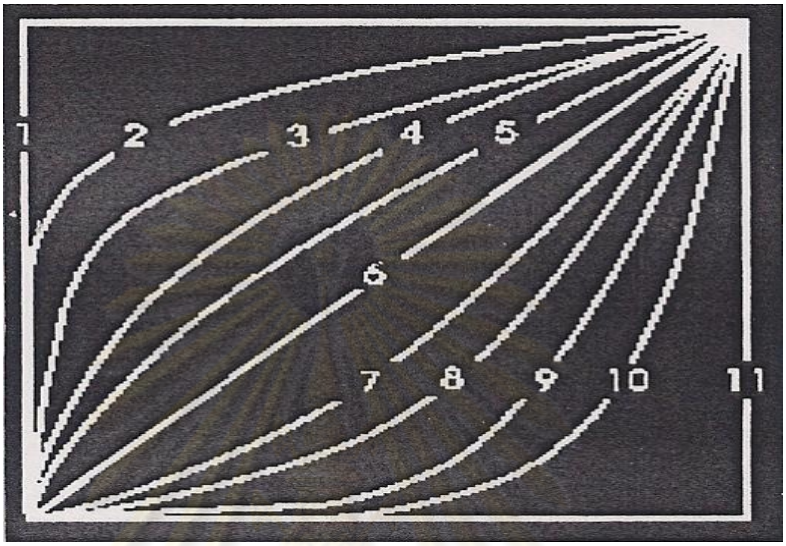


Figure 2.8 The gradient curve profiles on ACQUITY UPLC™ [18]

Figure 2.9 The chromatograms according to the gradient curve profiles on ACQUITY UPLC™ [18].

The gradient curve profiles are described in Table 2.1.

Table 2.1 The particular of curve 1 to 11 gradient profiles.

Curve	Effect
1	Immediately to specified condition
2-5	convex
6	linear
7-10	concave
11	Maintain start conditions

2.2.1.2 Sample Manager (SM)

It has been designed to accept 0.1 to 50 μL injection range with appropriate loops. The sample temperature can be controlled from 4 to 40 ^{o}C.

2.2.1.3 Column Manager

It is designed for the shorter columns such as 50, 100 and 150 mm. The column heater can be heated up to 65^{o}C.

2.2.1.4 Optical Detection (TUV and PDA)

It has been specially designed for low volume light guiding flow cells that have optimum path length and high light throughput. The analytical flow cell size 10 mm, 500 nL are used for high sensitivity and better chromatographic resolution.

2.2.2 The advantages of UPLC™ system

2.2.2.1 Speed

The analysis time is reduced because the column has been developed for smaller column diameter, particle size and shorter column. The speed of the UPLC™ system is faster than the conventional HPLC system about 9 times. The chromatograms on the conventional HPLC and UPLC™ systems are compared in Figure 2.10.

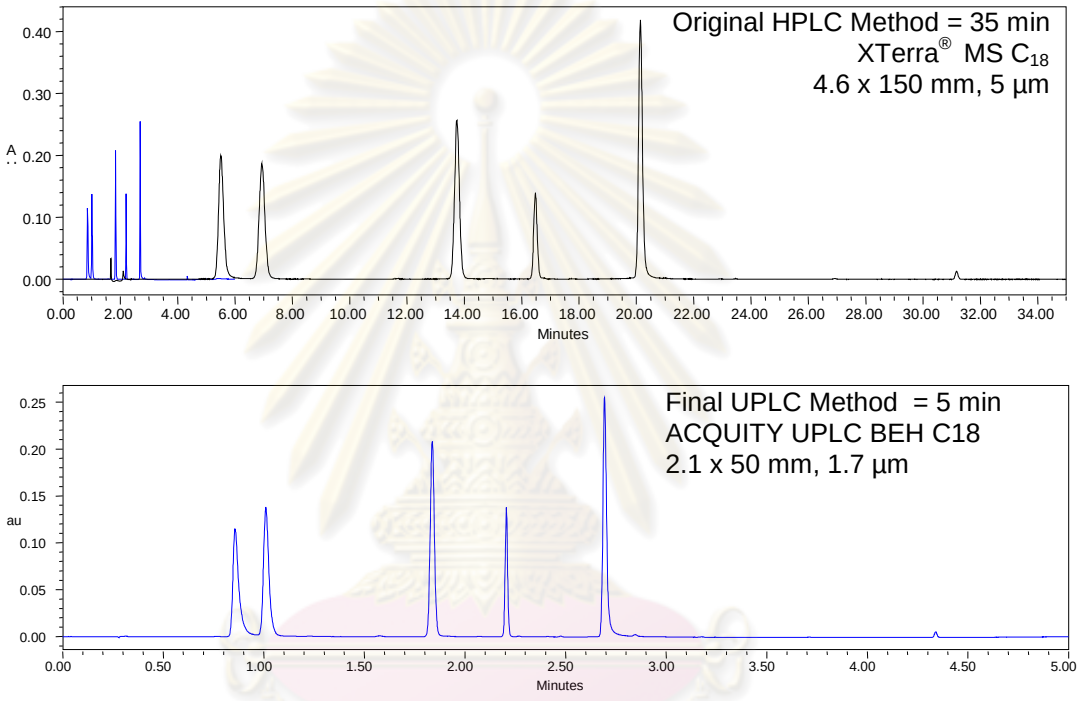


Figure 2.10 Comparison of the analysis time running on the conventional HPLC system and the UPLC™ system [19].

2.2.2.2 Sensitivity

The sensitivity can be improved when the particle size of column is decreased because the surface area of the stationary phase is increased and then the interaction between the analyte and the stationary phase is enhanced. The Figure 2.11 demonstrates the sensitivity when using columns with different particle sizes at the same column length and the same the flow rate.

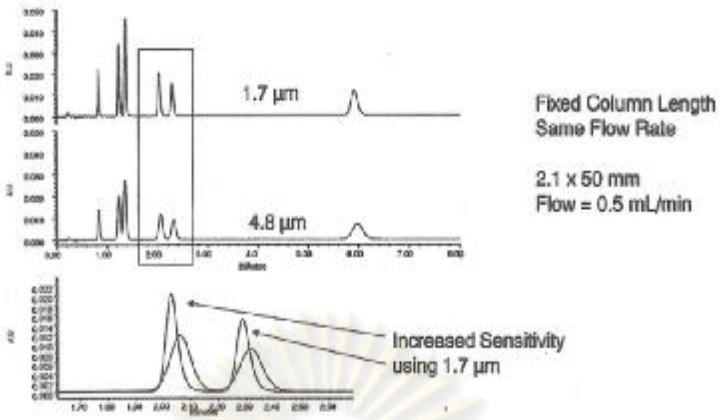


Figure 2.11 Comparison of sensitivities when using columns with different particle sizes [20].

2.2.2.3 Resolution

The competency of separation depends on the number of theoretical plates of the column. The ACQUITY UPLC® columns contain smaller particle size i.e., 1.7 to 1.8 µm, resulting to that the plate numbers of the ACQUITY UPLC® columns are about 3 times more than those of the HPLC columns. The comparison of the chromatogram resolutions on the conventional HPLC system and the UPLC™ system are shown in Figure 2.12.

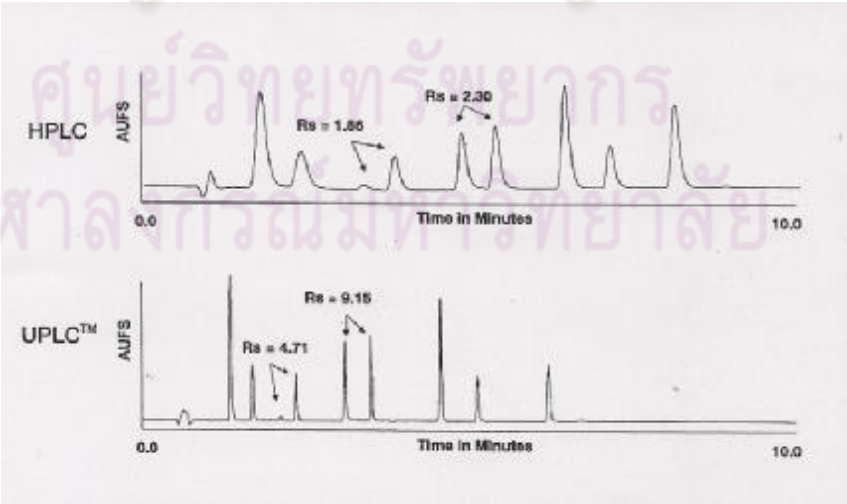


Figure 2.12 The ability of separation between the HPLC column and UPLC® column [21].

The efficiency of the column is measured by the number of theoretical plates and can be normalized with the length of the column to give the height equivalent to the theoretical plate (HETP or H). The Van Deemter equation describes the various factors, as shown in Figure 2.13

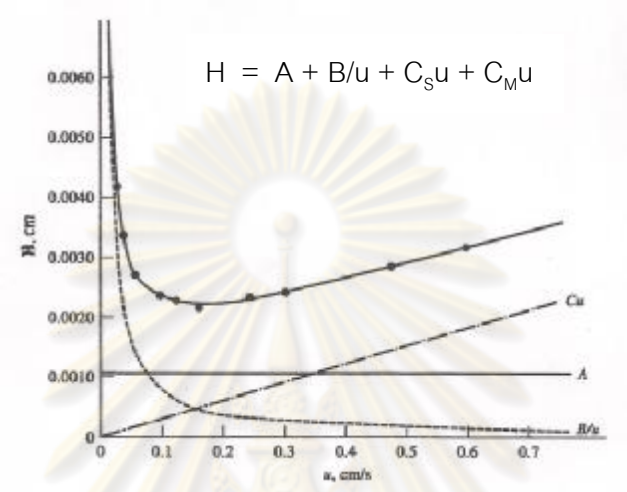


Figure 2.13 The Van Deemter plot and Van Deemter equation [22].

Where ; H = HETP (plate height)

A = Eddy diffusion term

B = Longitudinal diffusion term

u = Linear of velocity of mobile phase (cm/s)

C_S = Mass transfer coefficient for stationary phase

C_M = Mass transfer coefficient for mobile phase

- A-term (eddy diffusion), this term accounts for the effects of packing size and geometry. The column packing consists of particles with flow channels in between due to the different in packing and particle shape. The speed of the mobile phase in various flow channels differs and analyte molecules travel along the different flow paths through the channels. Factors that affecting A-term are particle size, particle

shape, particle pore structure, quality of the column packing and wall effects (material, roughness and diameter).

- B-term (longitudinal diffusion), this term represents broadening due to diffusion of individual analyte molecules in the mobile phase. The factor affecting B-term is flow rate, when increase the flow, the time for diffusion is reduced. The B-term on UPLC™ system is shown in Figure 2.14.

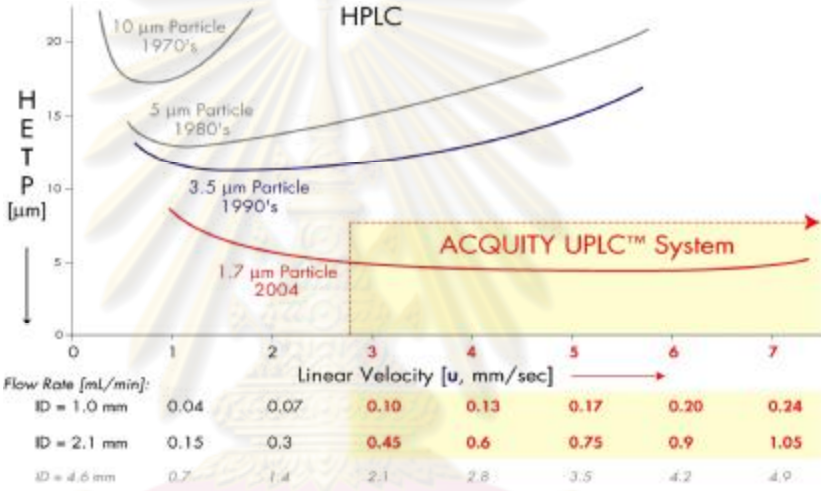


Figure 2.14 Comparison of the B-term between HPLC system and UPLC™ system [23].

Figure 2.14 described the optimum linear velocity to maximize the chromatographic efficiency of different particle size.

- C-term (Mass transfer coefficient for stationary phase and mobile phase), this term corresponds to the time for a solute to reach an equilibrium between the mobile phase and the stationary phase. The factors that affecting C-term are thickness and viscousity stationary phase.

2.3 Method validation

The method validation is required to approve that the method is suitable to be used in a cosmetic laboratory. The analytical parameters considered in the validation procedure are as follows :

2.3.1 System suitability ; should declare on the following terms

2.3.1.1 Theoretical plates (N)

The number of theoretical plates (N) expresses column efficiency. The plate number can be measured from the peak width and retention time as shown in Figure 2.15.

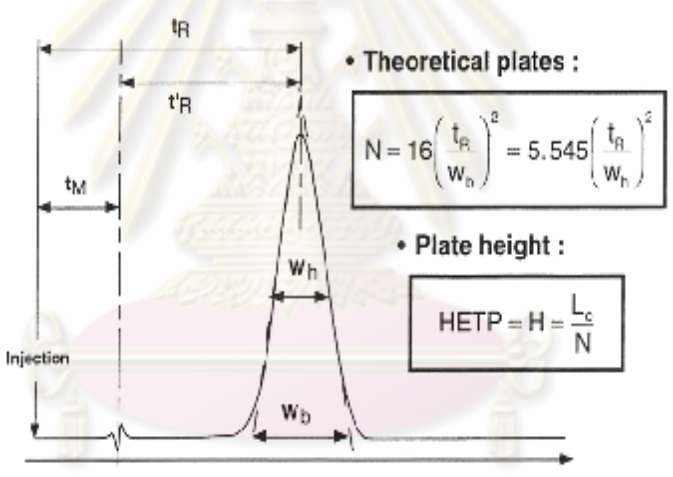


Figure 2.15 The theoretical plate (N) [24].

2.3.1.2 Tailing factor or asymmetry factor

The tailing factor or peak asymmetry can be measured by one of two ways. The tailing factor is measured at 5% of the peak height as shown in Figure 2.16.

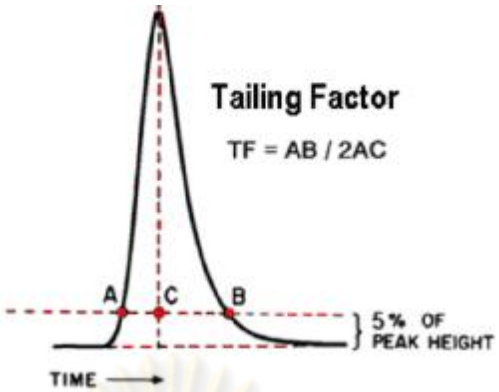
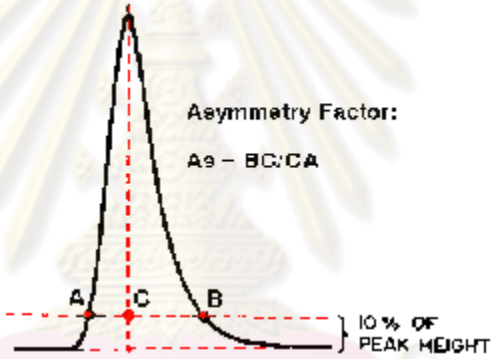


Figure 2.16 The tailing factor [25].

The asymmetry factor is measured at 10% of the peak height as shown in Figure 2.17.



When : - A_S = 0.9-1.1 to assume the gaussian peak

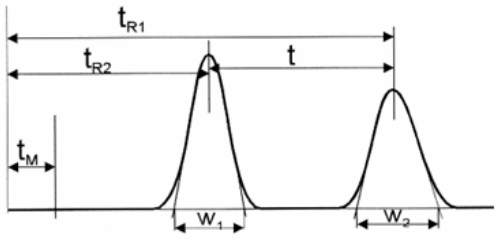
- $A_S > 1$ to assume the tailing peak

- $A_S < 1$ to assume the fronting peak

Figure 2.17 The asymmetry factor [26].

2.3.1.3 Resolution (R_s)

The resolution describes the separation of band centers. The resolution of two species, A and B, is defined as Figure 2.18.



$$R = \frac{\Delta t}{\frac{1}{2}(w_1 + w_2)}$$

$$R = \left(\frac{k'}{1+k'}\right)\left(\frac{\alpha - 1}{\alpha}\right)\frac{\sqrt{N}}{4}$$

Figure 2.18 The resolution (R_S) [27].

2.3.1.4 Retention factor (k´)

k´ is the ratio of the amount of time that a solute spends in stationary and mobile phase. This is calculated from the retention time of a peak and the dead time of the system as shown in Figure 2.19.

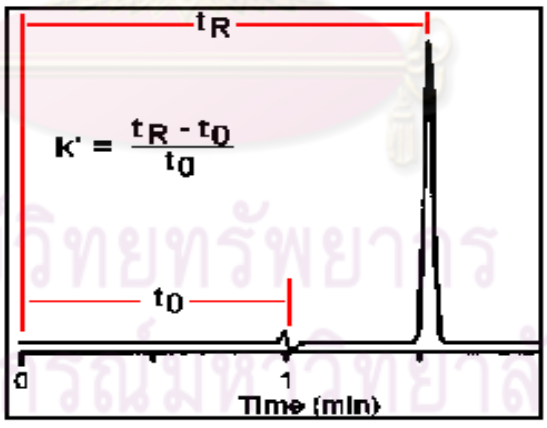


Figure 2.19 The retention factor (k´) [28].

2.3.2 Specificity or selectivity

The specificity or selectivity is the ability to accurately and specifically

measure the analyte in the presence of components that may be expected to be present in the sample matrix.

2.3.3 Linearity and range

The linearity is the ability to obtain test results directly or by a well defined mathematics transformation proportional to the concentration of analyte in the sample within given range. Linearity is usually expressed in term of variance around the slope of the regression line.

2.3.4 Precision

The precision is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision is usually expressed as the standard deviation or relative standard deviation.

2.3.5 Accuracy

The accuracy is the closeness of test result obtained by the method to the true value. Accuracy may often be expressed as %recovery by the assay of known, added amounts of the analyte.

2.3.6 Limit of detection (LOD)

The limit of detection is the lowest concentration of analyte in the sample that can be detected.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and apparatus

3.1.1 The UPLC™ system

- Pump : Waters ACQUITY ™ Ultra Performance Chromatography ; Binary Solvent Manager (BSM) (Waters, USA)

- Autosampler : Waters ACQUITY ™ Ultra Performance Liquid Chromatography ; Sample Manager (SM) (Waters, USA)

- Detector : ACQUITY ™ Ultra Performance Liquid Chromatography ; Photodiode Array, 283 nm (Waters, USA)

- Column

  : ACQUITY UPLC ® HSS T3, 1.8 μm, 2.1x100, 150 mm (Waters, USA)

  : ACQUITY UPLC ® BEH Shield RP, 1.7 μm, 2.1x100 mm (Waters, USA)

  : ACQUITY UPLC ® BEH C18, 1.7 μm, 2.1x150 mm (Waters, USA)

  : ACQUITY UPLC ® BEH C8, 1.7 μm, 2.1x150 mm (Waters, USA)

3.1.2 Glasswares and equipment

- Piston pipette, 10-100 μL and 100-1000 μL (Biohit, Finland)

- Volumetric flask individual certificate 10, 25 and 1000 mL (Brand, USA)

- 2 mL-vials with PTFE/silicone septa caps (Waters, USA)
- Ultrasonic bath (Crest, USA)
- Vortex mixer (Genie, USA)
- Syringe of filtering PVDF membrane filter 0.2 μm diameter 13 mm (Orange Scientific, Belgium)
- Analytical balance accurate 0.01 mg (Metter-Toledo AX105 DR, USA)

3.2 Chemical reagents, standards and samples

3.2.1 Methanol, HPLC grade (Fisher Scientific, Canada)

3.2.2 Acetic acid, AR grade (Merck, Germany)

3.2.3 Beta-arbutin, with Certificate of Analysis (Bioland, USA)

3.2.4 Alpha-arbutin, with Certificate of Analysis (Pentapharm, Switzerland)

3.2.5 Deionized water (Branstead, USA)

3.2.6 Laboratory-made of the skin whitening creams, lotions and gels (label as LM1, LM2 and LM3, respectively) containing beta- and alpha-arbutin each about 2% were prepared in Division of Cosmetic and Hazardous Substances, Department of Medical Sciences, Ministry of Public Health.

3.2.7 Five commercial skin whitening cosmetic samples purchased from local markets containing different whitening agents and different percent amounts.

- C1 : Laneige Perfect Renew Essence (Amorepacific, Korea)
- C2 : Etude O_2 White lotion (Etude, Korea)
- C3 : Smooth E White Baby Face Serum (Smooth E, Thailand)

- C4 : DHC α-Arbutin White Cream (DHC, Japan)

- C5 : Mistine re-Touch (Mistine, Thailand)

3.3 Preparation of mobile phase

3.3.1 Acetic acid 0.1% v/v (solvent A)

A 1.0 mL of glacial acetic acid was pipette into a 1000 mL of volumetric flask and made up to the volume with deionized water and mixed well.

3.3.2 Methanol (solvent B)

The compositions of solvent A and solvent B were programmed via gradient elution profiles from the UPLC™ system.

3.4 Preparation of mixed standard beta- and alpha- arbutin stock solution of 1,000 µg/mL

Each standard of beta- and alpha-arbutin were accurately weighed about 0.025 g to the nearest of 0.1 mg into a 25 mL of volumetric flask, dissolved with methanol, mixed on a vortex mixer and made up to the volume with methanol.

3.5 Preparation of standard solution for calibration curve

A series 20, 60, 100, 200 and 300 µL of mixed standard stock solution was pipetted into each 10 mL of volumetric flask and made up to the volume with 95 : 5 (0.1%acetic acid : methanol) and mixed well. The concentrations of working standard solutions are 2, 6, 10, 20 and 30 µg/mL, respectively. Label as S1, S2, S3, S4 and S5. The standard solution were filtered through 0.2 µm PVDF into a 2-mL vial prior to analysis.

3.6 Preparation of synthetic samples solution for precision

The arbutins in synthetic samples of LM1, LM2 and LM3 were determined for five replicates labeled as A, B, C, D and E. The sample was accurately weighed (about 0.5 g) into 25 mL of volumetric flask (the amount of the sample was calculated from the label to ensure that the final concentration of sample solution should be within calibration curve) dissolved with methanol 10 mL and was mixed on vortex mixer, sonicated for 5 min and made up with methanol and mixed well. An aliquot of 100 μL was pipette into a 10 mL of volumetric flask. Make up with 95 : 5 (0.1% acetic acid : methanol) and mixed well. The sample solution was filtered through 0.2 μm PVDF syringe filter into a 2-mL vial prior to analysis.

3.7 Preparation of spiked synthetic samples solution for accuracy (%recovery three levels)

Syntheic samples of LM1, LM2 and LM3 spiked with standard arbutins at the level of 50%, 100% and 150% of labeled amount were determined for five replicates at each level. The samples were prepared in the same procedure as described in 3.6. The %recovery to spiked arbutins of three levels were determined as follow:

$$\%\text{recovery} = [(S-U) \times 100] / C \qquad (3.1)$$

Where S is concentration of beta- or alpha-arbutin in spiked sample, %w/w. U is concentration of beta- or alpha-arbutin in unspiked sample, %w/w and C is concentration of standard added, %w/w.

3.8 Preparation of commercial cosmetic samples solution (for determination of beta- and alpha-arbutin)

The arbutins in commercial cosmetic samples of C1, C2, C3, C4 and C5 were determined for two replicates labeled as A and B of each sample and prepared as described in 3.6. Spiked commercial cosmetic samples with standard arbutins at the level of 50% or 100% of labeled amount were determined for two replicates. The

samples were prepared in the same procedure as described in 3.6. The recovery to spiked arbutins of three levels were determined as in 3.7.

3.9 Preparation of spiked standard stock solution for limit of detection

A blank cosmetic sample (the sample that beta- and alpha-arbutin were not found) was accurately weighed (about 0.5 g) into 25 mL of volumetric flask. An aliquot of 30 µL of 1,000 µg/mL standard stock solution was spiked into the sample, then dissolved with methanol and mixed on the vortex mixer, sonicated for 5 min and made up to the volume with methanol. The sample solution was filtered through 0.2 µm PVDF syringe filter into a 2-mL vial prior to analysis.

3.10 The UPLC™ optimization

The important factors affecting on the separation performance of beta- and alpha-arbutin such as water, aqueous solution, organic solvents, columns, gradient conditions, flow rate and temperature were investigated in order to determine the optimum analysis conditions. The factors were described as follows :

3.10.1 Solvents for preparation of standards and samples

3.10.1.1 In this experiment various solvents affecting the solubility of beta- and alpha- arbutin in various matrices (cream, lotion and gel) were investigated such as water, 0.1% acetic acid, methanol, ethanol, isopropanol and acetonitrile.

3.10.2 Columns

The chemical structures of both arbutins were found that they were relatively a polar. Several types of columns were tested for optimized separation.

3.10.2.1 The ACQUITY UPLC® HSS T3 (C18 High Strength Silica) 1.8 µm particle size, i.d. 2.1 x 100 and 150 mm (Figure 3.1).

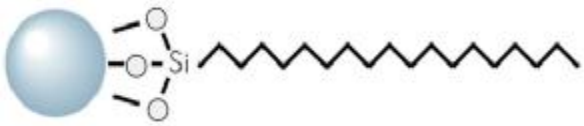


Figure 3.1 The feature of the ACQUITY UPLC® HSS T3 column.

It is a reversed phase column designed for separation of polar organic compounds. It has been developed for 100% aqueous compatibility and more efficiency than ACQUITY UPLC® BEH C18 column.

3.10.2.2 The ACQUITY UPLC® BEH Shield RP18 (C18 Bridged Ethyl Hybrid + polar group) 1.7 μm particle size, i.d. 2.1 x 100 mm column (Figure 3.2).

Figure 3.2 The feature of the ACQUITY UPLC® BEH Shield RP18 column.

The stationary phase has been developed to combine the hydrophobicity of an alkyl ligand with the hydrophilicity of an embedded polar group for 100% aqueous compatibility.

3.10.2.3 The ACQUITY UPLC® BEH C18 (C18 Bridged Ethyl Hybrid) 1.7 μm particle size, i.d. 2.1 x 150 mm column (Figure 3.3).

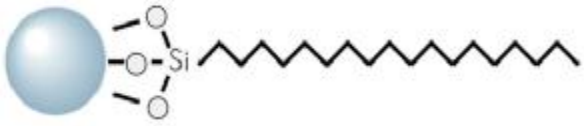


Figure 3.3 The feature of the ACQUITY UPLC® BEH C18 column.

Bridged Ethyl Hybrid (BEH) has been developed by embedded ethane group (CH_2-CH_2) into the silica backbone to improved efficiency, strength and be used for wide pH range 1-12. The figure of ethane group addition was shown in Figure 3.4.

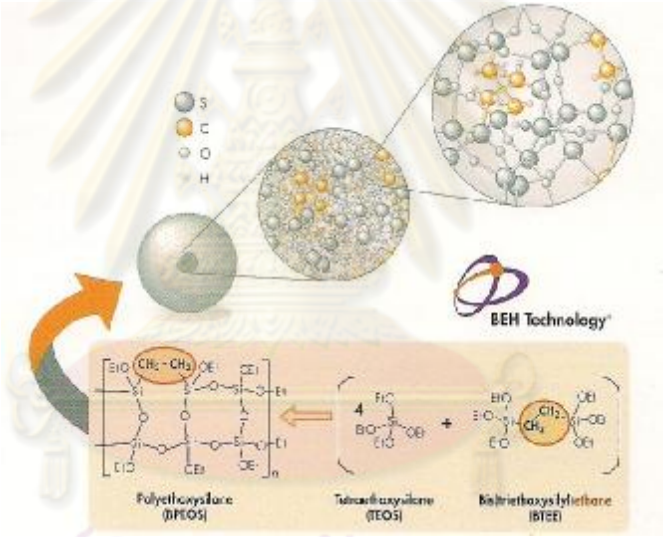


Figure 3.4 The feature of bridged ethyl hybrid (BEH) [29].

3.10.2.4 ACQUITY UPLC BEH C8 (C8 Bridged Ethyl Hybrid) 1.7 μm particle size, 2.1 x 100 and 150 mm (Figure 3.5).

The stationary phase is the same material as ACQUITY UPLC® BEH C18 but has a short chain of carbon. So, it is suitable for polar compound more than BEH C18.

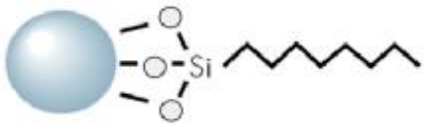


Figure 3.5 The feature of the ACQUITY UPLC® BEH C8 column.

3.11 Method validation

3.11.1 System suitability

The first level of standard solution 2 µg/mL (S1) was repeatedly injected. The retention times of five replicates, standard deviation of peak areas, number of the theoretical plates (N), retention factor (k´), resolution (R_S) and tailing factor were determined. The acceptable criteria were shown in Table 3.1.

Table 3.1 The system suitability criteria of chromatographic system.

Whitening agents	%RSD of peak area	Theoretical plate (N)	Tailing factor	Resolution (R_S)	Retention Factor (k´)
Beta-arbutin	≤ 3	≥ 10,000	≤ 2	≥ 1.5	≥ 2
Alpha-arbutin	≤ 3	≥ 10,000	≤ 2	≥ 1.5	≥ 2

The criteria of %RSD according to the Horwitz trumpet (Figure 3.6) showed that the relative standard deviation of a method varied with the concentration (c), which can be approximated by the empirical equation RSD = $\pm 2^{(1-0.5 \log c)}$.

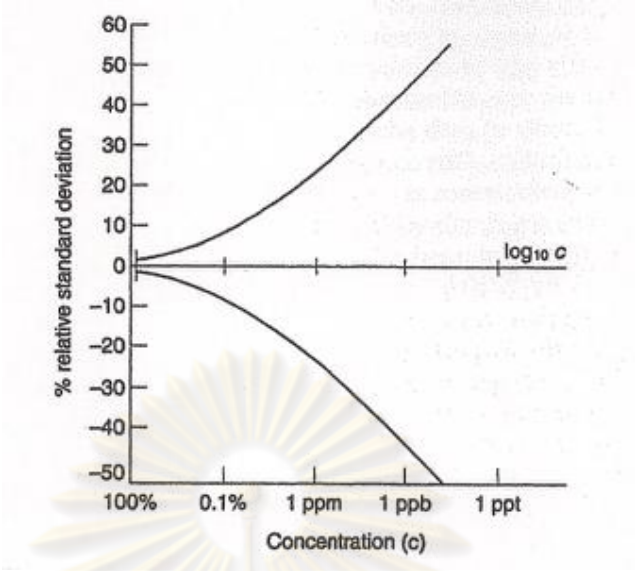


Figure 3.6 The Horwitz trumpet [30].

3.11.2 Specificity or Selectivity

Mixtures of standard beta- and alpha- arbutin were injected. Each substance was eluted at different time meaning to show that the compounds did not interfere with each other.

3.11.3 Linearity and range

3.11.3.1 System linearity

Mixtures of standard solutions (prepared as described in 3.5, S1, S2, S3, S4 and S5) were injected for five replicates at each point. Relative standard deviation (%RSD) of peak areas should not differ than 2%. The calibration curve of peak area versus concentrations and the regression line was constructed by a method of the least squares. The following linear regression equation was obtained from the calibration curve.

$$A_o = b_1C_o + b_o$$

When : b_1 = slope

b_o = intercept

C_o = concentration in μg/mL

A_o = peak area

The correlation coefficient from a fitted linear regression line was calculated and the value should be close to 1.0.

3.11.3.2 Method linearity

Spiked sample solutions (prepared as described in 3.7 for %recovery) were injected for duplicates. The linear relationship between the concentration of standard found versus the concentration of standard spiked was determined. The correlation coefficient (r) was calculated and the value should be close to 1.0.

3.11.4 Precision

3.11.4.1 System precision or reproducibility

Inject the sample solution that prepared as described in 3.6 were injected. The %RSD of peak areas for five replicates should not differ than 3% (n=5).

3.11.4.2 Method precision

3.11.4.2.1 Repeatability (intra day)

The sample solutions that prepared as described in 3.6 were injected. The %RSD of peak areas for five replicates should not differ than 3%.

3.11.4.2.2 Intermediate precision (inter day)

The sample solutions prepared as described in 3.6 were injected for five days. The *p*-value from ANOVA single factor was calculated. The criteria of *p*-value should not less than 0.05.

3.11.5 Accuracy

The sample solutions prepared as described in 3.9 were injected. The %recovery for 3 levels should be within a range of 90 to 110.

3.11.6 Limit of Detection (LOD)

Inject the sample solution that prepared as described in 3.10 for duplicate injections. The LOD should be the lowest concentration that can be detected.

3.12 The determination of beta- and alpha-arbutin in commercial cosmetic samples

Inject the sample solution that prepared as described in 3.8 and 3.9 in order to determination of beta- and alpha-arbutin.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Study of solvents for preparation of standards and samples

The chemical structures of beta- and alpha-arbutin are high polar organic compounds that are very well soluble in water and alcohol but the cosmetic products usually contain a variety of components that are not easily dissolved in water. So that, various organic solvents were investigated as shown in Table 4.1.

Table 4.1 Dissolution of standard arbutins and various samples.

Substances	Solubility				
	Water	Methanol	Ethanol	Isopropanol	Acetonitrile
Beta-arbutin	✓	✓	✓	✓	✓
Alpha-arbutin	✓	✓	✓	✓	✓
Cream sample	✗	✓	✓	✓	✓
Lotion sample	✗	✓	✓	✓	✓
Gel sample	✗	✓	✓	✓	✓

Note ✓ good soluble and ✗ not soluble

Both standard arbutins are well soluble in water but the cosmetic samples could not be dissolved because the matrices of cosmetic samples might have consisted of relatively non polar components. Methanol, ethanol, isopropanol and acetonitrile were organic solvents that showed good dissolution of all standards and cosmetic samples.

The methanol was selected for this study because it was available in the laboratory and low cost when compared to the other solvents.

4.2 UPLC™ Method development.

Beta- and alpha-arbutin are isomers having the same chemical structures and similar properties that are hardly separated by a conventional HPLC column, probably due to their similar interactions between the mobile phase and the stationary phase, where the resolving power of the HPLC column is insufficient. Considering the chemical structures of beta- and alpha-arbutin on chair form (Figure 4.1), the different is that the phenol group of beta-arbutin is on the axial position whereas phenol group of alpha-arbutin is on the equatorial position. The different in the conformation may lead to the difference in the strength of the chemical interaction on the stationary phase. Therefore, the higher separation power of UPLC™ column may be considered.

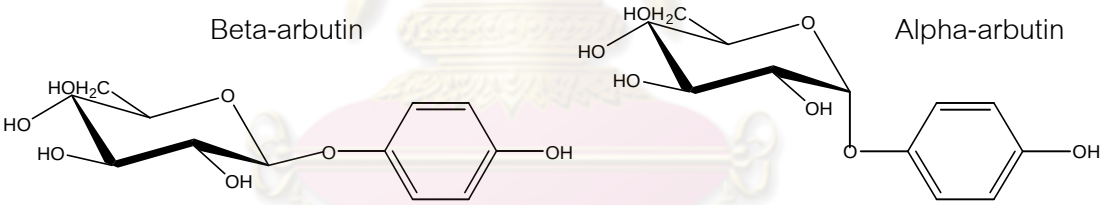


Figure 4.1 Comparison of chair-from between beta- and alpha-arbutin.

There are many kinds of the ACQUITY UPLC® columns available for reversed-phase mode. There were four choices of columns for this study, i.e., ACQUITY UPLC® HSS T3, 1.8 μm, ACQUITY UPLC® BEH Shield RP18, 1.7 μm, ACQUITY UPLC® BEH C18, 1.7 μm and ACQUITY UPLC® BEH C8, 1.7 μm. Since arbutins are well soluble in water, the isocratic mode with high aqueous percentage was first investigated. Two

columns, which were designed for 100% aqueous compatibility, i.e., HSS T3 and BEH Shield RP18 columns, were studied.

4.2.1 Isocratic mode using 100% aqueous as a mobile phase

The ACQUITY UPLC® HSS T3, 1.8 μm, 2.1 x 100 mm and ACQUITY UPLC® BEH Shield RP18, 1.7 μm, 2.1 x 100 mm columns and 100% of 0.1% acetic acid as a mobile phase of flow rate 0.5 mL/min were employed for this trial. The peaks of beta- and alpha-arbutin standards were not well separated (R_s = 0.96 and 1.32, respectively), as shown in Figure 4.2.

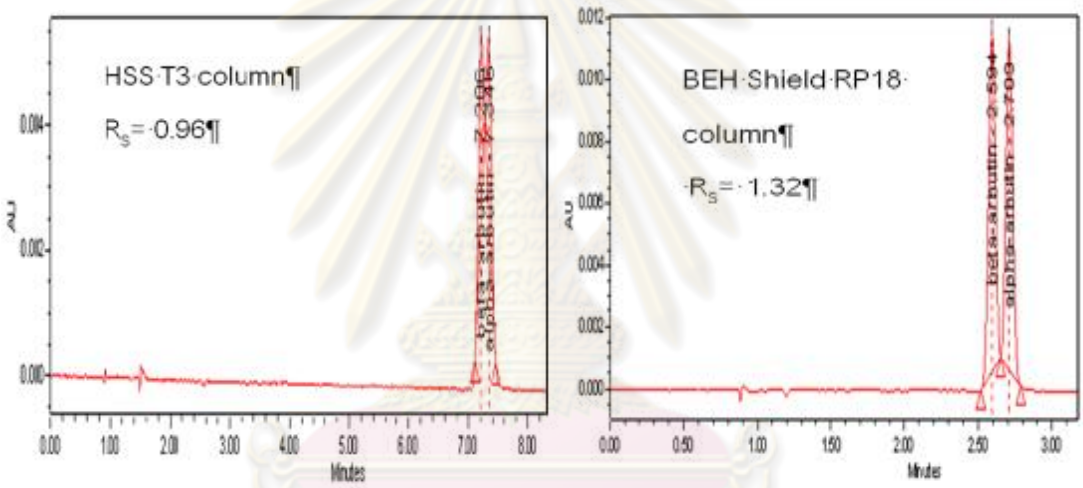


Figure 4.2 Separation of beta- and alpha-arbutin using 100% aqueous solution as a mobile phase on HSS T3 and BEH Shield RP18 columns.

4.2.2 Isocratic mode using various organic compositions as a mobile phase

The mobile phases of various ratio of 0.1% HOAc and MeOH were studied. The ratio of 0.1% HOAc : MeOH were varied at 97%, 95% and 93% of 0.1% HOAc. The flow rate was also reduced from 0.5 mL/min to 0.3 mL/min. The results were shown in Figure 4.3. The ratio of 95% of 0.1% HOAc : 5% MeOH exhibited the best resolution.

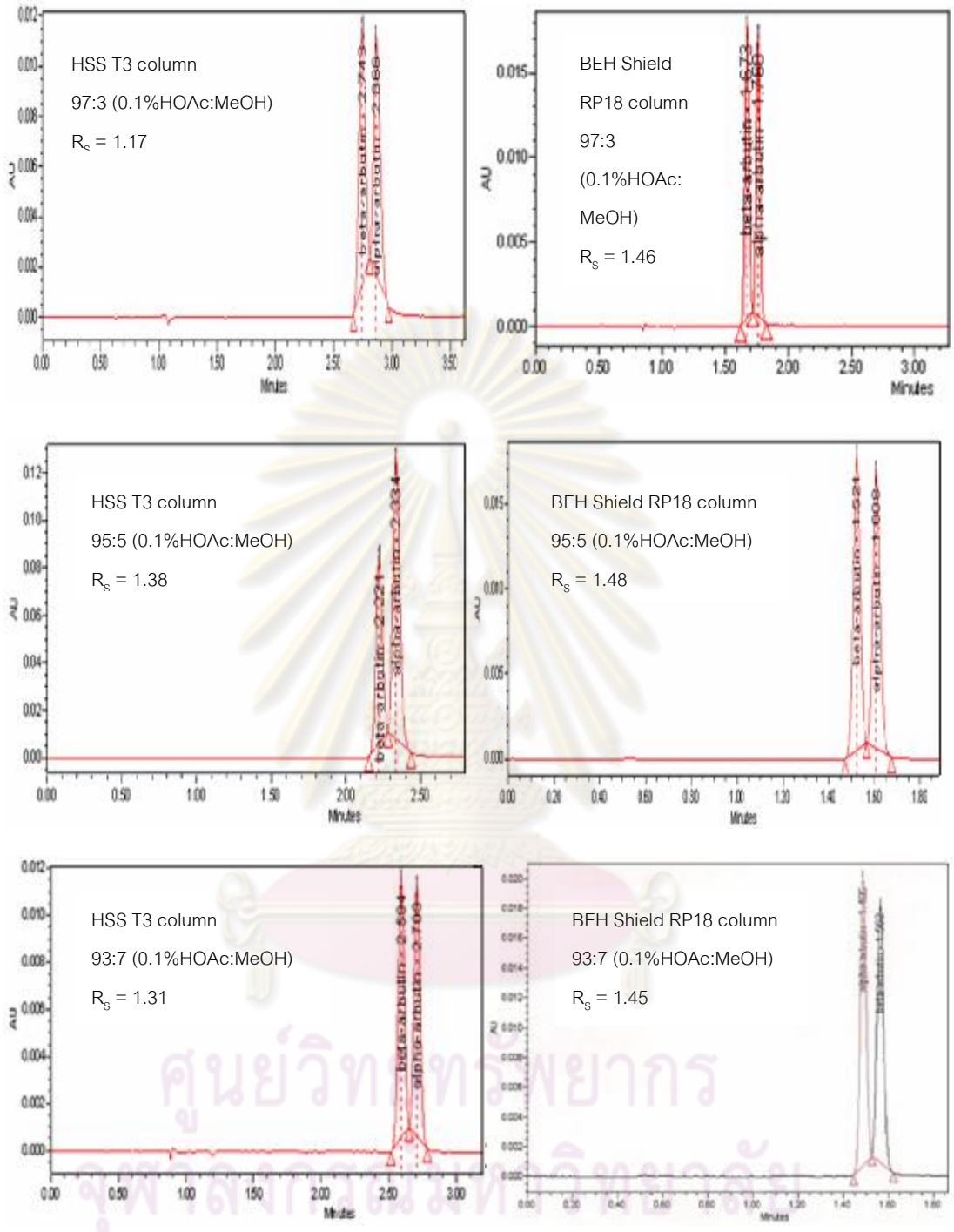


Figure 4.3 Separations of beta- and alpha-arbutin using various organic compositions as a mobile phase on HSS T3 and BEH Shield RP18 columns

4.2.3 Temperature of sample manager

According to the report by the Pentapharm Co., Ltd. (alpha-arbutin manufacturers, Switzerland), alpha-arbutin was well soluble in cold water. So, the temperature of sample manager (autosampler) was set at 5-8 °C for investigation. However, the results were found that the temperature of the sample manager did not affect on the R_S of beta- and alpha-arbutin.

4.2.4 Column length

Theoretically, when the column length is extended, the theoretical plate (N) of column is increased and the R_S is improved. Therefore, in this study the longer column of 150 mm was tested. Unfortunately, ACQUITY UPLC® BEH Shield RP18, 1.7 μm was not available for this length. The isocratic mode with mobile phases of various ratio of 0.1% HOAc and MeOH, i.e., 93%, 94%, 95%, 97% and 100% of 0.1% HOAc at the flow rate of 0.3 mL/min was investigated. The results showed in Figure 4.4. The resolution for separation of both arbutins was improved compared to the column length of 100 mm. The best R_S (1.61) was also observed when using 95 : 5 of 0.1% HOAc: MeOH as a mobile phase.

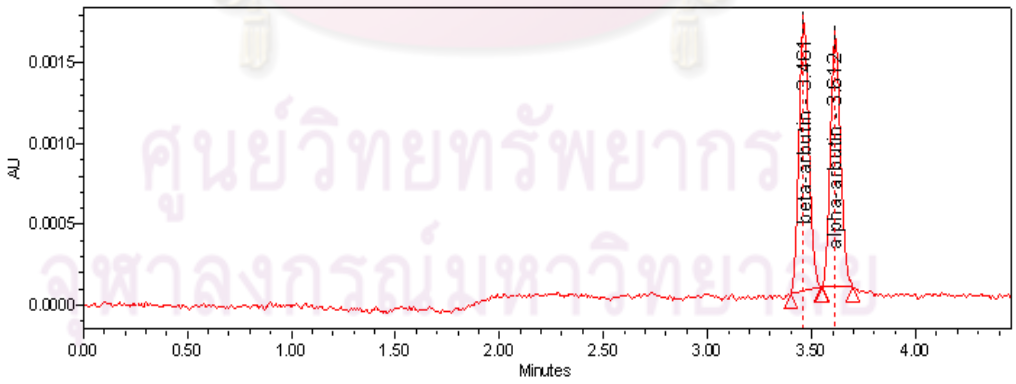


Figure 4.4 Chromatogram of beta- and alpha-arbutin on ACQUITY UPLC® HSS T3, 2.1 x 150 mm column.

Despite ACQUITY UPLC® HSS T3 column is designed for using with 100% aqueous mobile phase, the lifetime of the column is important because it is

expensive. So, the gradient elution was studied. This allowed a flush step after the analytes were completely eluted to prolong the column life time.

4.2.5 Gradient mode

The various parameters of gradient mode such as, time, flow rate, ratio of mobile phase (0.1% HOAc : MeOH) and gradient elution curve profile) were optimized. The gradient mode was applied for four kinds of columns; i.e., ACQUITY UPLC® HSS T3, 1.8 μm, 2.1 x 150 mm, ACQUITY UPLC® BEH Shield RP18, 1.7 μm, 2.1 x 100 mm, ACQUITY UPLC® BEH C18, 1.7 μm, 2.1 x 150 mm and ACQUITY UPLC® BEH C8, 1.7 μm, 2.1 x 150 mm. The mobile phase composition for gradient elution was started at 95% 0.1%HOAc : 5%MeOH, where the optimized R_s was obtained according to the result of the isocratic mode. Then the composition of mobile phase, time and gradient elution curve profile were adjusted for complete separation of the analytes Finally, the ratio of mobile phase was set to flush or regenerate the column for about 1.5 min. After that, the system would be return to the initial step to equilibrate the system. The optimized gradient elution conditions of HSS T3 and BEH Shield RP18 columns were shown in Table 4.2. The chromatograms were also shown in Figure 4.5.

Table 4.2 The optimization of gradient elution profile to obtain highest R_S when using HSS T3 and BEH Shield RP18 columns.

Time (min)	Flow rate (mL/min)	% 0.1% HOAc	% Methanol	Curve
initial	0.3	93	7	-
3.0	0.3	97	3	5
3.4	0.3	90	10	8
4.2	0.3	93	7	5

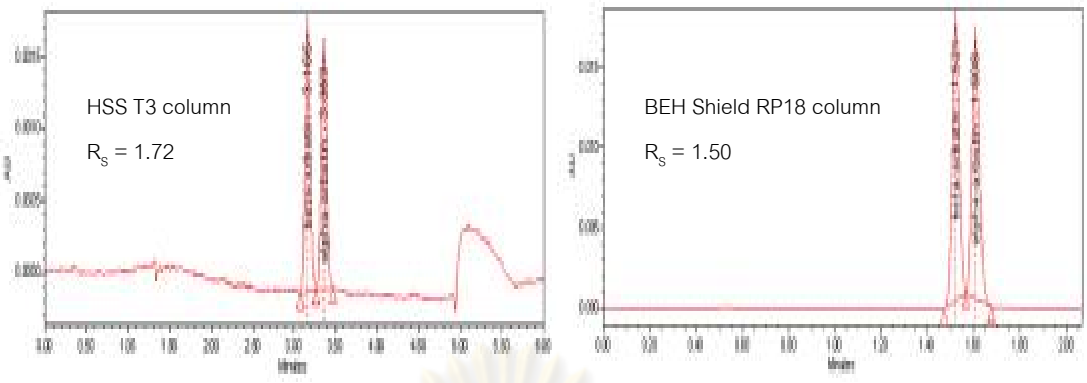


Figure 4.5 Separation of beta- and alpha-arbutin using the optimized gradient elution profile for HSS T3 and BEH Shield RP18 column.

According to the results, the BEH Shield RP18 column was not chosen because the R_S was less than HSS T3 column. The gradient mode was not studied for the longer column (150 mm) of BEH shield RP 18 because it was unavailable in the laboratory. Nevertheless, the trend of BEH Shield RP 18 for separate beta- and alpha-arbutin and decrease the analysis time if can find the 150 mm column length in the future.

In addition, ACQUITY UPLC® BEH C18, 1.7 μm, 2.1 x 150 mm and ACQUITY UPLC® BEH C8, 1.7 μm, 2.1 x 150 mm columns were also studied in gradient mode. Both columns were not designed for using with high aqueous mobile phase because the column would be damaged. So that, the variation of gradient condition ratio between 0.1% HOAc and MeOH included the time and curve were investigated. Finally, the suitable gradient elution profile of both columns and chromatograms were shown in Table 4.3 and Figure 4.6.

Table 4.3 The optimization of the gradient conditions for highest R_S when using BEH C18, and BEH C8 columns.

Time (min)	Flow rate (mL/min)	% 0.1% HOAc	% Methanol	Curve
initial	0.3	98	2	-
2	0.3	100	0	5
3.0	0.3	80	20	8
4.2	0.3	98	2	5

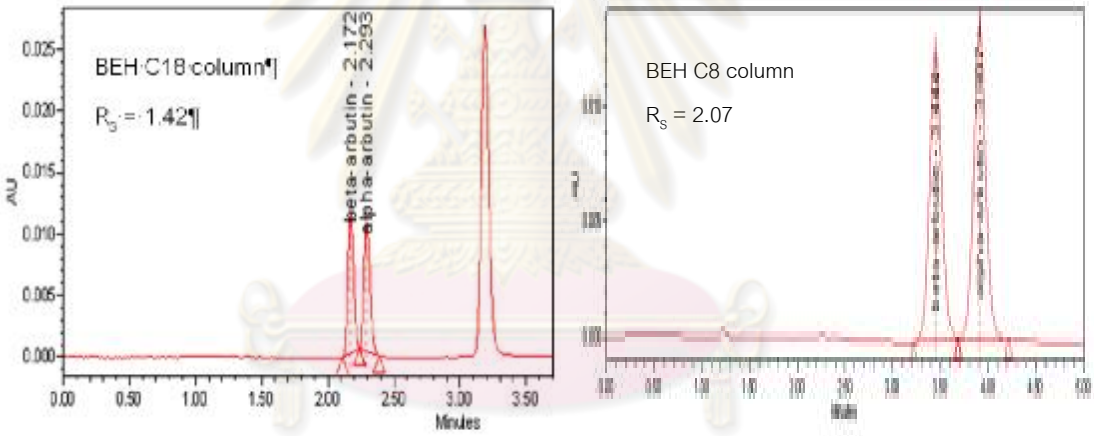


Figure 4.6 Separation of beta- and alpha-arbutin using the optimized gradient elution profile for BEH C18 and BEH C8 column.

The ACQUITY UPLC® BEH C8, 1.7 µm, 2.1 x 150 mm should have been chosen for method validation because it obtained the best R_S when compared with the other columns. However, during the validation, it was found that the efficiency of separation decreased rapidly and was not within acceptable criteria. The high aqueous content might have affected the packing material inside the column (i.e. silica). To resolve the problem, one should increase the ratio of the organic solvent to reactivate

the packing material inside the column resulting in extensively long analysis time. So, it was not suitable for this research. Then, the HSS T3 column was chosen for method validation.

4.3 Method validation

4.3.1 System suitability

Chromatograms of five injections of 2 µg/mL standard beta- and alpha-arbutin were shown in Figure 4.7.

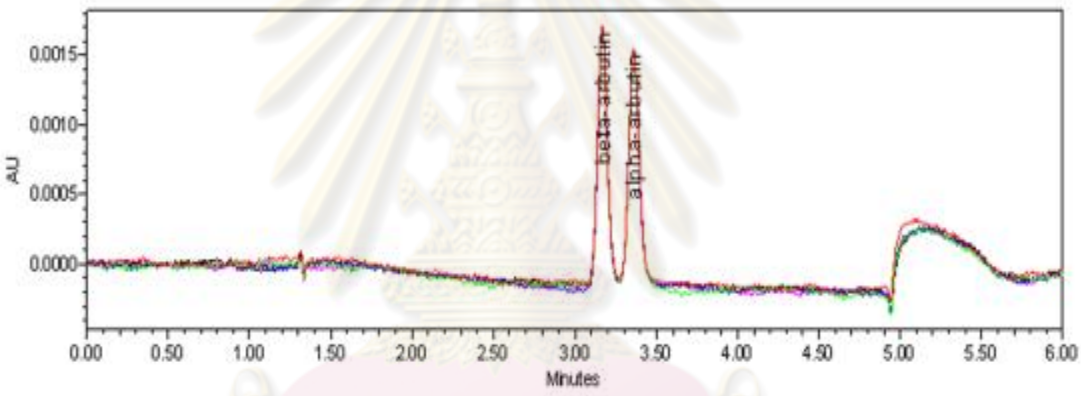


Figure 4.7 Chromatograms of five injections of 2 µg/mL standard beta- and alpha-arbutin.

The parameters of system suitability were summarized in Table 4.4 and Table 4.5, respectively.

Table 4.4 System suitability parameters of standard beta-arbutin.

	Name	Retention time (t_R) (min)	Peak area	Theoretical plate (N)	Retention factor (k´)	Tailing factor	Resolution ($R_{S)}$
1	Beta-arbutin	3.1655	7697	12443	11.66	1.07	-
2	Beta-arbutin	3.1653	7584	12762	11.66	1.06	-
3	Beta-arbutin	3.1652	7592	12721	11.66	1.05	-
4	Beta-arbutin	3.1657	7638	12756	11.66	1.06	-
5	Beta-arbutin	3.1648	7661	12705	11.66	1.04	-
Mean		3.1653	7634	12677		1.06	
SD			47.2	133.1			
%RSD			0.6	1.0			

Table 4.5 System suitability parameters of standard alpha-arbutin.

	Name	Retention time (t_R) (min)	Peak area	Theoretical plate (N)	Retention factor (k´)	Tailing factor	Resolution ($R_{S)}$
1	Alpha-arbutin	3.3593	7320	13013	12.44	1.06	1.71
2	Alpha-arbutin	3.3585	7101	12934	12.43	1.10	1.71
3	Alpha-arbutin	3.3590	7228	12763	12.44	1.12	1.72
4	Alpha-arbutin	3.3598	7302	12892	12.44	1.09	1.72
5	Alpha-arbutin	3.3572	7215	13013	12.43	1.11	1.71
Mean		3.3588	7233	12923	12.44	1.10	
SD			87.0	103.5			
%RSD			1.2	0.8			

All parameters of the system suitability were within the acceptable criteria. The %RSD of areas and theoretical plates (N) of both standards were less than 3.0, the tailing factor was less than 2, the retention factor (k´) was more than 2 and the resolution (R_S) was more than 1.5.

4.3.2 Selectivity or specificity

The selectivity of the method was demonstrated that the retention times of beta-arbutin, alpha-arbutin and hydroquinone were different and not interfered. The retention time of beta- and alpha-arbutin and hydroquinone were 3.16, 3.36 and 4.12 min, respectively as shown in Figure 4.8.

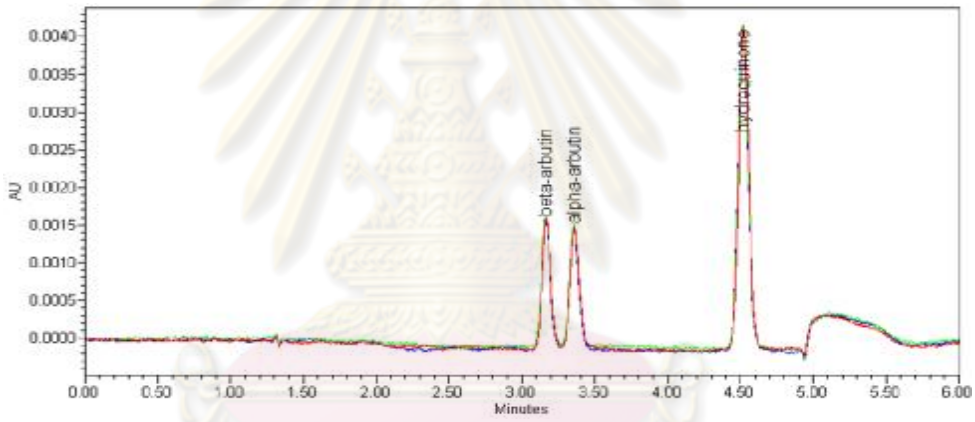


Figure 4.8 The selectivity of beta-arbutin, alpha-arbutin and hydroquinone.

4.3.3 Linearity and range

4.3.3.1 System linearity

The standard S1, S2, S3, S4 and S5 (prepared as described in 3.5) were injected for five injections at each point to construct the calibration curve between peak areas and concentrations. The regression line by method of least squares was established. The coefficient of determination (R^2) of the beta-and alpha-arbutin from

a fitted linear regression line were 0.9999 and 0.9999, respectively as shown in Figure 4.9 and 4.10.

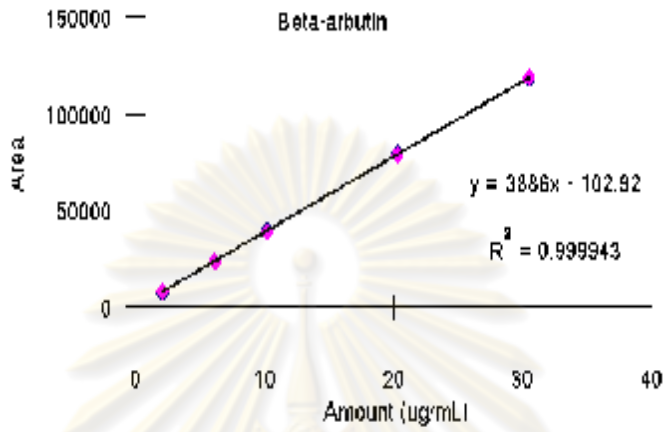


Figure 4.9 Calibration curve of standard beta-arbutin.

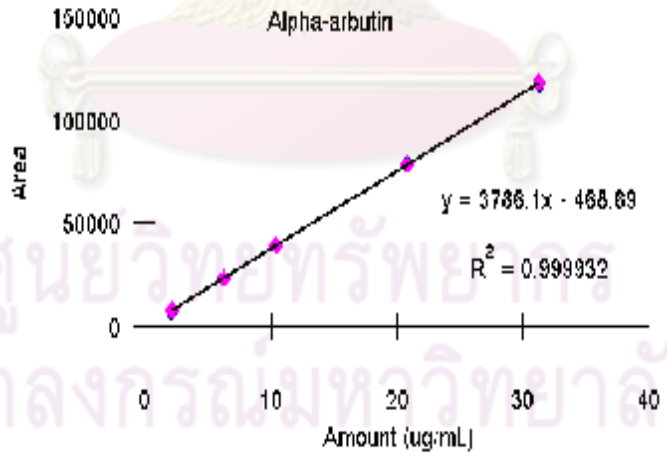


Figure 4.10 Calibration curve of standard alpha-arbutin.

4.3.3.2 Method linearity

The correlation coefficient (r) of spiked and found standards in the cosmetic samples at three levels (50%,100% and 150%) was determined. The

correlation coefficient of beta-arbutin in cream (Figure 4.11), lotion (figure 4.12) and gel (Figure 4.13) was 0.9995, 0.9997 and 0.9995, respectively. The correlation coefficient of beta-arbutin in cream (Figure 4.14), lotion (Figure 4.15) and gel (Figure 4.16) was 0.9999, 0.9995 and 0.9997, respectively. The detail of values was shown in Appendix B.

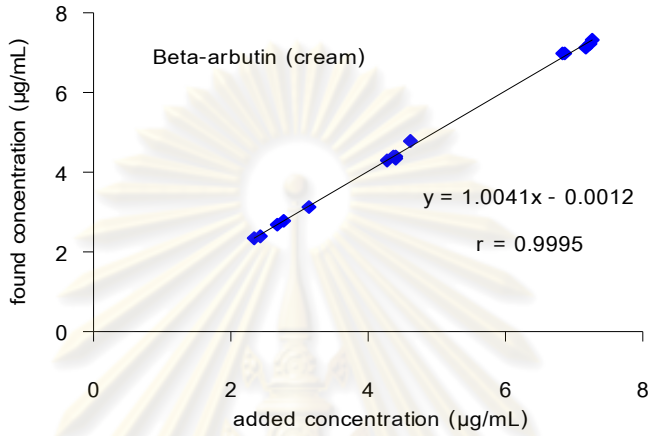


Figure 4.11 Method linearity of beta-arbutin in cream cosmetic sample.

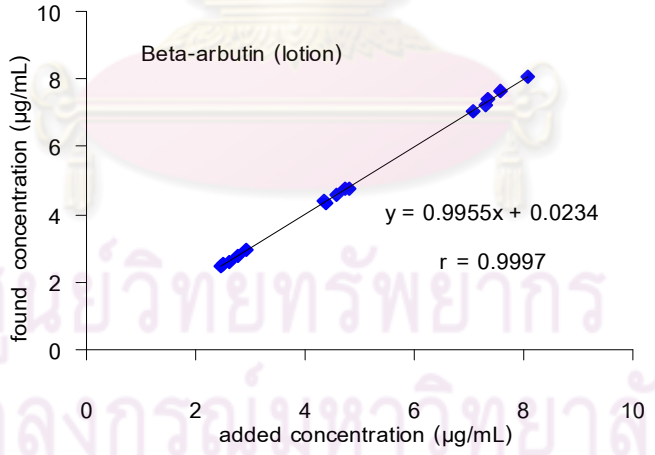


Figure 4.12 Method linearity of beta-arbutin in lotion cosmetic sample.

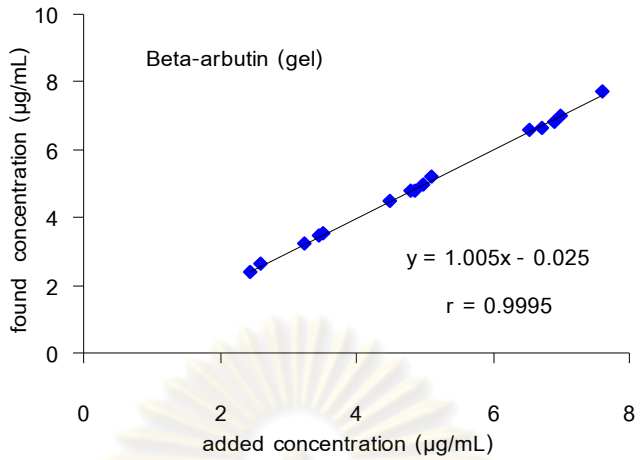


Figure 4.13 Method linearity of beta-arbutin in gel cosmetic sample.

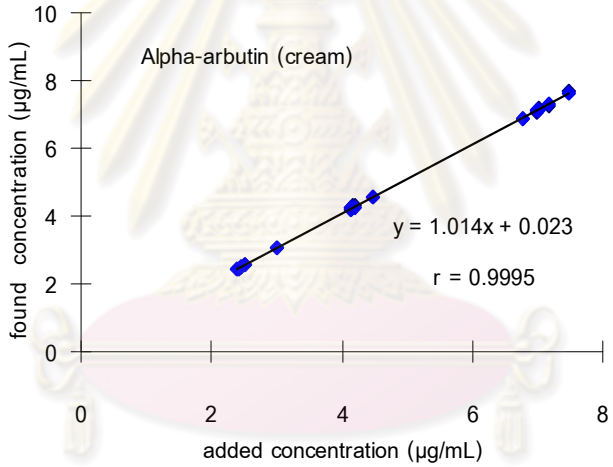


Figure 4.14 Method linearity of alpha-arbutin in cream cosmetic sample.

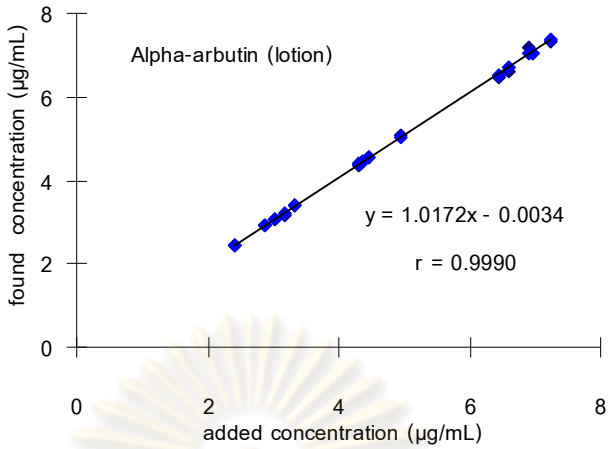


Figure 4.15 Method linearity of alpha-arbutin in lotion cosmetic sample.

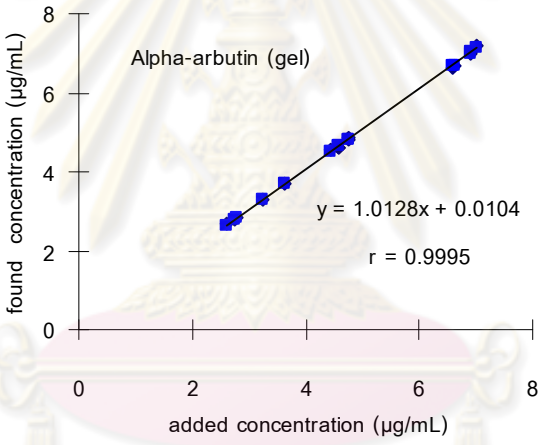


Figure 4.16 Method linearity of alpha-arbutin in gel cosmetic sample.

4.3.4 Precision

4.3.4.1 System precision or reproducibility

The sample solutions of the several matrices were injected in duplicate. The %Relative Percent Different (%RPD) of beta-arbutin in cream, lotion and gel were 0.37, 0.59 and 0.22, respectively. The %RPD of alpha-arbutin in cream lotion and gel were 0.12, 0.19 and 0.38, respectively. The results were shown in Table 4.6

Table 4.6 System precision for determination of beta- and alpha arbutin in various cosmetic samples.

System Precision : Duplicate				
Cream				
Vial	Beta-arbutin		Alpha-arbutin	
	%RPD	Maximum	%RPD	Maximum
1	0.11	0.37	0.04	0.12
2	0.16		0.05	
3	0.37		0.06	
4	0.09		0.07	
5	0.23		0.12	
Lotion				
Vial	Beta-arbutin		Alpha-arbutin	
	%RPD	Maximum	%RPD	Maximum
1	0.23	0.59	0.19	0.22
2	0.06		0.22	
3	0.07		0.18	
4	0.13		0.15	
5	0.59		0.19	
Gel				
Vial	Beta-arbutin		Alpha-arbutin	
	%RPD	Maximum	%RPD	Maximum
1	0.15	0.22	0.22	0.38
2	0.14		0.14	
3	0.05		0.16	
4	0.22		0.01	
5	0.01		0.38	

4.3.4.2 Repeatability (intra day)

The analysis of beta- and alpha-arbutin in cream, lotion and gel cosmetic sample for five replicates, %RSD of beta-arbutin in cream, lotion and gel are 0.34, 0.50 and 0.49, respectively and alpha-arbutin in cream, lotion and gel are 0.15, 0.14 and 0.29, respectively. The results as shown in Table 4.7 and Table 4.8.

Table 4.7 Repeatability (intra-day) of beta-arbutin for several matrices cosmetic samples.

Day	Beta-arbutin					
	%RSD		%RSD		%RSD	
	cream	maximum	lotion	maximum	gel	maximum
1	0.336	0.34	0.498	0.50	0.192	0.49
2	0.132		0.042		0.111	
3	0.206		0.052		0.247	
4	0.234		0.112		0.165	
5	0.212		0.074		0.492	

Table 4.8 Repeatability (intra-day) of alpha-arbutin for several matrices cosmetic samples.

Day	Alpha-arbutin					
	%RSD				%RSD	
	cream	maximum	Lotion	maximum	gel	maximum
1	0.14	0.15	0.100	0.14	0.12	0.29
2	0.078		0.053		0.221	
3	0.149		0.139		0.294	
4	0.043		0.073		0.088	
5	0.02		0.098		0.082	

4.3.4.2 Intermediate precision (inter day)

The Intermediate precision of determination of beta- and alpha-arbutin in cream, lotion and gel cosmetic samples for five replicates was represented by %RSD. The %RSD of determination of beta-arbutin in cream lotion and gel for five days were 0.34, 0.50 and 0.49, respectively. The %RSD of determination of alpha-arbutin in cream, lotion and gel for five days were 0.15, 0.14 and 0.29, respectively. The accepted criteria of the *p*-value from analysis of variance (ANOVA-single factor) should be more than 0.05. The *p*-value of beta-arbutin in cream lotion and gel are 0.34, 0.63 and 0.11, respectively and the p-value of alpha-arbutin in cream, lotion and gel are 0.67, 0.81 and 0.32, respectively. The detail of values was shown in Appendix A.

4.3.5 Accuracy

The %recovery for three levels of beta- and alpha-arbutin in cream, lotion and gel cosmetic samples were between 98-102%. The detail of values was shown in Table 4.9.

Table 4.9 The %recovery of beta- and alpha-arbutin in various cosmetic samples.

% RECOVERY						
Cream sample						
Added level	Bata-arbutin			Alpha-arbutin		
	%recovery	range (%)	mean	%recovery	range (%)	mean
50%	98.72-101.03	98.38-101.70	99.83	98.20-100.81	98.20-100.81	99.62
100%	98.38-100.89			99.52-100.52		
150%	98.51-101.70			98.55-100.23		
Lotion sample						
Added level	Bata-arbutin			Alpha-arbutin		
	%recovery	range (%)	mean	%recovery	range (%)	mean
50%	98.50-101.33	98.12-101.33	99.77	98.03-100.72	98.03-101.60	99.31
100%	98.38-100.95			98.32-100.78		
150%	98.12-100.56			98.02-101.60		
Gel sample						
Added level	Bata-arbutin			Alpha-arbutin		
	%recovery	range (%)	mean	%recovery	range (%)	mean
50%	98.34-100.82	98.02-101.57	99.70	98.25-100.30	98.25-100.69	99.20
100%	98.02-101.57			98.14-100.69		
150%	98.85-101.46			98.07-100.20		

4.3.6 Limit of detection (LOD)

Limit of detection (LOD) from spiked mixture standards in cosmetic sample was 0.005 % w/w.

4.4 The results of method validation

The results of method validation were summarized in Table 4.10

Table 4.10 The summary of the method validation parameters

Parameter	Beta-arbutin			Alpha-arbutin		
1. System suitability						
- Theoretical plate (N)	12677			12856		
- Tailing factor	1.06			1.10		
- Resolution (R_S)	-			1.71		
- Retention factor (k´)	11.66			12.44		
2. Specificity selectivity						
- retention time (min)	3.16			3.36		
3. Linearity and Range						
- Range, μg/ml	2-30			2-30		
3.1 System linearity						
: coefficient of determination (R^2)	0.9999			0.9999		
3.2 Method linearity	cream	lotion	gel	cream	lotion	gel
: correration coefficient (r)	0.9995	0.9997	0.9995	0.9993	0.9995	0.9997
4. Precision						
4.1 Precision (system)						
: standard solution, %RSD of peak area (n=5)	1.41			1.05		
: sample solution,	cream	lotion	gel	cream	lotion	gel
%RPD of peak area (n=5)	0.37	0.59	0.22	0.12	0.19	0.38
4.2 Precision (method)						
4.2.1 Repeatability (intra-day) %RSD (5 replicates)	0.34	0.50	0.49	0.15	0.14	0.29
4.2.2 Intermediate precision (inter-day), *p*-value (5 days)	0.34	0.63	0.11	0.67	0.81	0.32
5. Accuracy : %recovery (3 levels, 5 replicates/level)	98.38-101.70	98.12-101.33	98.02-101.57	98.20-100.81	98.03-101.60	98.25-100.69
6. Limit of detection (LOD) (%w/w)	0.005			0.005		

4.5 Application of the method to the commercial cosmetic samples

Five commercial cosmetic samples from several markets which contained different percent amount of beta-arbutin or alpha-arbutin were tested. The samples were prepared as described in from 3.13. The samples that were labeled as arbutin, were found as beta-arbutin (C1 = 2.09% w/w and C2 = 2.13% w/w) and the samples that were labeled as alpha-arbutin, were found as alpha-arbutin (C3 = 1.04%w/w and C4 = 0.95% w/w). One cosmetic sample that was labeled as alpha-arbutin was not found as alpha-arbutin. The results were summarized in Table 4.11. The chromatograms and spectra of all cosmetic samples were shown in Appendix C.

Table 4.11 Determination of arbutins in the commercial cosmetic samples

Sample name	Whitening agent	%Found (w/w)	%RPD	%Recovery
C1	Beta-arbutin	2.09	0.78	100.59
C2	Beta-arbutin	2.13	0.23	100.15
C3	Alpha-arbutin	1.04	0.52	100.26
C4	Alpha-arbutin	0.95	1.14	100.73
C5	Alpha-arbutin	-	-	-

CHAPTER V

CONCLUSIONS

The method for simultaneous determination of beta- and alpha-arbutin in whitening cosmetics using ultra performance liquid chromatography (UPLC™) was first developed in this study. Using this new UPLC™ technology instead of the typical HPLC markedly increased speed, sensitivity and resolution of the analysis.

The low cost and readily available methanol was found to be the best solvent which can dissolve the standard beta- and alpha-arbutin in a variety of cosmetic matrices without any interference to the chromatography system. In addition, 0.1% acetic acid was used in the mobile phase since it demonstrated stronger interaction resulting in better resolution than water.

Variation of flow rates, gradient ratio in mobile phase and the column types and lengths have been studied in order to achieve the best separation of the isomer substances as beta- and alpha-arbutin. It was found that the BEH C8 column performed best but was not stable for the high aqueous mobile phase. In addition, increasing the length of the BEH shield RP18 column to 150 mm, improved the plate number and the alpha- and beta-arbutin separation.

The HSS T3 column which has been designed for a 100% aqueous mobile phase, produced the acceptable R_S for beta- and alpha-arbutin separation within a short analysis time. The optimized conditions were 0.1% acetic acid and methanol as mobile phase in a nonlinear gradient condition at flow rate of 0.3 mL/min.

Method validation demonstrated that the linear working range of beta- and alpha-arbutin were 2 to 30 μg/ mL. Accuracy (%recovery) and precision (%RSD) of the method were demonstrated by spiking standards at 3 different levels into cream, lotion and gel cosmetic samples. Percents recovery of beta-arbutin were 98.38-101.70, 98.12-

101.33 and 98.02-101.57 and alpha-arbutin were 98.20-100.81, 98.03-101.60 and 98.25-100.69 from each matrix, respectively. %RSD of alpha-arbutin were 0.34, 0.50 and 0.49 and %RSD of beta-arbutin were 0.15, 0.14 and 0.29 from each matrix, respectively. The limit of detection for both arbutins were 0.005% w/w.

The overall performance of this method is considered to be effective, accurate, precise, specific reliable and rapid including the ease of sample preparation which can be applied to the alpha- and beta-arbutin determination in routine cosmetics analysis.

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[28] Getting Start in HPLC [on line]. Available from http://www.lcresources.com/resources/getstart/section3A-k [2009, 12, 7]

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APPENDICES

APPENDIX A

Processing Method:	Arbutins	System:	UPLC1
Processing Method ID:	22784	Channel:	283.0nm@1
Calibration ID:	24181	Date Calibrated:	7/30/2009 11:52:30 AM

R^2 0.999854 R 0.999927 Intercept = -1241.9834 Slope = 3947.5042 Standard Error = 635.4951

Regression Equation = Y = 3.95e+003 X - 1.24e+003

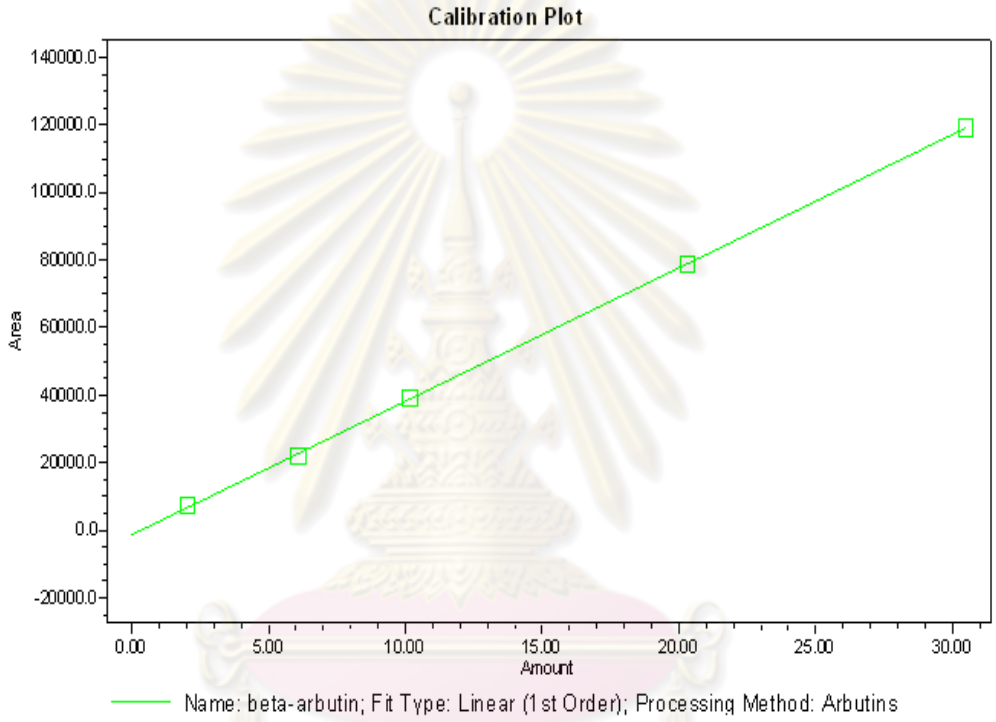

Figure A-1 The linear regression of standard beta-arbutin for commercial cosmetic sample.

Processing Method:	Arbutins	System:	UPLC1
Processing Method ID:	22784	Channel:	283.0nm@1
Calibration ID:	24181	Date Calibrated:	7/30/2009 11:52:30 AM

R^2 0.999905 R 0.999952 Intercept = -1400.3862 Slope = 3814.9196 Standard Error = 499.3821

Regression Equation = Y = 3.81e+003 X - 1.40e+003

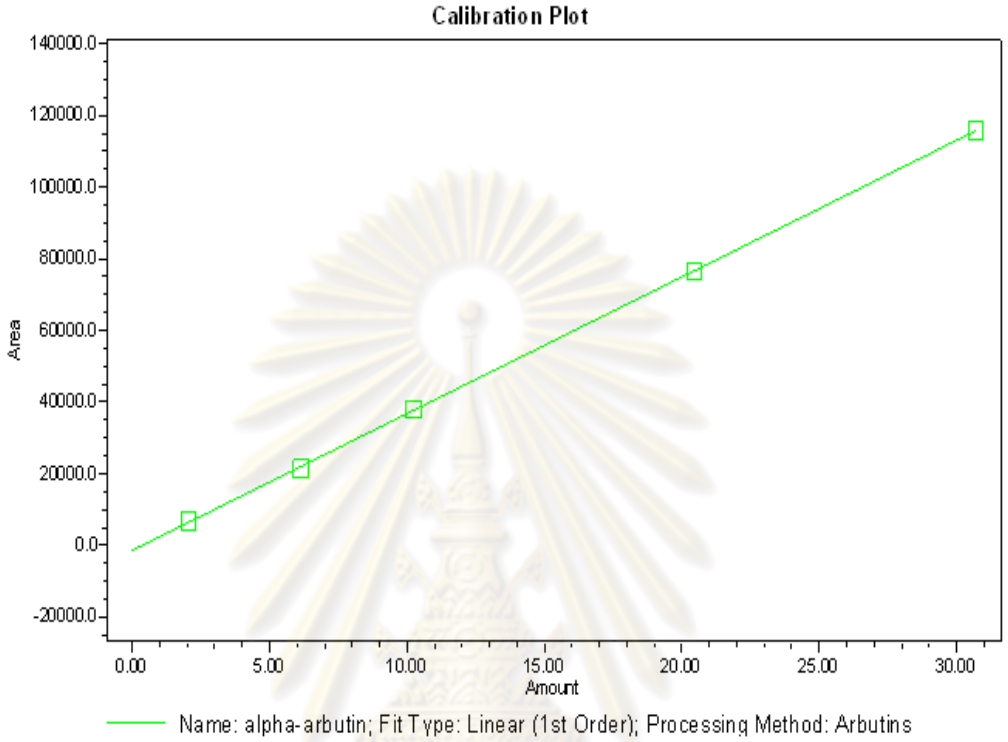

Figure A-2 The linear regression of standard alpha-arbutin for commercial cosmetic sample.

APPENDIX B

Table B-1 The data for method linearity of beta-arbutin in cream cosmetic sample.

Lavel added	Added (μg/ml)	Found av. (μg/ml)	Found (μg/ml)	SD (%w/w)	Mean (%w/w)	Mean±SD (%w/w)	Sam.conc. (μg/ml)
50%	2.7649	2.6715	2.7859	0.31	2.67	2.67 ± 0.31	5.1415
	2.3386		2.3643				4.6589
	2.4183		2.3943				4.7835
	3.1274		3.1247				4.7217
	2.6892		2.6884				4.5512
100%	4.3983	4.4400	4.3596	0.18	4.44	4.44 ± 0.18	4.5749
	4.6175		4.7583				4.7482
	4.2828		4.3163				4.9473
	4.4103		4.3977				4.7539
	4.3704		4.3681				4.8558
150%	6.8326	7.1073	6.9561	0.15	7.11	7.11 ± 0.15	4.8799
	6.8684		6.9602				4.5686
	7.1832		7.1096				5.2714
	7.2429		7.2169				4.7260
	7.2748		7.2938				4.8983

Table B-2 The data for method linearity of beta-arbutin in lotion cosmetic sample.

Lavel added	Added (μg/ml)	Found av. (μg/ml)	Found (μg/ml)	SD (%w/w)	Mean (%w/w)	Mean±SD (%w/w)	Sam.conc. (μg/ml)
50%	2.4686	2.6706	2.4647	0.21	2.67	2.67 ± 0.21	4.3932
	2.7557		2.7870				4.8206
	2.9352		2.9809				4.7857
	2.5005		2.5380				4.5951
	2.6082		2.5825				4.6036
100%	4.8135	4.5614	4.7577	0.21	4.56	4.56 ± 0.21	4.5691
	4.7377		4.7850				4.6410
	4.3748		4.3350				4.4959
	4.5702		4.5501				4.6350
	4.3270		4.3792				4.4271
150%	7.3459	7.4733	7.4015	0.41	7.47	7.47 ± 0.41	4.4017
	8.0717		8.0735				4.3733
	7.0947		7.0411				4.7605
	7.3100		7.1999				4.6008
	7.5932		7.6505				4.5645

Table B-3 The data for method linearity of beta-arbutin in gel cosmetic sample.

Lavel added	Added (μg/ml)	Found av. (μg/ml)	Found (μg/ml)	SD (%w/w)	Mean (%w/w)	Mean±SD (%w/w)	Sam.conc. (μg/ml)
50%	3.5219	3.0486	3.5311	0.51	3.05	3.05 ± 0.51	4.9738
	3.4621		3.4582				4.8976
	3.2270		3.2241				4.3347
	2.5976		2.6206				5.0344
	2.4342		2.4091				4.8023
100%	5.0916	4.8372	5.1885	0.25	4.84	4.84 ± 0.25	4.7636
	4.4820		4.5058				4.6341
	4.7928		4.7658				4.5998
	4.9800		4.9407				4.5610
	4.8525		4.7854				4.3955
150%	7.0039	6.9584	6.9902	0.45	6.96	6.96 ± 0.45	4.5855
	6.7051		6.6647				4.5564
	7.5895		7.7156				4.6704
	6.5218		6.5662				4.8885
	6.9003		6.8551				4.9293

Table B-4 The data for method linearity of alpha-arbutin in cream cosmetic sample.

Lavel added	Added (μg/ml)	Found av. (μg/ml)	Found (μg/ml)	SD (%w/w)	Mean (%w/w)	Mean±SD (%w/w)	Sam.conc. (μg/ml)
50%	2.4464	2.6032	2.5156	0.27	2.60	2.60 ± 0.27	5.0786
	2.5089		2.5427				4.6020
	2.4112		2.4665				4.7251
	2.3878		2.4103				4.6640
	3.0131		3.0808				4.4956
100%	4.1308	4.3232	4.2167	0.14	4.32	4.32 ± 0.14	4.5190
	4.4747		4.5642				4.6902
	4.1464		4.2405				4.8868
	4.1933		4.3091				4.6958
	4.1737		4.2854				4.7964
150%	6.7687	7.1999	6.8618	0.30	7.20	7.20 ± 0.30	4.8202
	6.9758		7.0539				4.5128
	7.1634		7.2357				5.2070
	7.0344		7.1749				4.6682
	7.4838		7.6732				4.8385

Table B-5 The data for method linearity of alpha-arbutin in lotion cosmetic sample.

Lavel added	Added (μg/ml)	Found av. (μg/ml)	Found (μg/ml)	SD (%w/w)	Mean (%w/w)	Mean±SD (%w/w)	Sam.conc. (μg/ml)
50%	2.4112	3.0053	2.4553	0.35	3.01	3.01 ± 0.35	4.2715
	3.3296		3.3880				4.6870
	3.0326		3.0606				4.6531
	2.8646		2.9441				4.4678
	3.1655		3.1784				4.4761
100%	4.9319	4.5639	5.0721	0.29	4.56	4.56 ± 0.29	4.4425
	4.4668		4.5410				4.5125
	4.3144		4.3514				4.3714
	4.3222		4.4221				4.5066
	4.3652		4.4330				4.3045
150%	6.9133	6.9298	7.1765	0.37	6.93	6.93 ± 0.37	4.2797
	7.2415		7.3383				4.2522
	6.5811		6.6078				4.6286
	6.4287		6.4762				4.4733
	6.9484		7.0502				4.4380

Table B-6 The data for method linearity of alpha-arbutin in gel cosmetic sample.

Lavel added	Added (μg/ml)	Found av. (μg/ml)	Found (μg/ml)	SD (%w/w)	Mean (%w/w)	Mean±SD (%w/w)	Sam.conc. (μg/ml)
50%	3.2319	3.0481	3.2801	0.43	3.05	3.05 ± 0.43	4.8262
	3.6188		3.6944				4.7523
	2.7708		2.8149				4.2061
	2.7590		2.7820				4.8850
	2.6105		2.6690				4.6599
100%	4.7560	4.6841	4.8434	0.13	4.68	4.68 ± 0.13	4.6223
	4.5645		4.6019				4.4966
	4.5763		4.6135				4.4634
	4.4278		4.5599				4.4257
	4.7482		4.8018				4.2651
150%	6.9093	6.9035	7.0340	0.23	6.90	6.90 ± 0.23	4.4495
	7.0149		7.1822				4.4212
	6.9211		6.9621				4.5318
	6.6123		6.7070				4.7435
	6.5772		6.6321				4.7830

Table B-7 The ANOVA for single factor of beta-arbutin in cream cosmetic sample.

Beta-arbutin in cream sample (%w/w)						
Replicate/day	1	2	3	4	5	mean
1	2.274	2.277	2.271	2.278	2.267	2.27
2	2.257	2.270	2.268	2.272	2.271	
3	2.275	2.275	2.275	2.266	2.270	
4	2.273	2.276	2.262	2.275	2.269	
5	2.275	2.273	2.271	2.267	2.259	

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	11.354	2.2708	6.02E-05
Column 2	5	11.371	2.2742	7.7E-06
Column 3	5	11.347	2.2694	2.33E-05
Column 4	5	11.358	2.2716	2.63E-05
Column 5	5	11.336	2.2672	2.32E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000135	4	3.374E-05	1.199005	0.341955	2.866081
Within Groups	0.000563	20	2.814E-05			
Total	0.000698	24				

Table B-8 The ANOVA for single factor of beta-arbutin in lotion cosmetic sample.

Beta-arbutin in lotion sample (%w/w)						
Replicate/day	1	2	3	4	5	mean
1	2.172	2.172	2.171	2.171	2.171	2.17
2	2.172	2.173	2.173	2.172	2.169	
3	2.160	2.171	2.171	2.168	2.173	
4	2.169	2.171	2.173	2.166	2.171	
5	2.147	2.171	2.171	2.169	2.172	

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	10.82	2.164	0.0001145
Column 2	5	10.858	2.1716	8E-07
Column 3	5	10.859	2.1718	1.2E-06
Column 4	5	10.846	2.1692	5.7E-06
Column 5	5	10.856	2.1712	2.2E-06

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000215	4	5.364E-05	2.1559486	0.111295	2.866081
Within Groups	0.000498	20	2.488E-05			
Total	0.000712	24				

Table B-9 The ANOVA for single factor of beta-arbutin in gel cosmetic sample.

Beta-arbutin in gel sample (%w/w)						
Replicate/day	1	2	3	4	5	mean
1	2.174	2.176	2.164	2.169	2.177	2.17
2	2.175	2.174	2.174	2.172	2.174	
3	2.171	2.179	2.178	2.175	2.152	
4	2.165	2.173	2.174	2.178	2.177	
5	2.174	2.175	2.174	2.175	2.176	

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	10.859	2.1718	1.67E-05
Column 2	5	10.877	2.1754	5.3E-06
Column 3	5	10.864	2.1728	2.72E-05
Column 4	5	10.869	2.1738	1.17E-05
Column 5	5	10.856	2.1712	0.0001167

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.56E-05	4	1.39E-05	0.3913288	0.812352	2.866081
Within Groups	0.00071	20	3.552E-05			
Total	0.000766	24				

Table B-10 The ANOVA for single factor of alpha-arbutin in cream cosmetic sample.

Alpha-arbutin in cream sample (%w/w)						
Replicate/day	1	2	3	4	5	mean
1	2.247	2.243	2.244	2.244	2.244	2.24
2	2.242	2.243	2.239	2.242	2.243	
3	2.241	2.244	2.246	2.244	2.244	
4	2.249	2.243	2.243	2.245	2.243	
5	2.245	2.240	2.247	2.245	2.243	

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	11.224	2.2448	1.12E-05
Column 2	5	11.213	2.2426	2.3E-06
Column 3	5	11.219	2.2438	9.7E-06
Column 4	5	11.22	2.244	1.5E-06
Column 5	5	11.217	2.2434	3E-07

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.3E-05	4	3.26E-06	0.652	0.632176	2.86608071
Within Groups	1E-04	20	5E-06			
Total	0.000113	24				

Table B-11 The ANOVA for single factor of alpha-arbutin in lotion cosmetic sample.

Alpha-arbutin in lotion sample (%w/w)						
Replicate/day	1	2	3	4	5	mean
1	2.127	2.124	2.120	2.124	2.128	2.12
2	2.123	2.125	2.124	2.124	2.124	
3	2.124	2.125	2.127	2.123	2.123	
4	2.123	2.125	2.127	2.123	2.127	
5	2.126	2.127	2.123	2.126	2.126	

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	10.623	2.1246	3.3E-06
Column 2	5	10.626	2.1252	1.2E-06
Column 3	5	10.621	2.1242	8.7E-06
Column 4	5	10.62	2.124	1.5E-06
Column 5	5	10.628	2.1256	4.3E-06

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.04E-06	4	2.26E-06	0.594737	0.670515	2.86608071
Within Groups	7.6E-05	20	3.8E-06			
Total	8.5E-05	24				

Table B-12 The ANOVA single factor of alpha-arbutin in gel cosmetic sample.

Alpha-arbutin in gel sample (%w/w)						
Replicate/day	1	2	3	4	5	mean
1	2.090	2.089	2.081	2.093	2.095	2.09
2	2.093	2.096	2.087	2.092	2.098	
3	2.096	2.086	2.096	2.095	2.093	
4	2.092	2.096	2.093	2.091	2.095	
5	2.096	2.095	2.093	2.091	2.095	

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	10.467	2.0934	6.8E-06
Column 2	5	10.462	2.0924	2.13E-05
Column 3	5	10.45	2.09	3.6E-05
Column 4	5	10.462	2.0924	2.8E-06
Column 5	5	10.476	2.0952	3.2E-06

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.1E-05	4	1.78E-05	1.266762	0.315847	2.86608071
Within Groups	0.00028	20	1.4E-05			
Total	0.000351	24				

Table B-13 The details of standard beta- and alpha-arbutin.

	Brand		Grade	Lot No.		%Purity	Mfg.	Exp.	Supplier
Beta-arbutin, β-at	Bioland		Cosmetic	ARP-810978		99.60%	9-Jul-08	8-Jul-10	Sinthai co.,ltd
Alpha-arbutin, α-at	Pentapharm		Cosmetic	41209201		97.70%	1-Feb-08	1-Feb-11	sinthai
Whitening agents	Flask no.	Weight (g)	True weight (g)	1	2	3	4	6	Flask no.
				0.02	0.06	0.10	0.20	0.30	Volume (ml)
Beta-arbutin, β-at	9	0.02550	0.02540	2.0318	6.0955	10.1592	20.3184	30.4776	Concentration (µg/mL)
Alpha-arbutin, α-at		0.02618	0.02558	2.0462	6.1387	10.2311	20.4623	30.6934	

Table B-14 The result of four commercial cosmetic samples.

Sample name	Whitening agent	Sample weight (g)	Spike std. (g)	Peak Area	Found conc. (ug/ml)	% Found (%w/w)	Mean (%w/w)	% RPD	Spike std. (%w/w)	% recovery	% LB (%w/w)	% LL (%w/w)	% UL (%w/w)
Laneige A	beta-arbutin (0.1 ml)	0.54582	-	16828	4.5776	2.0967	2.0886	0.78			2.00	1.70	2.36
Laneige B	beta-arbutin (0.1 ml)	0.54654	-	16713	4.5483	2.0805							
Laneige C	beta-arbutin (0.1 ml)	0.50713	0.01304	36127	9.4665	4.6667			2.571333	100.2632			
Laneige D	beta-arbutin (0.1 ml)	0.50224	0.01289	35863	9.3996	4.6788			2.566502	100.9234			
Etude A	beta-arbutin (0.1 ml)	0.55357	-	17403	4.7233	2.1331	2.1356	0.23			2.00	1.70	2.36
Etude B	beta-arbutin (0.1 ml)	0.59280	-	18770	5.0695	2.1380							
Etude C	beta-arbutin (0.1 ml)	0.50547	0.01194	34417	9.0333	4.4678			2.362158	98.7339			
Etude D	beta-arbutin (0.1 ml)	0.52982	0.01507	40794	10.6488	5.0247			2.844362	101.5746			
Smooth E A	alpha-arbutin (0.1 ml)	0.50963	-	14715	4.2244	1.0361	1.0388	0.52			2.00	1.70	2.36
Smooth E B	alpha-arbutin (0.1 ml)	0.50456	-	14637	4.2040	1.0415							
Smooth E C	alpha-arbutin (0.1 ml)	0.57867	0.00748	36568	9.9526	2.3493			1.292619	101.3833			
Smooth E D	alpha-arbutin (0.1 ml)	0.50372	0.00778	40137	10.8882	2.5701			1.544509	99.1448			
DHC A	alpha-arbutin (0.1 ml)	0.50216	-	13053	3.7886	0.9431	0.9485	1.14			2.00	1.70	2.36
DHC B	alpha-arbutin (0.1 ml)	0.54216	-	14383	4.1373	0.9539							
DHC C	alpha-arbutin (0.1 ml)	0.58724	0.00945	44723	12.0901	2.5735			1.609223	100.9804			
DHC D	alpha-arbutin (0.1 ml)	0.52869	0.00850	39967	10.8436	2.5638			1.607747	100.4698			

Table B-15 The results of commercial cosmetic samples that did not detected beta- or alpha-arbutin.

Sample name	Whitening agent	Sample weight (g)	Spike std. (g)	Peak Area	Found conc. (ug/ml)	% Found (%w/w)	Mean (%w/w)	% RPD	Spike std. (%w/w)	% recovery	% LB (%w/w)	% LL (%w/w)	% UL (%w/w)
Mistine A	beta-arbutin (0.1 ml)	0.62924	-	-	-	-	-	#DIV/0!					
Mistine B	beta-arbutin (0.1 ml)	0.67956	-	-	-	-	-						
Mistine C	beta-arbutin (0.1 ml)	0.60182	0.00003	927	0.5495	0.005	-			#DIV/0!			
Mistine A	alpha-arbutin (0.1 ml)	0.62924	-	-	-	-	-						
Mistine B	alpha-arbutin (0.1 ml)	0.67956	-	-	-	-	-						
Mistine C	alpha-arbutin (0.1 ml)	0.60182	0.00003	954	0.6171	0.005	-			#DIV/0!			

APPENDIX C

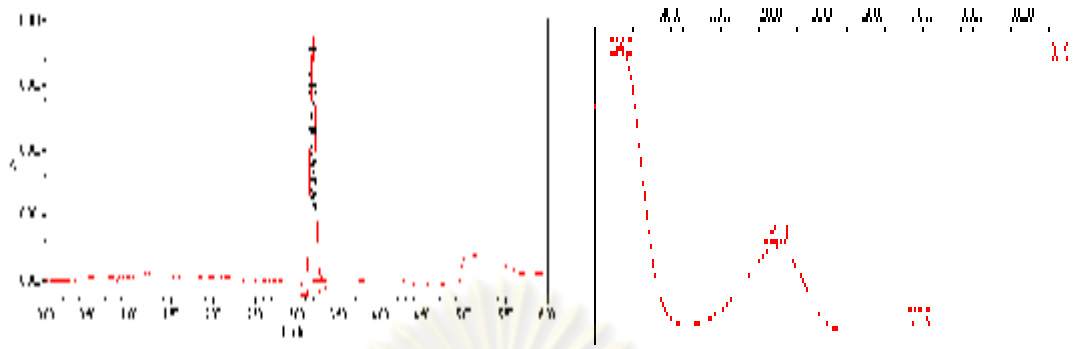

Figure C-1 The chromatogram and spectra of cosmetic sample name as C1.

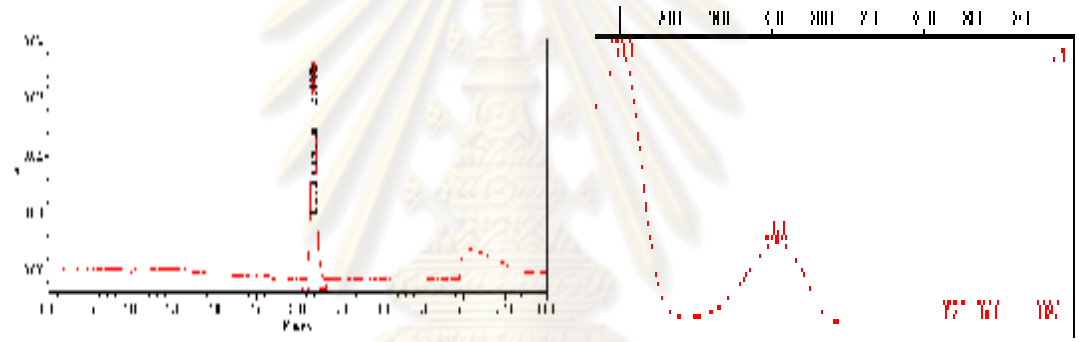

Figure C-2 The chromatogram and spectra of cosmetic sample name as C2.

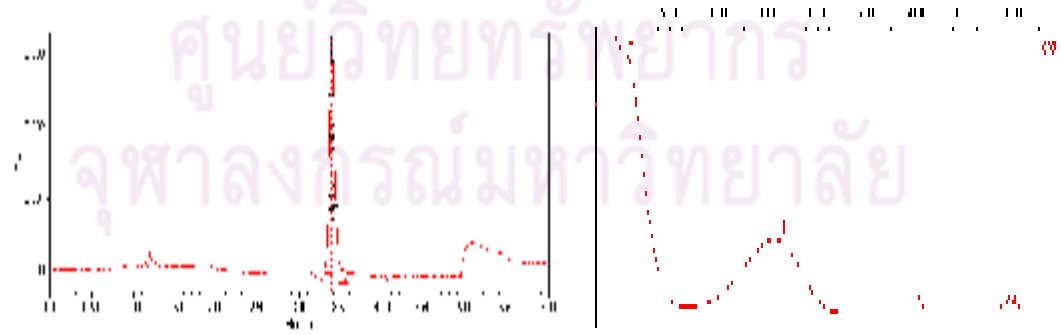

Figure C-3 The chromatogram and spectra of cosmetic sample name as C3.

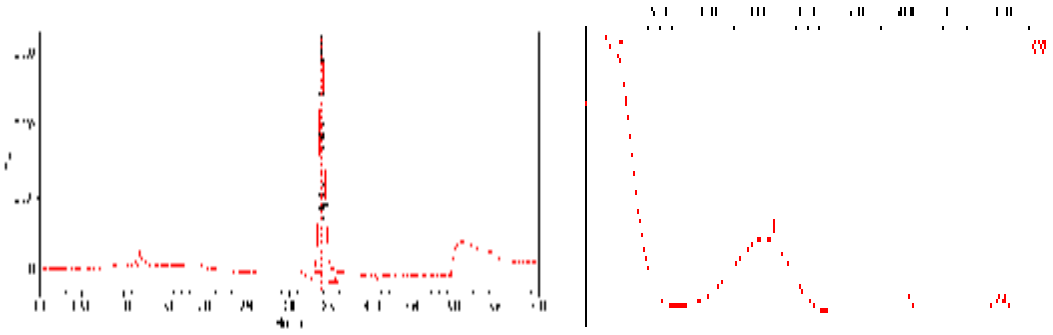

Figure C-4 The chromatogram and spectra of cosmetic sample name as C4.

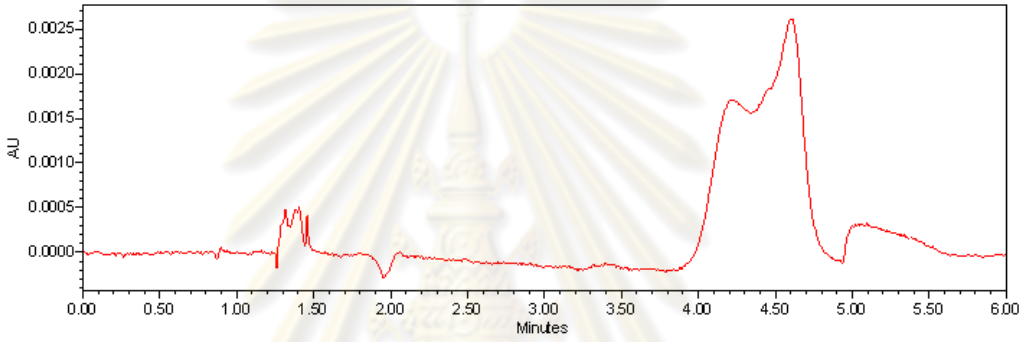

Figure C-5 The chromatogram of cosmetic sample name as C5 that did not contained beta- and alpha-arbutin.

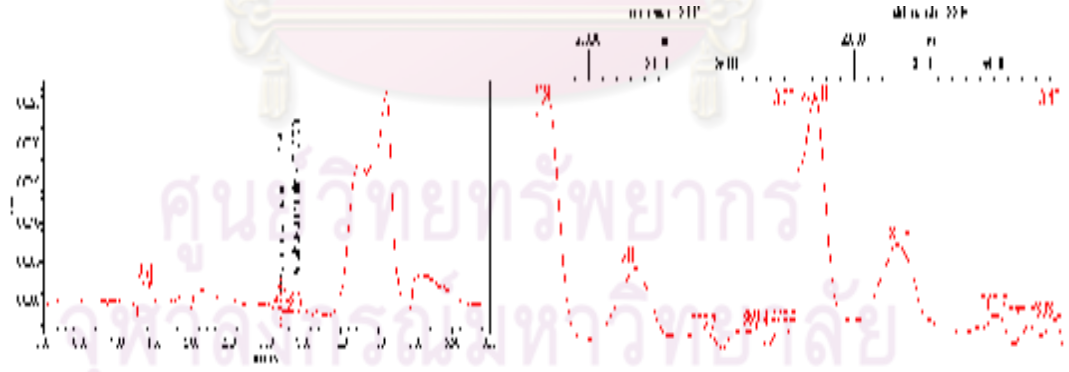

Figure C-6 The chromatogram and spectra of cosmetic sample name as C5 that spiked standard beta- and alpha-arbutin for limit of detection (LOD).

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Presentation :

1. M. Mala and P. Varanusupakul, "UPLC method development for simultaneous determination of alpha- and beta-arbutins in cosmetic products", Present at The International Symposium on Pharmaceutical and Biomedical Analysis (PBA 2009), 11-14 October 2009, Orlando, Florida, USA.
2. M. Mala and P. Varanusupakul, "An UPLC method for simultaneous determination of alpha- and beta- arbutins in cosmetics", Present at The Pure and Applied Chemistry International Conference 2010, 21-23 January 2010, Ubonratchathani, Thailand (International).

Publication :

1. M. Mala and P. Varanusupakul, "A HPLC method using UPLC™ column for simultaneous determination of alpha- and beta-arbutin in cosmetics" Pure and Applied Chemistry International Conference 2010 Proceeding, 41-43.