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Development and validation of a modified QuEChERS method coupled with LC-MS/MS to determine arbutin in pear peels

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Abstract A new effective method was developed to determine the concentration of arbutin in pear peels using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The original QuEChERS was modified to enable the extraction of the polar arbutin molecule. Use of an initial 50:50 acetonitrile:water extraction solvent led to the highest extraction efficiency. The arbutin extracted from pear peels was found to be identical to the β-arbutin standard, as confirmed by NMR and LC-MS/MS analyses. For quantitative analysis, the mass spectra of the precursor ion $[M+NH_4]^+$ at *m/z* 290.0 and the product ion of arbutin at *m/z* 163.0 were used. The limit of detection, limit of quantification, linearity, precision, accuracy, and recovery of the proposed method were evaluated. We successfully applied this method to pear samples and it may be suitable for the quantitative analysis of arbutin in other similar plant materials.

Keywords: arbutin, pear peels, QuEChERS, LC-MS/MS, quantitative analysis

Introduction

Arbutin (4-hydroxyphenyl D-glucopyranoside) is a glycosylated hydroquinone originally extracted from the bearberry fruit and later purified from a variety of plants, including plants from the *Lamiaceae, Ericaceae*, *Saxifragaceae,* and *Rosaceae* families (1-3). There are two isomers of arbutin, viz. the α- and β-form (4). α-Arbutin can be enzymatically synthesized from hydroquinone and sugar (5,6), whereas β-arbutin naturally occurs in various plants (7). Of these two forms, the α-form has been shown to be more effective than the β-form for the inhibition of tyrosinase, as measured in mammalian and mouse melanoma tyrosinases (8-10). Since arbutin exhibits excellent efficiency in the removal of pigmentation such as melasma and freckles from human skin, it has seen wide cosmetic use as a skin-lightening agent. This effect is based on the fact that when skin is stimulated by external stresses, such as UV light or air pollution, free radicals are generated inside the skin, activating melanocytes. Tyrosinase, which is present in melanocytes, is a key enzyme involved in the synthesis of 3-(3,4-dihydroxyphenyl)-alanine from tyrosine. Arbutin effectively inhibits tyrosinase activity by competing with tyrosine (11). As a result, synthesis of melanin is inhibited, producing a skin-whitening effect (1,12). In medicine, leaves and leaf extracts containing arbutin have been used to treat urinary tract infections, cystitis, kidney stones, and as a diuretic (13,14). Arbutin can also be converted to hydroquinone (HQ), a compound known for antibacterial, astringent, and disinfectant properties (15,16). For example, arbutin is rapidly metabolized and excreted as HQ, HQ glucuronide, and HQ sulfate. As a result of this metabolism, arbutin exhibits hepatotoxic, nephrotoxic, and carcinogenic effects in animal experiments (17). Therefore, the use of arbutin should be strictly regulated.

Numerous methods have already been developed for the determination of arbutin in plant extracts, including spectrophotometry (18), capillary zone electrophoresis (19), GC-MS (20,21), and RP-HPLC (9,22-25). However, an efficient analytical method for the quantification of arbutin using LC-MS/MS has not been developed yet. LC-MS/MS analysis provides high analytical sensitivity due to the increased signal-to-noise ratio for the detection of target samples in different matrices. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method is a European accredited assay that is used for analysis of mycotoxins and pesticide residues in food destined for human consumption and livestock feed (26-29). It is a fast, easy, economical, and efficient pre-processing method for sample preparation. In addition, the subsequent analysis can be performed without a concentration step, which is the most time-consuming step in conventional preparation methods. The QuEChERS method was originally optimized mostly for analysis of hydrophobic molecules using an organic solvent. This may limit the type of samples to be used to analyze using the original QuEChERS method.

In this study, to establish a rapid method for the determination of arbutin, we have modified the QuEChERS method for specialized application to arbutin, which is a highly polar molecule. Furthermore, a specific LC-MS/MS analysis was developed, since it is a highly selective and sensitive tool for the quantitative analysis of various materials, from small molecules to large macromolecules.

It has been known that Asian pear peels contain arbutin and chlorogenic acid as major phenolic constituents (30). The pear peels are thrown out in large quantities as byproducts during the processing of pear products. In addition, the large amount of immature pears are removed from the tree to increase the size and quality of the remaining fruits. We have thus established a novel method for the extraction and quantitative analysis of arbutin in pear byproduct samples.

Materials and Methods

Chemicals and reagents LC-MS-grade acetonitrile (Merck, Darmstadt, Germany), ammonium formate (Thermo Fisher Scientific, Waltham, MA, USA), standard β-arbutin (Sigma-Aldrich, St. Louis, MO, USA), sodium chloride (JUNSEI, Tokyo, Japan), and spin-X micro-centrifuge filters (0.2-μm nylon filtered, Costar; Corning Inc., Corning, NY, USA) were used. Pear samples (pear peels, immature pear fruit) were collected from the farm at the Chonnam National University in Korea. The fruits were hand-peeled with a peel thickness of approximately 1 mm and the peels were stored at −80°C. Dried leaves of loquat were obtained from a loquat farm in Wando province in Korea and stored at −80°C.

Optimization of extraction conditions To optimize the extraction solvent composition, various mixtures of water and acetonitrile (0:100, 25:75, 50:50, 75:25, 100:0, v/v) were tested. In each test, a 10 g sample was homogenized with 10 mL of extraction solvent for 1 min. Then, 1 g of sodium chloride was added to each homogenized sample and vortexed for 3 min. The samples were centrifuged at 3,180× *g* for 3 min before the supernatant was collected in a 15-mL conical tube. The supernatant was diluted 100 times with water and filtered through a spin-X micro-centrifuge filter for the preparation of LC-MS/MS samples. For the recovery test, each sample was spiked with standard arbutin before homogenization. An un-spiked sample was used as the control.

Reversed phase-high performance liquid chromatography (RP-HPLC) Arbutin was purified for NMR analysis using an RP-HPLC (Shimadzu LC-10ADvp) according to the following procedure.

Arbutin was first extracted using the modified QuEChERS method, and the extract was then lyophilized. The extracted arbutin was dissolved in 1 mL of distilled water containing 0.05% trifluoroacetic acid (TFA). The mobile phases were distilled water with 0.05% TFA (mobile A) and acetonitrile with 0.05% TFA (mobile B). The sample was eluted using an isocratic 2% mobile phase B, and monitored at 280 nm. The prep-column was a Shim-pack C_{18} (20×250 mm, Shimadzu, Kyoto, Japan).

LC-MS/MS analysis For LC-MS/MS analysis, a Shimadzu LC-10ADvp system coupled to an API 2000 (AB SCIEX, Framingham, MA, USA) with a triple quadrupole was used. The column was an Agilent ZORBAX C_{18} (4.6×250 mm, 5-μm particle size, Agilent Technologies, Santa Clara, CA, USA) and the mobile phases were water containing 2 mM ammonium formate with 0.1% formic acid (mobile A) and acetonitrile with 0.1% formic acid (mobile B). The gradient program was as follows: from 2% to 8% mobile B for 6 min with a flow rate of 1 mL/min. The injection volume was 20 μL. The mass spectrometer was operated in the ESI positive ion and SRM modes. The declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), and collision cell exit potential (CXP) were carefully optimized. The mass parameters were as follows: curtain gas=10, spray voltage=5500, temperature=500°C, and ion source gas=50 psi.

^{1}H NMR spectroscopy The chemical structure of the purified arbutin was confirmed using ^{1}H NMR spectroscopy. The samples were prepared by dissolving 5 mg of standard and 5 mg of purified arbutin separately in vials containing 0.5 mL of deuterium oxide. The ^{1}H NMR spectra were measured with a Bruker Avance 600 MHz NMR spectrometer (Bruker Biospin, Billerica, MA, USA) at 25°C.

Method validation The LC-MS/MS analysis was validated in terms of sensitivity, linearity, recovery rate (R, %), repeatability (RSD, %), limit of detection (LOD), and limit of quantification (LOQ). The calibration curve was constructed over a concentration range of 1.6-25.6 μg/mL. Five different points were plotted. The coefficient of correlation (R^2) was used to judge the linearity. The LOD and LOQ were defined as the lowest concentrations with signal-to-noise ratios of 3 and 10, respectively. The recovery test was performed to verify the accuracy. Three different concentrations (0.2, 0.5, 0.8 mg/g) were used to spike samples containing a known quantity of extract.

Results and Discussion

Optimization of extraction The original QuEChERS method was developed for the determination of mycotoxins and residual pesticides that were normally solubilized in organic solvents, such as acetonitrile, and could be efficiently extracted from various sample matrices. Unlike mycotoxins and pesticides, arbutin is a very polar molecule that is much more soluble in water than in organic solvents. Therefore, we needed to find the optimal solvent system for arbutin extraction from pear peels. Figure 1 shows the overall procedure of arbutin extraction and preparation optimized in this work. For the extraction solvent used in the homogenization of the sample, we tested 5 different solvents with various proportions of

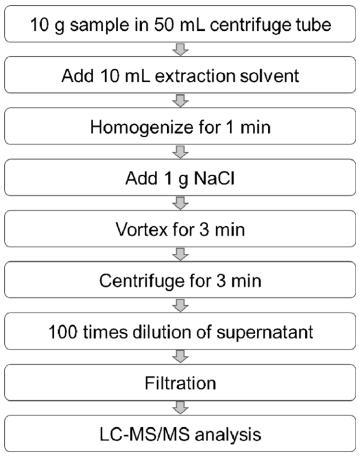

Fig. 1. Procedure for extraction of natural arbutin from pear peels for purification and LC-MS/MS analysis.

water and acetonitrile (0:100, 25:75, 50:50, 75:25, and 100:0, v/v). Arbutin spiked samples (1 mg/g of arbutin in 10 g of pear peels) were homogenized with 10 mL of extraction solvents. The recovery ratio of arbutin is very low for the solvents with 0 and 25% water (data not shown). The recovery was maximized at 50% water and then the recovery slightly decreased when the solvent contained 75 and 100% water. In addition, the impurity was minimized at acetonitrile:water (50:50, v/v) condition. In practical, we suggest that the extraction condition can be used from 50 to 100 % water. In this study, we fixed the water content at 50% for the following steps. Sodium chloride (NaCl) was added after homogenization to enhance the recovery of arbutin (data not shown). In the original QuEChERS method, $MgSO_4$ removes the residual water in the organic phase after phase separation. However, since water and organic phases were not separated, we did not use $MgSO_4$ in the modified method (Fig. 1). After centrifugation, the supernatant was used for RP-HPLC, NMR, and LC-MS/MS experiments.

Identification of the extracted arbutin The arbutin extracted by the optimized QuEChERS was further purified by RP-HPLC and characterized by LC-MS and 1D ^{1}H NMR experiments. The purified arbutin from the pear peels was compared with a β-arbutin standard. Due to its high polarity, arbutin was quickly eluted at around 6.7 min from the C_{18} column of the RP-HPLC system (Fig. 2A) (30). Both the extracted sample and β-arbutin standard showed the same retention time. LC-MS analysis of the extracted and standard arbutin indicated the same mass (290 *m/z),* corresponding to $[M+NH_4]^+$ (Fig. 2A). The ^{1}H NMR spectra of the extracted and standard arbutin are almost identical (Fig. 2B and 2C). Consequently, the compound extracted from the pear peels was identified as β-arbutin.

LC-MS/MS analysis of arbutin To establish the LC-MS/MS conditions, we used β-arbutin as a standard. LC-MS/MS analysis with SRM or multiple reaction monitoring (MRM) mode enables the sensitive and specific detection of targeted compounds. The pear peel extract was diluted and directly analyzed by the LC-MS/MS system. The mass parameters were optimized to obtain the highest signals for the ion $[M+NH_4]^+$. The precursor ion was found at 290.0 *m/z*. As a result of the MS/MS analysis, the product ion values were determined to be 144.9, 163.0 and 180.2 *m/z*. The proposed fragmentation pathways and structures of the product ions are shown in Fig. 3. Product ions with the indicated structures and masses (*m/z*) were formed by the loss of HQ and water molecules. Arbutin was hydrolyzed at the glycosidic bond between sugar and HQ, which produced glucose (181 *m/z*). Two fragments were then sequentially formed by a neutral loss of water (31). Based on the results, the LC-MS/MS parameters for arbutin quantification with a specific SRM transition mode were optimized using 290.0 *m/z* as the precursor ion and 163.0 *m/z* as the product ion (data not shown).

Method validation A standard calibration curve was constructed

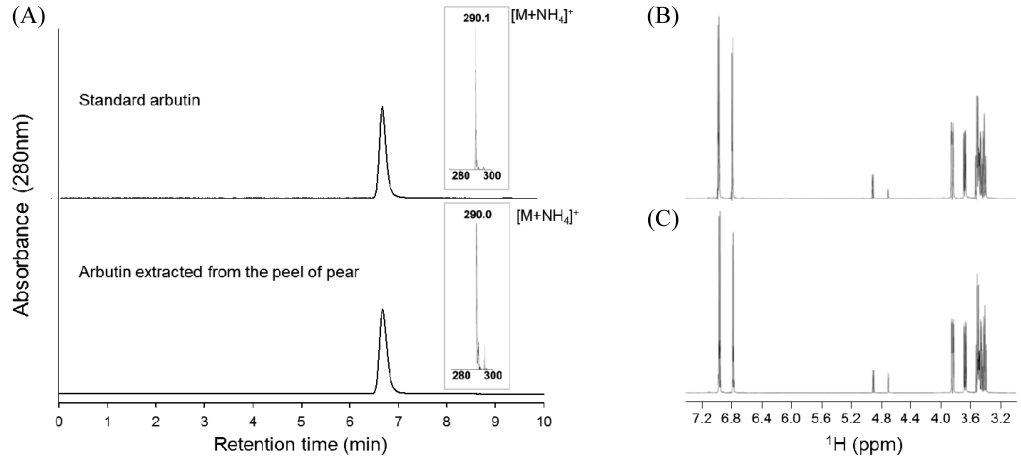

Fig. 2. Identification of arbutin from pear peels using HPLC, LC-MS, and ^{1}H NMR spectroscopy. (A) Reversed-phase HPLC elution profile of the pure β-arbutin standard and purified arbutin from the pear peels. Insets indicate the LC-MS spectra of each arbutin peak. ^{1}H NMR spectra of standard (B) and purified arbutin (C).

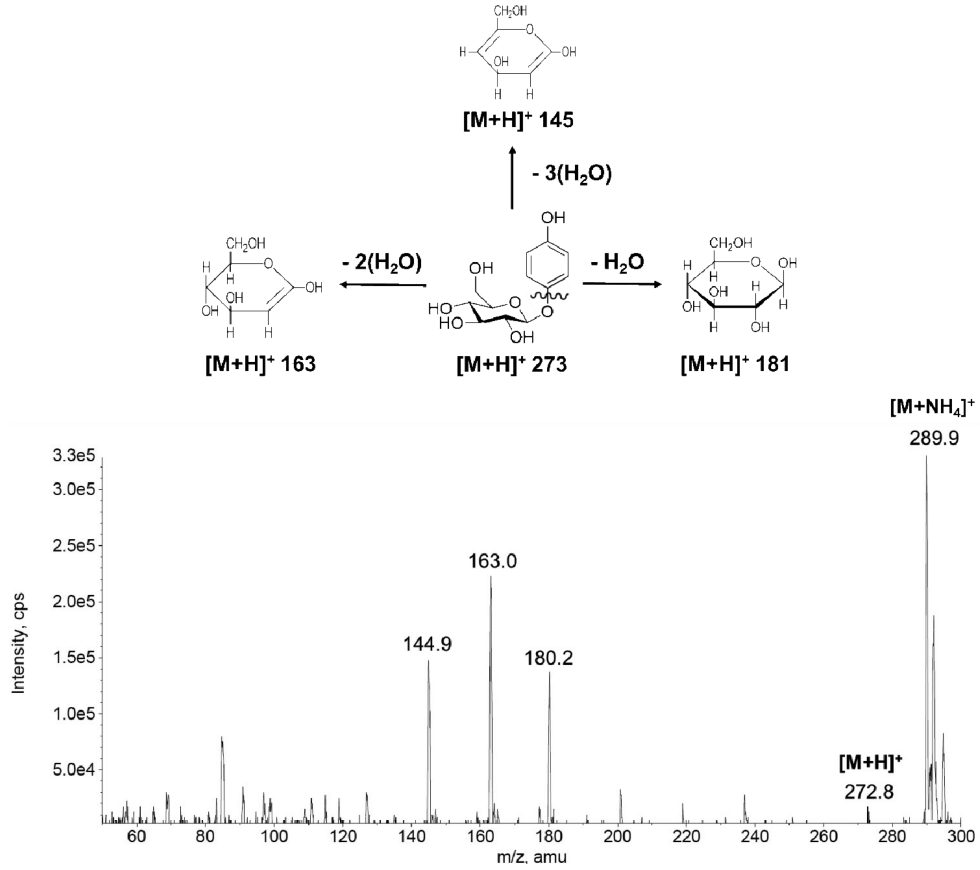

Fig. 3. Full-scan product ion spectra and the fragmentation schemes for arbutin.

using five different concentrations, viz. 1.6, 3.2, 6.4, 12.8, and 25.6 μg/mL, of the β-arbutin standard. Satisfactory linearity was obtained, where the correlation coefficient (R^2) was greater than 0.999 in SRM mode (data not shown). The LOD and LOQ were 0.2 and 0.8 μg/mL, respectively (Table 1). To determine the accuracy of the method, sample materials were spiked with standard arbutin solutions of three different concentrations (0.2, 0.5, 0.8 mg/g). The recovery and repeatability in terms of RSD (%) were recorded and are listed in Table 2. The recovery was calculated by comparing the actual and theoretical areas of the spiked and un-spiked samples. The mean recoveries ranged from 74.68% to 92.68% with RSD of 0.74% to 2.89%. These results indicate that the developed method is both precise and accurate.

Applications of the method Arbutin is naturally found in various plants including pears (13) and has been used as a specific marker in the evaluation of the authenticity of pear products (7). Various plants containing natural arbutin have also been suggested to be a good source of arbutin for commercial purposes. Therefore, methods for the extraction and purification of arbutin from pear and other plants have been developed using solvent fractionation, column chromatography, and HPLC (9,22-25). However, these previous procedures for the extraction and purification of arbutin are complex and time-consuming processes. In this study, we have developed a quick and simple method for the extraction and purification of arbutin by modifying the original QuEChERS method. In this method, only a single homogenization and centrifugation step is needed to obtain pure arbutin with high recovery for a final purification step or LC-MS/MS analysis. This optimized extraction method was applied to the quantitative analysis of arbutin in other plant samples including pear peels, immature pear fruit, and loquat leaves. The amounts of arbutin in the samples are summarized in Table 3. The pear peels and immature pear fruit contain about 0.916 and 0.918 mg/g of arbutin with good RSD values (%) of 5.86 and 8.21, respectively. However, the amount of arbutin in loquat leaves was very low (0.010 mg/g) while the RSD is slightly high (18.96%). We successfully applied the developed method to various types of plant materials such as fruit skin, whole fruit, and plant leaves. This suggests that the method

Table 1. Calibration curve, correlation coefficients, limit of detection (LOD), and limit of quantitation (LOQ) of arbutin obtained by LC-MS/MS

Analyte	Concentration range (μg/mL)	Calibration equation[1)]	Correlation factor R^2 (n=3)	LOD (μg/mL)	LOQ (μg/mL)
Arbutin	1.6-25.6	y=2775.3x+392.9	0.9994	0.2	0.8

[1)]Calibration equation; x is concentration of arbutin solution in μg/mL and y is peak area of the compound.

Table 2. Recovery of arbutin in pear peel extracts

Analyte	Amount added (mg/g, *n*=3)	Recovery (%)	RSD (%)
Pear peels	0.8	74.68	2.06
	0.5	78.39	0.74
	0.2	92.68	2.89

Table 3. Content of arbutin in plant samples

Samples	Species	Arbutin	
		X (mg/g)±SD[1)]	RSD (%)
Pear peels	*Pyrus pyrifolia*	0.916±0.054	5.86
Immature pear fruit	Nakai cv. Niitaka	0.918±0.075	8.21
Loquat leaves	*Eriobotrya japonica*	0.010±0.002	18.96

[1)]Arbutin content in material±SD (standard deviation) in mg/g dry weight (*n*=3)

could be applicable to the extraction and determination of arbutin from a broad variety of plant materials.

In conclusion, we modified the original QuEChERS method for the development of an efficient extraction and determination method for arbutin in pear peels. The mixture of water and acetonitrile (50:50, v/v) was found to be the best solvent with the highest recovery of arbutin. The whole extraction process was simplified by using only a single homogenization and centrifugation step and completing the extraction within 10 min. LC-MS/MS was used for the quantitative analysis of the extracted arbutin in various plant samples. The developed method is simple, rapid, sensitive, reproducible, and fully validated. This method was also clearly proven to be rapid and highly effective for the extraction and determination of arbutin in plants and fruits.

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Disclosure The authors declare no conflict of interest.

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