High-Performance Liquid Chromatographic Method for Separation and Quantitative Analysis of Arbutin in Plant Tissue Cultures

Nisit Kittipongpatana*, Aueporn Chaiwan, Umaporn Pusod and Ornanong S. Kittipongpatana

Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand

**Corresponding authors: E-mail: nisitk@pharmacy.cmu.ac.th*

ABSTRACT

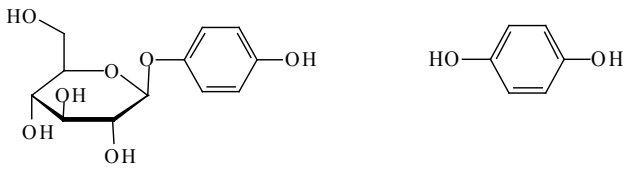
A simple and sensitive isocratic high-performance liquid chromatographic (HPLC) method for the quantitation of arbutin, a tyrosinase-enzyme inhibitor, commonly used as a whitening agent in cosmetic products, is reported. A system which consisted of a reverse-phase Apollo C-18 column (4.6x150 mm), a methanol-water (10:90) mobile phase, a flow rate of 0.9 mL/min and a UV detection at 280 nm provided a highly-sensitive and reproducible assay. Simultaneous separation and determination of arbutin from its precursor, hydroquinone, in plant cell suspension culture samples at amounts as low as 0.16 μg was achieved within 10 minutes. Sharp symmetrical peaks were obtained and a linear relationship between the amount of arbutin injected and the area under the peak was achieved over the range of 0.016 to 1.024 mg/mL with the relative standard deviation of less than 0.4%. Using this method of analysis, it was demonstrated that cell suspension cultures of Capsicum annuum L., Solanum aculeatissimum Jacq., and Datura fastuosa L. (Solanaceae) were able to biotransform exogenous hydroquinone into arbutin, while those of Ocimum basilicum L. (Lamiaceae) and Allamanda cathartica L. (Apocynaceae) did not show such ability.

Key words: High-performance liquid chromatography (HPLC), arbutin, hydroquinone, plant cell suspension culture

INTRODUCTION

Arbutin [1], a phenolic glucoside found in many plants such as bearberry (*Arctostaphylos uva-ursi*), cranberry (*Vaccinium macrocapron*) and blueberry (*Vaccinium corymbosum*) (Ericaceae), as well as bergenia (*Bergenia crassifolia*) (Saxifragaceae) and pears (*Pyrus communis*) (Rosaceae), is a known inhibitor of tyrosinase enzyme and has been employed as a whitening agent in many cosmetic preparations. It is structurally related to hydroquinone (Figure 1[2]), a compound which also possesses a tyrosinase-inhibitory effect but the legal use as an ingredient in topical cosmetic products has been prohibited in Thailand due to its toxicity and side effects. The advantages of arbutin over hydroquinone are that it is more stable,

causes much less or no irritation to the skin and does not have unpleasant odor or side effect. In addition to the skin-whitening effect, arbutin was also reported to possess anti-ageing, UV-B/ UV-C filtering and pain-relieving properties as well as to promote scar-free healing.



[1] [2]

Figure 1. Structures of arbutin [1] and hydroquinone [2].

Arbutin can be prepared by chemical synthesis from glucose (Huang et al., 2004) but, biosynthetically, plants with glucosyltransferase enzymes can convert hydroquinone into arbutin. Because the price of arbutin is significantly higher (~200 times) than that of hydroquinone, it is logical to develop a plant cell culture system that can convert the less-expensive hydroquinone into the higher-value arbutin. Such systems could be those of known arbutin-producing plant species or systems that derived from plants not known to accumulate arbutin but capable of performing substrate glucosylation. Although there is, to date, no report of arbutin-producing Thai plants, a number of plant species are known to carry out such task, including *Catharanthus roseus* (Inomata et al., 1991), *Rauwolfia serpentina* (Arend et al., 2000) and *Datura innoxia* (Suzuki et al., 1987). In these processes, a detection and quantitation method to follow the production of arbutin is as equally important as the production iself. Chromatographic methods, i.e., thin-layer chromatography (TLC) and, more commonly, high-performance liquid chromatography (HPLC) have been used for such purpose. A number of high-performance liquid chromatographic methods have been reported for qualitative and quantitative analysis of arbutin. The conditions and complexities of these methods were varied, based on the nature and compositions of the samples. Early-developed methods focused on the separation, detection and quantitation of arbutin in plant extract samples which contained many interferences and some methods required a long time of separation (Kraus and Stahl, 1979; Sticher et al., 1979; Fromard, 1983; Stambergova et al., 1985; Anetai and Yamagishi, 1986). Later, when the compound had been isolated, purified and formulated into products, methods were developed for the analysis of arbutin and related compounds (i.e., methylarbutin, hydroquinone) and other whitening agents (i.e., glycolic acid, ascorbic acid, magnesium ascorbyl phosphate, ascorbyl glucoside and kojic acid) in cosmetic products (Teglia, 1989; Huang et al., 2001; Chang and Chang, 2003; Huang et al., 2004). Recently, HPLC methods were developed for the determination of arbutin in gel formulation (Zhang et al., 2004), for the separation of arbutin from its impurities in chemical synthesis samples (Lian et al., 2005) and for detection and quantitation of arbutin and metabolites (hydroquinone glucuronide, hydroquinone sulfate) in urine samples following oral administration of bearberry leaf extracts (Quintus et al., 2005).

In this study, we present isocratic HPLC condition that is simple and rapid for simultaneous separation of arbutin from hydroquinone and quantitation of arbutin in plant cell suspension culture samples. The method allows rapid analysis of arbutin in plant tissue sample. This method was applied to the detection and quantitation of arbutin obtained from hydroquinone biotransformation, using cell suspension cultures of *Capsicum annuum* L., *Solanum aculeatissimum* Jacq., *Datura fastuosa* L., *Ocimum basilicum* L. and *Allamanda cathartica* L.

MATERIALS AND METHODS

Materials

Arbutin standard (minimum 98%) was purchased from Sigma Chemical Co. (St. Louis, USA). Hydroquinone was purchased from Fluka. All solvents used for chromatographic purposes were of HPLC grade. Other solvents were of ACS grade or equivalent.

Methods

Callus and cell suspension cultures

Callus cultures of *Capsicum annuum* L., *Solanum aculeatissimum, Datura fastuosa, Ocimum basilicum* L. and *Allamanda cathartica* L. were initiated by placing sterile explants, obtained either from aseptically-germinated seeds or chemically-disinfected explants, on Murashige and Skoog (M&S) medium containing 2% w/v (20 g/L) sucrose, 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L benzylaminopurine (BAP). After four to six weeks of growth and subculture, the callus cultures were transferred into liquid media of the same compositions where cell suspension cultures were established. After a few days, 5 mL of homogenous cells were transferred into culture bottles and fresh liquid media were added to 35 mL. The cultures were maintained on a gyratory shaker (120 rpm) for two weeks in the dark condition before biotransformation experiments.

Biotransformation of hydroquinone to arbutin

The 2-week-old cell suspension cultures of the plants studied were added with 15.57 mM hydroquinone. The cell suspension samples were harvested on day 5 after the addition of hydroquinone and were filtered through a filter paper to separate the cells from the liquid media. The liquid media were adjusted to 35 mL with distilled water, passed through a 0.2 μm membrane filter into sample vials and finally subjected to the analysis of arbutin by HPLC.

High-performance liquid chromatography conditions

HPLC was performed using a Hewlett Packard HP 1100 Liquid Chromatography System which consisted of a double-piston pump, an autosampler and a variable wavelength UV/VIS detector. The HPLC column was Apollo C-18 (4.6 x 150 mm, 5 μm particle diameter, 100 Å average pore size) (Alltech Associates, Inc., USA). The methanol: water (90:10) mobile phase was pumped through the column at a flow rate of 0.9 mL/min. Arbutin and hydroquinone were both detected by UV

absorption at 280 nm. Each injection volume was 10 μL. The standard solutions of arbutin and hydroquinone were prepared in deionized water.

Recovery percentage

Five milligrams of standard arbutin was added into a 2-week-old *C. annuum* cell suspension culture (35 mL). The cell suspension sample was then harvested, without the addition of hydroquinone, and filtered to separate the liquid from the cells. The liquid medium was adjusted to 35 mL with distilled water and passed through a 0.2 ?m membrane filter. The filtrate was subjected to HPLC analysis and the amount of arbutin in the sample was determined using the standard curve. The percent recovery of arbutin was calculated. This experiment was repeated three times.

Quantitation of arbutin in cell suspension samples and reproducibility

The HPLC injection consisted of 10 μL of the liquid medium samples. The area under the curve (AUC) which appeared at the same retention time (RT) as that of the authentic standard was recorded. This AUC was used to calculate the amount of arbutin in the sample by using the linear equation obtained from the composite standard curve. The reproducibility of quantitative analysis was verified by carrying out five replicated injections of each sample. The coefficient of variation of each determination was calculated.

RESULTS AND DISCUSSION

Both arbutin and hydroquinone, which exhibit good UV absorption at 280 nm, are phenol derivatives and therefore are weakly acidic. Arbutin is more polar than hydroquinone due to the presence of the glucose moiety. This polar group can interact with silanol groups on the bonded phase of HPLC column which results in the peak-tailing phenomenon. A reverse-phase column packed with a base-deactivated silica, which decreased free silanol concentration, was selected as stationary phase to overcome this problem. This column is less active towards polar compounds (Steffeck et al., 1995) and significantly yields a symmetrical arbutin peak which allows higher accuracy of peak integration in the quantitation process. The use of aqueous methanol was found to yield better and more sensitive separation than aqueous acetonitrile. The optimum strength of 10% methanol is shown to be adequately effective for the separation of the two compounds in a 10-minute run, while the use of higher concentration of methanol resulted in a shorter run time and the compromised resolution between the two peaks. The separation of arbutin and hydroquinone is based on the difference in the polarity and, under the developed conditions, arbutin elutes earlier at a retention time (RT) of ~4.0 minutes, while the less-polar hydroquinone showed a peak at RT ~5.8 minutes (Figure 2).

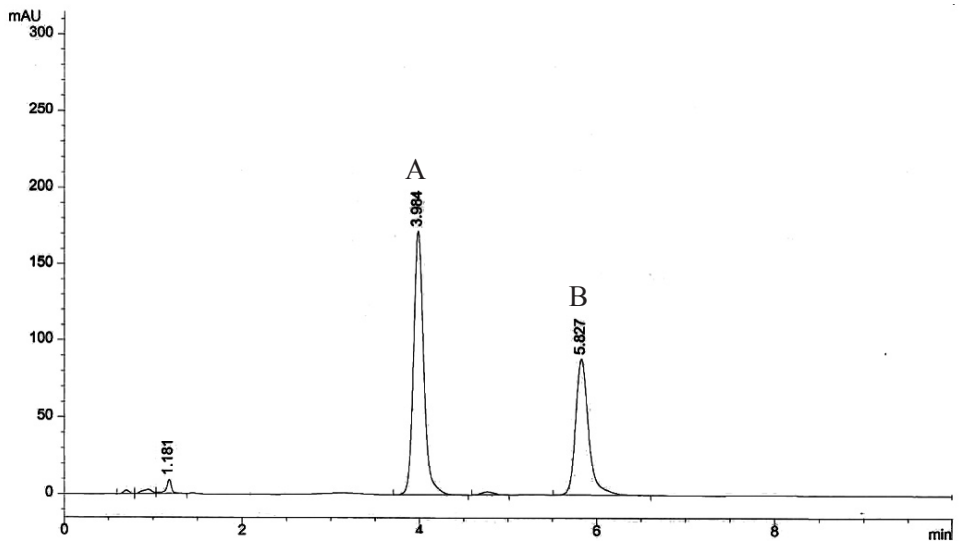


Figure 2. HPLC chromatogram of standard arbutin (2.56 μg) (A) and hydroquinone (B) on an Apollo C-18 column (4.6x250 mm), mobile phase - methanol/ water 90/10; flow rate - 0.9 mL/min; detection - UV, 280nm

The HPLC chromatogram of the liquid medium obtained from the cell culture without the addition of hydroquinone (Figure 3) shows no significant peaks at the RT of arbutin or hydroquinone.

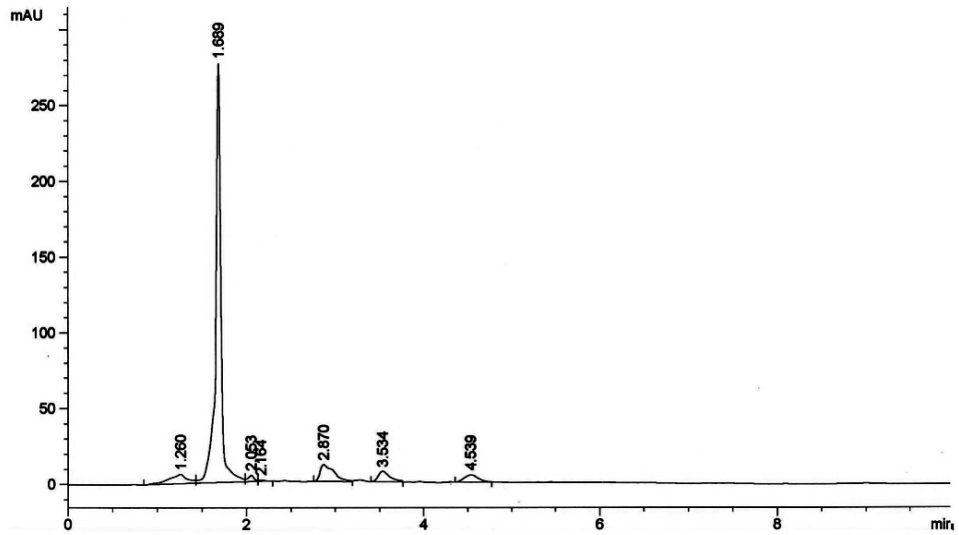


Figure 3. HPLC chromatogram of blank liquid medium (A) and liquid medium obtained from a 2-week-old cell suspension culture of *C. annuum* with no addition of hydroquinone (B).

The liquid medium sample of *C. annuum* cell suspension culture treated with 15.57 mM hydroquinone for 5 days before harvest (Figure 4) shows the presence of arbutin, as a product of biotransformation, at RT ~3.9 min. The remaining hydroquinone which was not biotransformed eluted at RT ~5.8 min. The overall separation was completed within 8 minutes and was considerably more rapid than the previously described methods (Sticher et al., 1979; Zhang et al., 2004) without compromising the sensitivity.

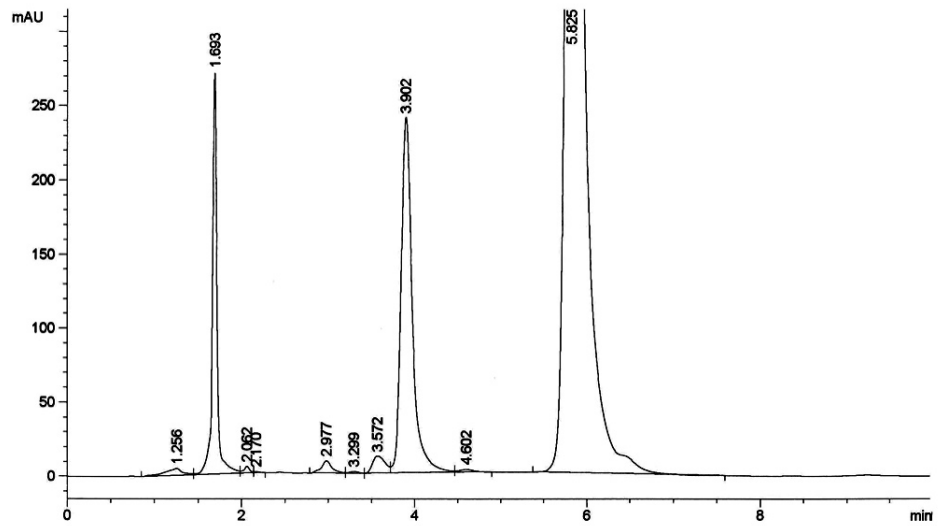


Figure 4. HPLC chromatogram of liquid culture media of *C. annuum* cell suspension culture treated with 15.57 mM hydroquinone for 5 days before harvest. Arbutin and the non-biotransformed hydroquinone are shown at RT 3.902 and 5.825 min, respectively.

Slight shifts in the retention time of arbutin (between 3.9-4.0 min) and hydroquione (between 5.8-5.9 min) peaks were observed in different runs but the deviations were well within ±5% of the RT registered for the standards. The addition of buffer or pH-modifying agent into the mobile phase was therefore not necessary. The finalized, optimum conditions of the method for the detection and quantitation of arbutin in plant cell suspension culture samples are: Apollo C-18 column (4.6 x 150 mm, 5 μm particle diameter, 100 Å average pore size), a methanol: water (90: 10) mobile phase, a flow rate of 0.9 mL/min, UV detection at 280 nm, an injection volume of 10 μL, and analysis carried out at ambient temperature.

The limit of detection (LOD) of arbutin using this HPLC method was found to be 0.05 μg (50 ng) at a signal-to-noise ratio 3:1. The lowest amount reproducibly detected and quantitated, however, was 0.16 μg (signal-to-noise ratio 10: 1), which was comparable to or better than that of the previously reported methods (Kraus and Stahl, 1979; Chang and Chang, 2003). Standard curves (Figure 5) were established using seven concentrations of standard arbutin on three separate days. A linear relationship between the amount of arbutin injected and the AUC was found

over the range of 0.016 to 1.024 mg/mL (0.16 to 10.24 μg injection). The correlation coefficients were 0.9999 (n=3), 0.9997 (n=1) and 0.9999 (n=1) for Days 1, 2 and 3, respectively. For the standard curve established on Day 1, the coefficients of variation were 0.15%, 0.22% and 0.36% for the injection amounts of 0.16 μg, 1.28 μg and 5.12 μg, respectively. The day-to-day coefficient of variation (CV) was 4.75%. The composite linear equation obtained from the regression analysis was y = 4946.3x + 1.3696, where y is the area under the peak and x is the amount of arbutin injected (μg).

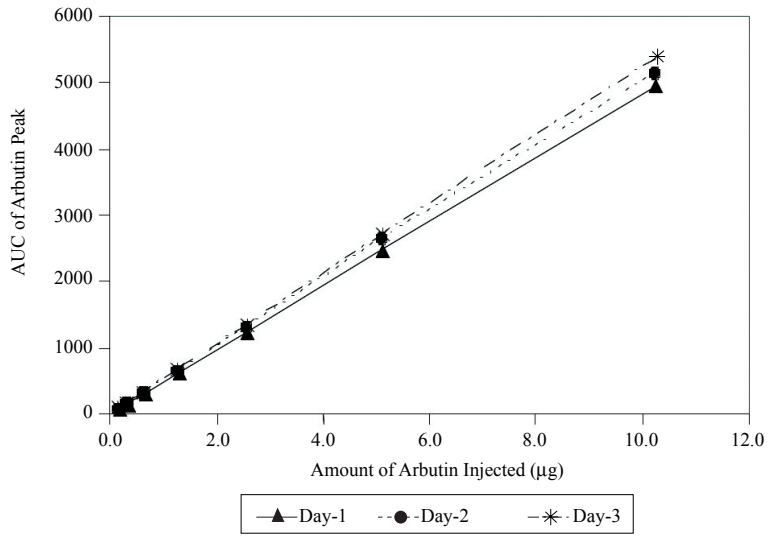


Figure 5. Composite standard curve for arbutin quantitation by HPLC established on three separate days, using seven injected amounts of standard arbutin (0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 10.24 μg).

The validity of the separation and quantitation methods was demonstrated through a percent recovery determination, using pure arbutin as a sample. The percent recovery of arbutin determined by HPLC was 97.28±1.89 % (n=3) (Table 1). The results suggested that arbutin added into the media was not absorbed into the cells nor was it significantly adsorbed onto the filter paper and the membrane filter in the sample preparation process. A "spiking" experiment, performed by adding a small, known amount of standard arbutin into a sample of filtered culture medium, yielded the expected increase in the AUC for the arbutin peak.

Table 1. Percent recovery of arbutin from cell suspension culture of C. annuum determined by using the established HPLC method.

No.	Amount Originally Added (mg)	AUC from HPLC Chromatogram	Amount Recovered (mg)	% Recovery
1	5.00	675.64	4.77	95.42
2	5.00	702.31	4.96	99.20
3	5.00	688.39	4.86	97.23

For an application of the method, samples prepared from five cell suspension cultures of different plant species and treated with hydroquinone for five days were analyzed for their arbutin content. The AUC of the arbutin peak was used to calculate the amount of arbutin in the sample, using the composite standard curve. The reproducibility of the quantitation method was also shown by repeating the arbutin analysis of three arbutin-containing cell suspension culture samples, five times for each. The consistency in arbutin content within each analysis is shown in Table 2. The coefficient of variation of an individual sample analysis was less than 5%. The different average arbutin contents observed among the three samples could be expected since plant cell suspension cultures of each plant possessed different hydroquione-arbutin biotransformation ability.

Table 2. Arbutin content (mg/L) of cell suspension cultures of medicinal plants grown in M&S medium with 2% w/v sucrose, 2,4-D and BAP and fed with 15.57 mM hydroquinone 5 days before harvest.

Cell Suspension Cultures	Arbutin Content (mg/L) from Analysis No.						
	1	2	3	4	5	AVG±SD	% CV
Capsicum annuum L.	306.95	309.56	300.60	302.44	316.17	307.15±6.17	2.01
Datura fastuosa L.	13.47	14.59	14.01	14.39	15.31	14.35±0.69	4.77
Solanum aculeatissimum Jacq.	67.99	71.06	69.12	68.36	71.74	69.66±1.66	2.39
Ocimum basilicum L.	----------undetectable----------						
Allamanda cathartica L.	----------undetectable----------						

% CV = coefficient of variation

CONCLUSION

The developed HPLC method is simple, sensitive, rapid and reproducible for simultaneous detection and quantitation of arbutin, in the presence of hydroquinone, in plant cell suspension samples. Good separation between the two compounds, and from other components in the plant cell suspension samples, is obtained in a relatively short time of analysis, using an economical HPLC column, a simple mobile phase and with minimal sample preparation. The established method demonstrated that

cell suspension cultures of *C. annuum* L., *S. aculeatissimum* Jacq. and *D. fastuosa* L. were able to biotransform exogenous hydroquinone into arbutin, while those of *O. basilicum* L. and *A. cathartica* L. did not show such ability. The method has been proven to be useful and effective in the detection of arbutin at various ranges of amount and can be used routinely for the screening of arbutin biotransformation in plant cell suspension samples.

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REFERENCES

Anetai, M., and T. Yamagishi. 1986. Exploration of crude drugs produced in Hokkaido. III. Quantitative determination of arbutin in the genus *Sedum* L. by high performance liquid chromatography. Hokkaidoritsu Eisei Kenkyushoho, 36: 81-82.

Arend, J., H. Warzecha, and J. Stockigt. 2000. Hydroquinone: O-glucosyltransferase from cultivated *Rauvolfia* cells: enrichment and partial amino acid sequences. Phytochemistry 53(2): 187-93.

Chang, M.L., and C.M. Chang. 2003. Simultaneous HPLC determination of hydrophilic whitening agents in cosmetic products. Journal of Pharmaceutical and Biomedical Analysis 33(4): 617-626.

Fromard, F. 1983. Arbutin and methylarbutin in *Arctostaphylos uva-ursi* and some other Ericaceae. Qualitative and quantitative analyses in chromatography. Comptes Rendus du Congres National des Societes Savantes, Section des Sciences 108(3): 251-67.

Huang, S.C., C.C. Lin, M.C. Huang, and K.C. Wen. 2004. Simultaneous determination of magnesium ascorbyl phosphate, ascorbyl glucoside, kojic acid, arbutin and hydroquinone in skin whitening cosmetics by HPLC. Yaowu Shipin Fenxi. 12(1): 13-18.

Huang, S.L., Y.L. Zhu, Y.J. Pan, and S.H. Wu. 2004. Synthesis of arbutin by two-step reaction from glucose. Journal of Zhejiang University Science 5(12): 1509-1511.

Huang, S., B. Huan, Q. Chen, Y. Huang, and X. Tie. 2001. Determination of arbutin in cosmetics by HPLC. Xiangliao Xiangjing Huazhuangpin 1: 13-16.

Inomata, S., M. Yokoyama, S. Seto, and M. Yanagi. 1991. High-level production of arbutin from hydroquinone in suspension cultures of *Catharanthus roseus* plant cells. Applied Microbiology and Biotechnology 36(3): 315-19.

Kraus, L., and E. Stahl. 1979. High-performance liquid chromatography of arbutin and methylarbutin. I. Qualitative and quantitative analysis. Journal of Chromatography 170(1): 269-71.

Lian, H., Y. Wei, and D.Li. 2005. Analysis of para-dihydroxybenzene content in synthetic arbutin. Faming Zhuanli Shenqing Gongkai Shuomingshu, CN 1614414 A 20050511, 8 pp.

Quintus, J., K.A. Kovar, P. Link, and H. Hamacher. 2005. Urinary excretion of arbutin metabolites after oral administration of bearberry leaf extracts. Planta Medica 71(2): 147-152.

Stambergova, A., M. Supcikova, and I. Leifertova. 1985. Evaluation of phenolic substances in *Arctostaphylos uva-ursi.* IV. Determination of arbutin, methylarbutin and hydroquinone in the leaves by HPLC. Cesko-Slovenska Farmacie 34(5): 179-82.

Steffeck, R.J., S.L. Woo, R.J. Weigand, and J.M. Anderson. 1995. A Comparison of silica-based C-18 and C-8 HPLC columns to aid column selection. LC-GC. 13(9): 720-726.

Sticher, O., F. Soldati, and D. Lehmann. 1979. High-performance liquid chromatographic separation and quantitative determination of arbutin, methylarbutin, hydroquinone and hydroquinone monomethylether in *Arctostaphylos, Bergenia, Calluna* and *Vaccinium* species. Planta Medica 35(2): 253-61.

Suzuki, T., T. Yoshiaki, M. Tabata, and Y. Fujita. 1987. Potential of Datura inoxia cell suspension cultures for glucosylating hydroquinone. Plant Cell Reports 6: 275-8.

Teglia, A. 1989. Cosmetic products for lightening the skin: HPLC determination of hydroquinone and its monoglucoside (Arbutin). Cosmetics & Toiletries, Edizione Italiana 10(4): 6-7, 10-11, 15, 17, 19-21, 24-26.

Zhang, T., X. Li, and B. Zhou. 2004. Determination of arbutin in arbutin gel by HPLC. Guangdong Yaoxueyuan Xuebao 20(6): 635-636.