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Composition of Polyphenols and Antioxidant Activity of Rabbiteye Blueberry (*Vaccinium ashei*) in Nanjing

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ABSTRACT: The total phenolic content (TFC), total flavonoid content (TFC), antioxidant activity, and polyphenol composition of extracts of rabbiteye blueberry fruit polyphenols (BBFPs), rabbiteye blueberry pomace polyphenols (BBPPs), and rabbiteye blueberry leaves polyphenols (BBLPs) were investigated. The highest TPC and TFC were found in the extract of BBLPs, followed by the extracts of BBPPs and BBFPs. The extract of BBLPs exhibited the highest antioxidant activity according to five antioxidant methods. All three samples exhibited significantly higher antioxidant activity than the positive control rutin. HPLC−DAD−MS^n analysis showed that the extract of BBFPs contained nine different anthocyanins, while the extract of BBPPs contained only four of them. In addition, four caffeoylquinic acids, quercetin, and its three derivatives were detected in the extract of BBLPs. The results indicate that the fruits, pomace, and leaves of rabbiteye blueberry were good sources of polyphenols and natural antioxidants. These could be useful as a functional food ingredient beneficial to human health.

KEYWORDS: *blueberry, leaves, pomace, polyphenols, anthocyanins, antioxidant activity*

INTRODUCTION

Rabbiteye blueberry (*Vaccinium ashei*), which is good for health and collected and consumed throughout North America, has been introduced to Nanjing, China, in recent years. Many reports have suggested that blueberry fruits have a wide range of health benefits such as superb antioxidant activity, antidiabetic activity,[1] and the ability to protect against cancer and stroke.[2] Besides, the intake of extract of blueberry fruits can improve short-term memory, balance, and coordination in aging rats.[3] The polyphenols in blueberry fruits, especially anthocyanins, which are hydrophilic antioxidants,[4] are mainly responsible for those health benefits. Compared with some synthetic antioxidants, natural antioxidants have higher efficiencies and lower manufacturing costs; in addition, synthetic antioxidants may exhibit toxicity. Therefore, there is a need to search for more effective and economic natural antioxidants that could be incorporated into diets.

Blueberry pomace and leaves are byproducts of the blueberry industry. They are created in large quantities and rich in polyphenols,[5,6] which creates an opportunity for their use in the neutraceutical industry. Blueberry pomace is the residue that remains when the fruits are processed for the production of juice, wine, and other products. Usually, through processing, only a small amount of anthocyanins and polyphenols is present in the final products, while a large amount remains in the pomace,[6] which makes blueberry pomace a rich source of natural colorants and neutraceuticals. Blueberry leaves contain a much larger amount of polyphenols than fruits,[5] but no anthocyanins have been found in fresh green leaves.[7] Blueberry leaves have attracted significant attention since some researchers reported that the polyphenols (especially proanthocyanidin with a degree of polymerization of 8−9) extracted from the leaves of rabbiteye blueberry could suppress the expression of subgenomic hepatitis C virus RNA.[8] It has been reported that blueberry leaves have antioxidant capacity,[5] antileukemia activity,[9] antihypertensive activity,[10] and antiobesity activity.[11] Moreover, blueberry leaves, along with roots and stems, can be added to fruits to treat different diabetic symptoms as they can elicit a different spectrum of antidiabetic activities.[12]

The composition of polyphenols and the antioxidant capacity of blueberry fruits, pomace, and leaves have been well reported in Europe and North America. However, only a limited number of works dealt with Chinese blueberry.[4,13] In our previous study,[4] we identified four different phenolic acids (i.e., *p*-hydroxybenzoic acids, vanillic acid, caffeic acid, and ferulic acid), four flavonoids (i.e., rutin, quercetin, myricetin, and quercitrin), and three proanthocyanins (i.e., catechin, gallocatechin, and condensed tannins) in blueberry fruits in Nanjing. However, we identified only two different anthocyanins (i.e., malvidin 3-galactoside and malvidin 3-glucoside) in blueberry fruits. Hence, this work mainly focuses on the identification of anthocyanins in blueberry fruits and pomace, as well as the identification of polyphenols in blueberry leaves. In addition, the total phenolic content, total flavonoid content, and antioxidant activities of ethanol extracts of blueberry fruits, pomace, and leaves have been determined.

MATERIALS AND METHODS

Plant Material. Fresh ripe fruits and green leaves of Brightwell rabbiteye blueberry (*V. ashei*) were harvested in July 2012 from orchards surrounding Lishui in Nanjing. Blueberry pomace was obtained as the residue after a wine-making process using the same

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blueberry fruits. The collected fruits, leaves, and pomace were dried immediately using an Eyela FDU-1200 freeze-dryer (Tokyo Rikakikai) and then kept at −20 °C before being ground to a uniform particle size.

Chemicals and Reagents. 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH), quercetin, rutin, and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchromate-2-carboxylic acid) was obtained from Acros Organics (Morris Plains, NJ). Gallic acid was purchased from J&K Chemicals Ltd. (Beijing, China). HPLC solvents were purchased from EMD (Darmstadt, Germany).

Preparation of Extracts of BBFPs, BBPPs, and BBLPs. Fruit powder (200 g), pomace powder (200 g), and leaf powder (200 g) were pulverized and extracted with 3000 mL of 70% ethanol containing 0.5% glacial acetic acid for 24 h in the dark at 4 °C. The extract was collected and centrifuged at 10000*g* for 20 min. The supernatant was filtered through medium-speed filter paper. The filtrate was concentrated in a rotary evaporator (0.1 MP, 40 °C) and then dried using an Eyela FDU-1200 freeze-dryer (Tokyo Rikakikai) to produce extracts of blueberry fruit polyphenols (BBFPs), blueberry pomace polyphenols (BBPPs), and blueberry leaf polyphenols (BBLPs). The lyophilized powder was used in the subsequent antioxidant capacity determination and HPLC−DAD−MS^n analysis.

Determination of the Total Phenolic Content. The total phenolic content (TPC) was estimated using the Folin-Ciocalteu colorimetric method described by Slinkard and Singleton.[14] Briefly, appropriate dilutions of the extracted samples (0.2 mL) were oxidized with 2 mL of 0.5 N Folin-Ciocalteu reagent for 4 min at room temperature. Then the reaction mixture was neutralized via addition of 2 mL of 75 mg/mL saturated sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm using a spectrophotometer after incubation for 2 h at room temperature in the dark. Quantification was conducted on the basis of a standard curve of gallic acid. Results are expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW).

Determination of the Total Flavonoid Content. The total flavonoid content (TFC) was measured using a modified colorimetric method.[15] An appropriately diluted sample of 1 mL was mixed with 0.1 mL of 5% $NaNO_2$. After 6 min, 0.1 mL of a 10% $AlCl_3{\cdot}6H_2O$ solution was added. Then 1 mL of 1 M NaOH was added to the mixture after an additional 5 min. The reactive solution was mixed well and left to stand for 15 min, and the absorbance was measured at 510 nm. The total flavonoid content was calculated and expressed as quercetin equivalents [milligrams of quercetin equivalent (QE) per gram of DW].

ABTS Assay. The $ABTS^{\bullet+}$ free radical scavenging activity of each sample was determined according to the method described by Miller et al.[16] Different levels (100, 200, 300, 400, and 500 μg/mL) of samples and trolox standard in 80% ethanol were prepared and assayed. The absorbance at 734 nm of the resulting oxidized solution was detected. Results were expressed in terms of trolox equivalent antioxidant capacity (TEAC), i.e., milligrams of trolox equivalent (TE) per gram of sample (DW). Rutin was used as a positive control.

DPPH Assay. The scavenging activity for $DPPH^{\bullet}$ radical was determined via spectrophotometric analysis based on the method described by Brand-Williams et al.[17] Different levels (10, 20, 30, 40, and 50 μg/mL) of samples and rutin (positive control) in 80% ethanol were prepared and assayed. The scavenging activity was expressed as the percentage of scavenged DPPH radical in the assay system. The IC_{50} value, denoting the concentration of the sample required to scavenge 50% DPPH free radicals, was calculated by graphical regression analysis, and expressed as milligrams of DW per milliliter.

FRAP Assay. The ferric reducing ability of plasma (FRAP) assay was conducted using a modified version of the method described by Benzie and Strain.[18] In the FRAP assay, the antioxidant samples reduced Fe^{3+}-TPTZ to Fe^{2+}-TPTZ, which was a blue complex with an absorption maximum at 593 nm. The absorbance increase at 593 nm was proportional to the reducing activity of the samples. An aliquot of different levels (100, 200, 300, 400, and 500 μg/mL) of samples and rutin (positive control) was added to FRAP reagent. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity was calculated from the linear calibration curve and expressed as millimoles of $FeSO_4$ equivalent per gram of sample (DW).

Reducing Power Assay. The reducing power was determined according to the method described by Oyaizu[19] with some modifications. The presence of antioxidants causes the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form. Hence, measuring the formation of Perl's Prussian at 700 nm can monitor the Fe^{2+} content. Various concentrations (50, 100, 150, 200, and 250 μg/mL) of samples and rutin (positive control) were tested, and the absorbance at 700 nm was measured against a blank. The IC_{50} value was the concentration of sample needed when the absorbance at 700 nm was 0.5. It was calculated by graphical regression analysis and was expressed as milligrams of DW per milliliter.

ORAC Assay. In the oxygen radical absorbance capacity (ORAC) assay, AAPH reacts with the fluorescent probe (fluorescein) to form nonfluorescent product. The radical scavenging activity was assayed by the improved oxygen radical absorbance capacity (ORAC) method as described previously.[20] The fluorescence decay curves of fluorescein in the presence of trolox, BBFPs, BBPPs, BBLPs, and rutin (positive control) at different concentrations were generated with MikroWin Microplate Data Reduction 2000 (Mikrotek Laborsysteme GmbH, Overath, Germany). The ORAC value was calculated by the slope of the sample equation by the slope of trolox curve obtained for the same assay. The final ORAC value is expressed as millimoles of TE per gram of DW.

HPLC−DAD−MS^n Analysis of Polyphenolic Compounds. Samples were filtered through a 0.22 μm filter (Millipore). HPLC analysis was conducted on an Aglient 1100 HPLC system (Agilent Technologies) equipped with a binary pump and a diode-array detector (DAD). Chromatographic analysis was conducted using a 250 mm × 4.6 mm, 5 μm particle size, end-capped reverse-phase Zorbax SB-C18 column (Agilent Technologies). The running temperature was 35 °C, and the injection volume was 10 μL. For polyphenols, the detection was conducted at 320 nm at a flow rate of 0.6 mL/min. Mobile phase A was a mixture of 0.1% HAc (ethanoic acid) and ultrapure water, whereas mobile phase B was a mixture of 0.1% HAc and methanol. The gradient was as follows: 5 to 10% B (from 0 to 5 min), 10 to 20% B (from 10 to 25 min), 20 to 23% B (from 25 to 35 min), 23 to 28% B (from 35 to 45 min), 28 to 35% B (from 45 to 60 min), 35 to 50% B (from 60 to 75 min), 50 to 55% B (from 75 to 80 min), 55 to 75% B (from 80 to 85 min), 75 to 35% B (from 85 to 90 min), and 35 to 5% B (from 90 to 95 min). For anthocyanins, the detection was conducted at 520 nm at the same flow rate. Mobile phase A was a mixture of 6% HAc and ultrapure water, whereas mobile phase B was a mixture of 6% HAc and acetonitrile. The gradient was as follows: 5 to 10% B (from 0 to 5 min), 10 to 15% B (from 5 to 20 min), 15 to 20% B (from 20 to 35 min), 20 to 40% B (from 35 to 40 min), 40 to 80% B (from 40 to 45 min), 80 to 85% B (from 45 to 50 min), 85 to 5% B (from 50 to 55 min), and 5% B (from 55 to 60 min).

MS^n analysis was conducted using an LCQ ion trap mass spectrometer fitted with an electrospray interface (Agilent Technologies). Spectra were recorded between m/z 100 and 1200 in positive ion mode (for anthocyanins) and negative ion mode (for polyphenols), respectively. Identification of anthocyanins and polyphenols was achieved via ion molecular mass, MS^n, and UV−visible spectra.

Statistical Analysis. All tests were performed in triplicate, and the data are presented as means ± the standard deviation. The IC_{50} values were calculated from linear regression analysis. The results were subjected to least significant difference (LSD) in one-way analysis of variance (ANOVA) using the PASW statistics 18 software to analyze the difference. Differences with a P value of <0.05 were considered significant.

Table 1. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of Blueberry Fruits, Pomace, Leaves, and Their Ethanol Extracts[a]

sample	extraction yield[b] (%, w/w)	TPC[c] (mg of GAE/g)	TFC[d] (mg of QE/g)	sample	TPC (mg of GAE/g)	TFC (mg of QE/g)
BBFPs	12.45	216.43 ± 2.33 c	115.55 ± 2.91 c	fruits	26.94 ± 0.29 c	14.39 ± 0.36 c
BBPPs	14.78	278.41 ± 20.66 b	146.48 ± 2.06 b	pomace	41.15 ± 0.39 b	21.65 ± 0.30 b
BBLPs	21.43	339.10 ± 3.09 a	198.10 ± 1.98 a	leaves	81.82 ± 0.75 a	47.80 ± 0.48 a

[a]Data are presented as means ± the standard deviation (n = 3). Different letters in the same column indicate significant differences (P < 0.05). [b]Extracted from freeze-dried materials. [c]TPC expressed as milligrams of gallic acid per gram of dry weight (DW). [d]TFC expressed as milligrams of quercetin per gram of dry weight (DW).

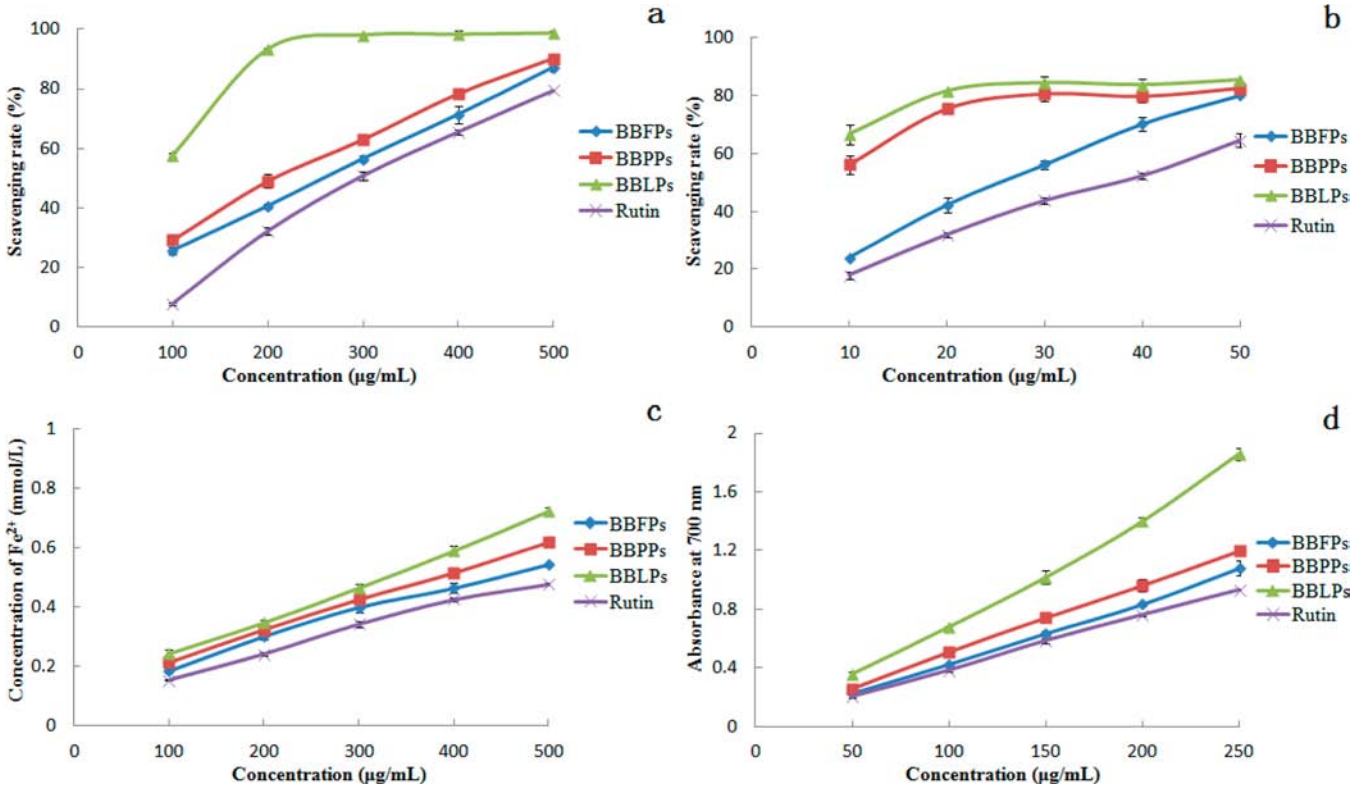

Figure 1. Antioxidant activity of different concentrations of extracts of BBFPs, BBPPs, and BBLPs and rutin using four kinds of assays: (a) $ABTS^{\bullet+}$ free radical scavenging activity, (b) $DPPH^{\bullet}$ free radical scavenging activity, (c) ferric ion reducing activity, and (d) reducing power.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). As shown in Table 1, all three samples had high TPC and TFC values. The highest TPC and TFC values were found in the extract of BBLPs, which were 339.10 ± 3.09 mg of GAE/g of DW and 198.10 ± 1.98 mg of QE/g of DW, respectively. The extract of BBLPs showed significantly (P < 0.05) higher values of TPC and TFC than the extracts of BBFPs and BBPPs.

The TPC and TFC of blueberry fruits, pomace, and leaves were calculated according to the extraction yield. The TPC of rabbiteye blueberry fruits (26.94 mg of GAE/g of DW) cultivated in Nanjing was higher than that of highbush blueberry fruits (25.6 mg of GAE/g of DW) reported by Lima et al.[21] and highbush blueberry fruits (11.7−19.6 mg of GAE/g of DW) reported by Kalt et al.[22] However, the TPC of the rabbiteye blueberry fruits in Nanjing (26.94 mg of GAE/g of DW) was lower than that of bilberry fruits (29.7 mg of GAE/g of DW) and highbush blueberry fruits (33 mg of GAE/g of DW) reported by Castrejón et al.[23] In addition, the TFC of rabbiteye blueberry in Nanjing (14.39 of QE/g of DW) is slightly higher than that of bilberry fruits (13.5 mg of QE/g of DW) and highbush blueberry fruits (7.4 mg of QE/g of DW) reported by Lima et al.[21]

For the blueberry leaves, both their TPC and TFC (81.82 mg of GAE/g of DW and 47.80 mg of QE/g of DW, respectively) were significantly higher than those in the fruits and pomace. In the leaf tissues of 87 highbush blueberries, the mean values of polyphenol contents were ∼30 times that observed in fruits on a fresh weight basis.[5] Previous research also showed that the green leaves of blueberry contained a much larger amount of flavonoids (quercetin and kaempferol) and hydroxycinnamic acid (*p*-coumaric and caffeic acid) than fruits.[24] In addition, the TPC and TFC of the leaves of rabbiteye blueberry in Nanjing were comparable to those of blackberry leaves (82.8−91.6 mg of GAE/g of DW) and strawberry leaves (55.2 mg of GAE/g of DW) reported by Wang and Lin.[25] Furthermore, Oszmianski et al.[26] reported that the polyphenol content of the blueberry leaves was much higher than those of any other leaves of tested berries (blackberry, raspberry, honeyberry, and strawberry). Wang and Lin[25] reported that the total phenolic content decreased while the content of anthocyanins and other flavonoids increased with maturity in fruit tissue. Like fruits,

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Table 2. Antioxidant Activity of Extracts of BBLPs, BBPPs, and BBLPs and Rutin Determined by an ABTS Assay, a DPPH Assay, a Ferric Reducing Ability of Plasma (FRAP) Assay, a Reducing Power Assay, and an Oxygen Radical Absorbance Capacity (ORAC) Assay[a]

sample	TEAC value (mg of TE/g of DW)	IC_{50} for DPPH (μg/mL)[b]	FRAP value (mmol of $FeSO_4$/g of DW)	IC_{50} for reducing power (μg/mL)[c]	ORAC value (mmol of TE/g of DW)
BBFPs	445.78 ± 4.52 c	26.78 ± 0.17 b	4.79 ± 0.07 c	118.00 ± 0.51 b	4.56 ± 0.04 c
BBPPs	521.85 ± 6.26 b	8.41 ± 0.03 c	5.33 ± 0.11 b	99.74 ± 0.27 c	9.64 ± 0.22 b
BBLPs	1281.55 ± 4.26 a	7.37 ± 0.04 d	6.39 ± 0.01 a	75.07 ± 0.26 d	11.06 ± 0.38 a
rutin	371.23 ± 2.48 d	36.94 ± 0.87 a	4.26 ± 0.04 d	128.35 ± 0.61 a	1.24 ± 0.03 d

[a]Data are presented as means ± the standard deviation (n = 3). Different letters in the same column indicate significant differences ($P < 0.05$). [b]The antioxidant activity was evaluated as the concentration of tested sample required to scavenge 50% of the DPPH free radicals. [c]The antioxidant activity was evaluated as the concentration of tested sample required when the absorbance at 700 nm was 0.500.

when leaves became older, the total polyphenol content decreased.[25]

The blueberry pomace also exhibited significantly ($P < 0.05$) higher TPC and TFC (41.15 ± 0.39 mg of GAE/g of DW and 21.65 ± 0.30 mg of QE/g of DW, respectively) than the fruits. The main components of blueberry pomace are seeds and pressed peels,[6] which contain larger amounts of anthocyanins, procyanidins, and polyphenols than flesh. Hence, the major part of polyphenols may be left in the pomace during common processing, making the pomace contain a larger amount of polyphenols than fruits. The TPC of the blueberry pomace in this study was higher than that of the pomace derived from a blueberry wine-making process (27.8 mg of GAE/g of DW) but lower than that of the pomace derived from a juice-making process (29.2 mg of GAE/g of DW) and a vinegar-making process (27.8 mg of GAE/g of DW) reported by Su et al.[6] This was because the blueberry pomace derived from different processes of the same fruits was different in total phenolic content, total anthocyanin content, and antioxidant activity.[6]

Antioxidant Activity of Extracts of BBLPs, BBPPs, and BBLPs. As shown in Figure 1a, the decrease in the level of the $ABTS^{\bullet+}$ free radicals was dependent on the concentration of the samples. At a concentration of 100 μg/mL, the extract of BBLPs could scavenge nearly 60% of the $ABTS^{\bullet+}$ free radicals, while the $ABTS^{\bullet+}$ radical scavenging activities of the extract of BBFPs, the extract of BBPPs, and rutin were relatively low (25.61, 29.26, and 7.65%, respectively). At a concentration of 500 μg/mL, the extracts of BBLPs, BBPPs, and BBFPs could scavenge nearly all $ABTS^{\bullet+}$ radicals (98.69, 90.03, and 87.12%, respectively), while the positive control rutin showed 79.43% of the $ABTS^{\bullet+}$ radical scavenging activity. The antioxidant activities decreased in the following order: extract of BBLPs > extract of BBPPs > extract of BBFPs > rutin. The TEAC values of the four samples were 1281.55 ± 4.26, 521.85 ± 6.26, 445.78 ± 4.52, and 371.23 ± 2.48 mg of TE/g of DW, respectively (Table 2).

The $DPPH^{\bullet}$ free radical scavenging activities of the four samples are shown in Figure 1b. For each sample, five concentrations were tested. The extract of BBLPs exhibited the highest $DPPH^{\bullet}$ scavenging activity, followed by the extracts of BBPPs and BBFPs. At a concentration of 50 μg/mL, the extracts of BBLPs, BBPPs, and BBFPs could scavenge approximately 80% of the $DPPH^{\bullet}$ free radicals, while the positive control rutin showed a relatively low $DPPH^{\bullet}$ free radical scavenging ability (64.58%). As shown in Table 2, the IC_{50} values were 26.78 ± 0.17, 8.41 ± 0.03, 7.37 ± 0.04, and 36.94 ± 0.87 μg/mL for the extracts of BBFPs, BBPPs, and BBLPs and rutin, respectively. The results revealed that all the extracts of BBFPs, BBPPs, and BBLPs had significantly ($P < 0.05$) higher $DPPH^{\bullet}$ free radical scavenging activities than rutin.

The FRAP values of five concentrations of each sample are given in Figure 1c. The FRAP values of the extracts of BBFPs, BBPPs, and BBLPs and rutin were 4.79 ± 0.07, 5.33 ± 0.11, 6.39 ± 0.01, and 4.26 ± 0.04 mmol of $FeSO_4$/g, respectively. Compared to the other three samples, the extract of BBLPs showed a significantly ($P < 0.05$) higher FRAP value. Also, the extracts of BBFPs and BBPPs showed significantly higher FRAP values than rutin.

As shown in Figure 1d, at a concentration of 250 μg/mL, the reducing power of the extract of BBLPs was 1.86 ± 0.04, which was significantly higher than that of the extract of BBFPs, the extract of BBLPs, and rutin (1.08 ± 0.05, 1.20 ± 0.02, and 0.93 ± 0.01, respectively). The reducing power decreased in the following order: extract of BBLPs > extract of BBPPs > extract of BBFPs > rutin. The result was similar to those of the TEAC assay, DPPH assay, and FRAP assay. Furthermore, the IC_{50} for reducing power was calculated. The IC_{50} values were 118.00 ± 0.51, 99.74 ± 0.27, and 75.07 ± 0.26 μg/mL, for the extracts of BBFPs, BBPPs, and BBLPs, respectively (Table 2).

In the ORAC assay, the three samples (i.e., the extracts of BBFPs, BBPPs, and BBLPs) were compared to the positive control rutin, and their ORAC values were 4.56 ± 0.04, 9.64 ± 0.22, 11.06 ± 0.38, and 1.24 ± 0.03 mmol of TE/g of DW, respectively. The extract of BBLPs showed the highest ORAC value. The ORAC values of the extracts of BBFPs, BBPPs, and BBLPs were significantly higher than that of rutin ($P < 0.05$), indicating that the extracts of BBFPs, BBPPs, and BBLPs were all good sources of antioxidants.

The DPPH assay can be used to evaluate antioxidants in lipophilic systems.[4] The FRAP assay and reducing power assay are mainly used in aqueous systems.[27] The ABTS assay is widely used in both aqueous and lipophilic systems.[4] The ORAC assay is the most utilized method, though many assays exist for the evaluation of antioxidant activity. It can be used in both aqueous and lipophilic systems. The ABTS assay, DPPH assay, and ORAC assay were based on the free radical ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, and AAPH free radical, respectively) scavenging activities of samples, while the FRAP assay and reducing power assay were based on the reduction capacity of the Fe^{3+} complex with respect to the ferrous form. All five methods mentioned above are valid, accurate, and reliable. The ORAC assay was conducted at 37 °C, while the other assays were conducted at room temperature. In addition, the ORAC assay was based on fluorescence decay, while the others were based on absorbance changes. In this study, the five methods were used to systematically assess the antioxidant capacity of the extracts of BBFPs, BBPPs, and BBLPs. The extract of BBLPs

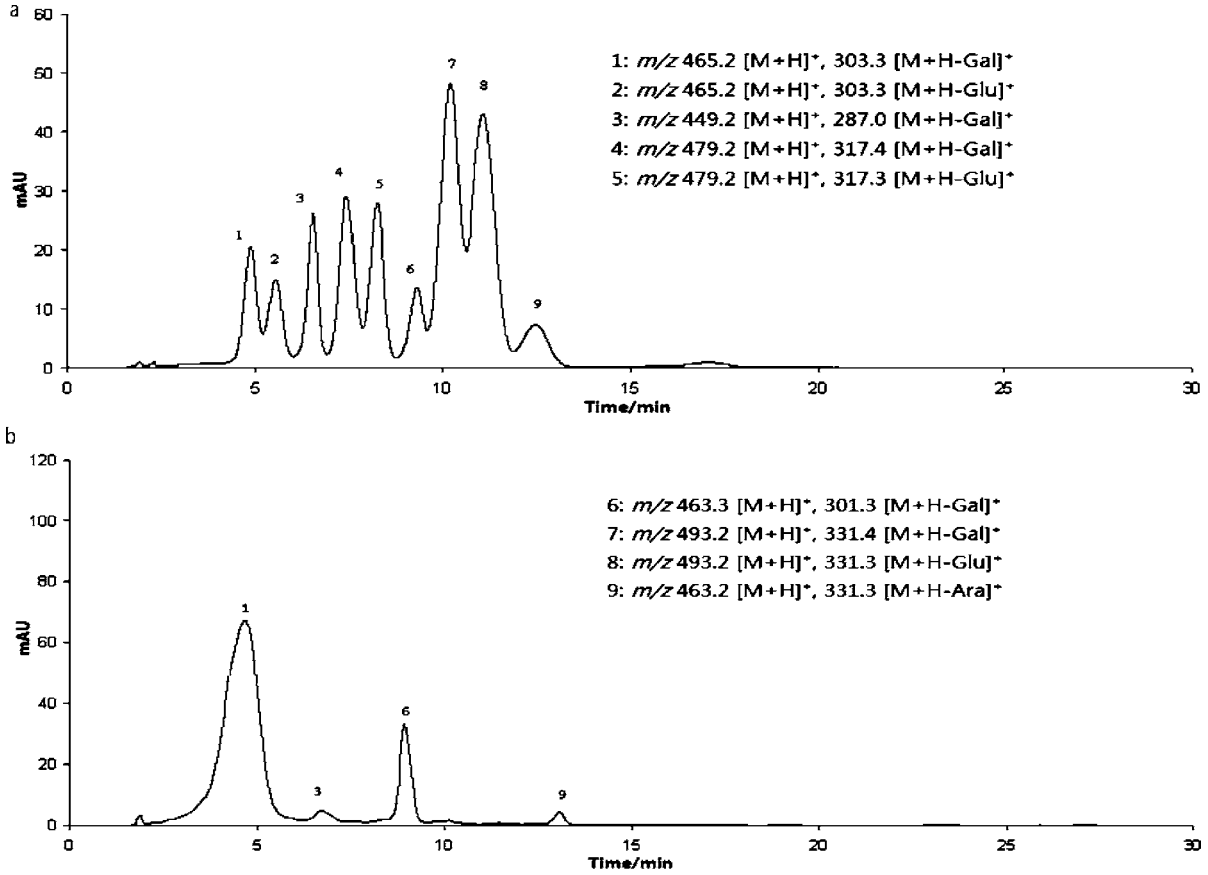

Figure 2. Chromatographic separation and UV detection (λ = 520 nm) of anthocyanins in extracts of BBFPs (a) and BBPPs (b). The following nine peaks were tentatively identified: peak 1, delphindin 3-galactoside; peak 2, delphindin 3-glucoside; peak 3, cyanidin 3-galactoside; peak 4, petunidin 3-galactoside; peak 5, petunidin 3-glucoside; peak 6, peonidin 3-galactoside; peak 7, malvidin 3-galactoside; peak 8, malvidin 3-glucoside; peak 9, malvidin 3-arabinose.

showed the highest antioxidant capacity, followed by the extracts of BBPPs and BBLPs. All three samples exhibited significantly ($P < 0.05$) higher antioxidant activity than the positive control rutin. Many bioactive functions of polyphenols, such as anticoronary heart disease, anticancer, and antidiabetes activities, have a direct relationship with its antioxidant activity. The results indicate that the fruits, leaves, and pomace of the rabbiteye blueberry in Nanjing were all good sources of antioxidants, which could be used for neutraceutical and therapeutic purposes.

Compared with berry fruits, berry leaves usually possess higher ORAC values. For 87 highbush blueberry plants cultivated in the United States, the mean ORAC value of their leaves was 490 mmol of trolox/g of FW, significantly higher ($P < 0.05$) than that of the fruits (15.9 mmol of trolox/g of FW).[5] Previous studies also reported that the ORAC values of leaves were higher than those of their corresponding fruits in four selected berries (thornless blackberry, red raspberry, black raspberry, and strawberry).[25] Previous studies found that there was a direct relationship between the antioxidant capacity and the total phenolic contents in blueberry fruits, leaves,[5] and pomace.[6] Hence, polyphenols in the extracts of BBFPs, BBPPs, and BBLPs made a major contribution to their antioxidant capacity.

Identification of Polyphenol Constituents by HPLC–DAD–MS^n Analysis. In this study, UV–visible spectra, LC–MS, and the subsequent fragmentations of the predominant ions in MS^2 and MS^3 spectra were used to analyze the composition of the samples. In addition, some literature data were used to support the identifications. As shown in Figure 2a, a total of nine different anthocyanins were detected in the extract of BBFPs, whereas only four anthocyanins were detected in the extract of BBPPs (Figure 2b). Peak identification was based on the comparison of their retention times and mass spectral data (Figure 3) with published data. The nine peaks were tentatively identified and are listed in Table 3. Acetylated anthocyanins were not detected in the extracts of BBFPs and BBPPs because acetylated anthocyanins are prevalent in wild blueberries but generally not in cultivated blueberries.[28] Generally, the anthocyanin content of wild blueberry fruits was higher than that of cultivated blueberry fruits.[28] The varieties of anthocyanins identified in the rabbiteye blueberry in Nanjing were slightly different from those in bilberry,[24] lowbush blueberry,[28,29] and rabbiteye blueberry reported by Prior et al.[28] As reported previously for many other phenolics, the composition of anthocyanins in blueberry fruits is dependent on their origin, their location, and the environmental conditions, and this fact affects the physiological benefits of anthocyanin-containing products. However, the profiles of anthocyanins in these blueberry fruits were all composed of delphinidin, cyanidin, petunidin, peonidin, and malvidin glycosides.

Anthocyanins are thermally sensitive compounds, which can degrade when exposed to heating or high temperature. During the blueberry wine-making process, heat was generated by mixing, crushing, pressing, drying, and shearing effects, causing

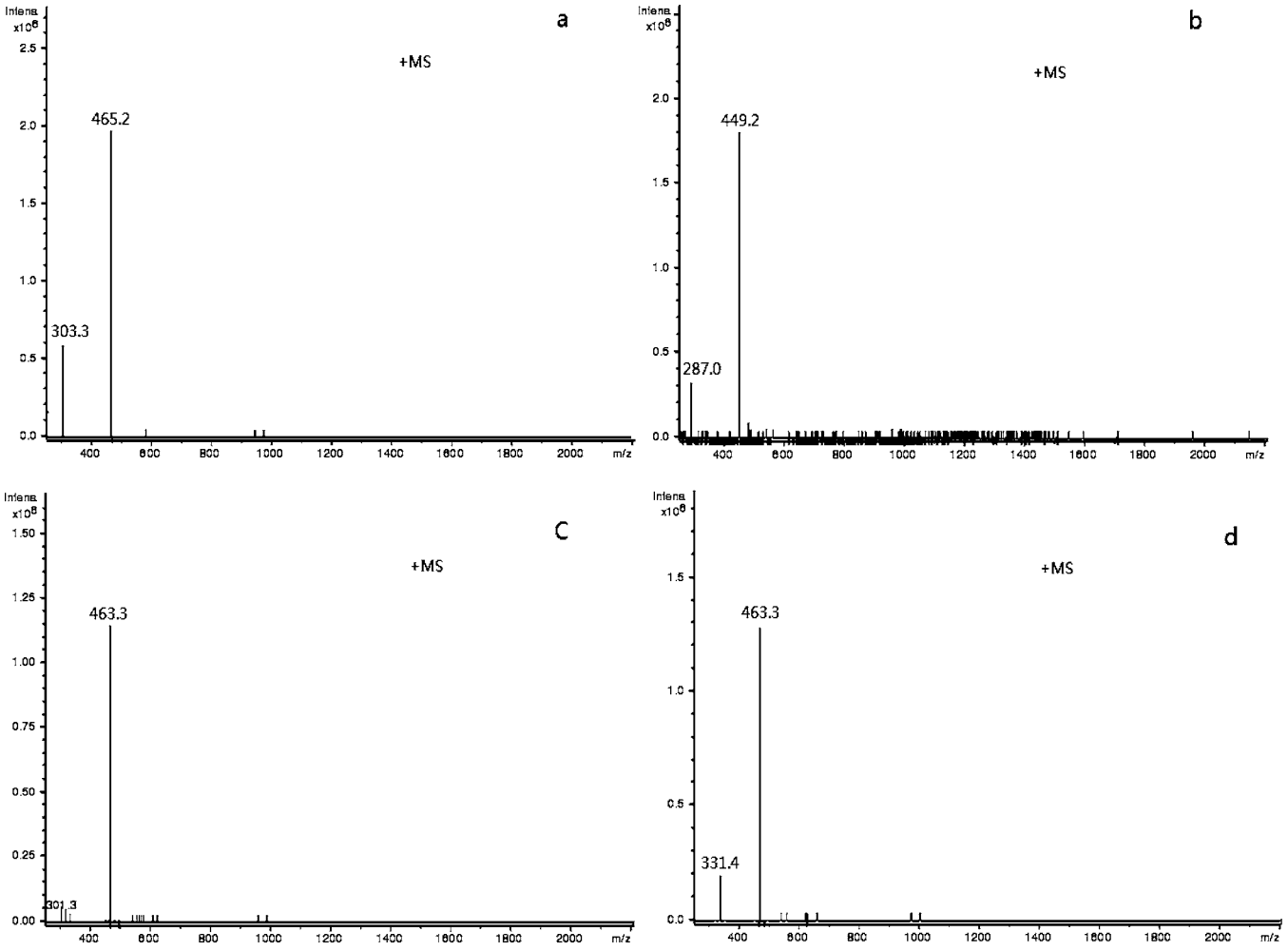

Figure 3. Positive ion MS of anthocyanins in extracts of BBFPs and BBPPs: (a) peak 1, (b) peak 3, (c) peak 6, and (d) peak 9.

a degradation of anthocyanins. In addition, polyphenol oxidase in fruit tissues and other enzymes generated by microorganisms during fermentation could also cause a loss of anthocyanins.[6] In addition, an extraction effect was also seen during the wine-making process. Hence, the five anthocyanins (i.e., delphindin 3-glucoside, petunidin 3-galactoside, malvidin 3-glucoside, malvidin 3-galactoside, and petunidin 3-glucoside) identified in the extract of BBFPs but not detected in the extract of BBPPs might have been lost during the blueberry wine-making process.

Typical HPLC profiles of polyphenols in the extract of BBLPs are illustrated in Figure 4. A total of eight peaks were identified by HPLC−DAD−MS^n with the support of literature data. The two peaks that had an $[M - H]^-$ ion at m/z 353 and an $[M - H - 162]^-$ ion at m/z 191 (quinic acid) and a λ_{max} at 320 nm were identified as 5-*O*-caffeoylquinic acid (neochlorogenic acid, peak 1) and 3-*O*-caffeoylquinic acid (chlorogenic acid, peak 2), respectively. Peak 5, which had an $[M - H]^-$ ion at m/z 515 and an $[M - H - 162]^-$ ion at m/z 353 (caffeoylquinic acid) and a λ_{max} at 330 nm, was identified as di-*O*-caffeoylquinic acid. Peak 6 had an $[M - H]^-$ ion at m/z 515 and a λ_{max} at 330 nm and yielded an m/z 353 ion with fragmentation (Figure 5c); therefore, it was identified as a di-*O*-caffeoylquinic acid, too. The retention times of the polyphenols increased in the following order: 3,4-di-*O*-caffeoylquinic acid < quercetin 3-*O*-glucuronide (peak 4) < 3,5-di-*O*-caffeoylquinic acid < 4,5-di-*O*-caffeoylquinic acid.[26] Therefore, peaks 5 and 6 were identified as 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid, respectively. Peak 8, which had an $[M - H]^-$ ion at m/z 301 and a λ_{max} at 370 nm, was identified as quercetin. Peak 3 had an $[M - H]^-$ ion at m/z 609 and a λ_{max} at 355 nm and yielded a quercetin ion at m/z 301 with further fragmentation, so it was identified as quercetin 3-*O*-rutinoside (Figure 5a). Peak 4 was identified as quercetin 3-*O*-glucuronide as it had an $[M - H]^-$ ion at m/z 477 and a λ_{max} at 360 nm and yielded a quercetin ion at m/z 301 with further fragmentation (Figure 5b). Peak 7, with an $[M - H]^-$ ion at m/z 591, was an unknown quercetin derivative. The ion at m/z 591 yielded an ion at m/z 447 with further fragmentation, and its MS^3 spectra produced a quercetin ion at m/z 301 (data not shown). However, this study cannot provide further information or suggestions about the remaining residue.

Caffeoylquinic acid (neochlorogenic acid, chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid) and quercetin and its derivatives were the most predominant polyphenol groups found in the blueberry leaves in Nanjing. The findings were consistent with previous reports. The major polyphenolic compounds in native blueberry in Europe (bilberry) were chlorogenic acid and quercetin 3-*O*-glucuronide.[26] Chlorogenic acid was the most abundant polyphenol in the leaves of Canadian lowbush blueberry followed by quercetin and its glycosides; a small amount of caffeic acid also

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Table 3. Polyphenols Identified in Fruits, Pomace, and Leaves of Rabbiteye Blueberry in Nanjing

	HPLC–DAD–MS data (m/z)	material
anthocyanins		
delphindin 3-galactoside	$[M + H]^+$ (465.2), $[M + H - Gal]^+$ (303.3)	fruits and pomace
delphindin 3-glucoside	$[M + H]^+$ (465.2), $[M + H - Glu]^+$ (303.3)	fruits
cyanidin 3-galactoside	$[M + H]^+$ (449.2), $[M + H - Gal]^+$ (287.0)	fruits and pomace
petunidin 3-galactoside	$[M + H]^+$ (479.2), $[M + H - Gal]^+$ (317.4)	fruits
petunidin 3-glucoside	$[M + H]^+$ (479.2), $[M + H - Glu]^+$ (317.3)	fruits
peonidin 3-galactoside	$[M + H]^+$ (463.3), $[M + H - Gal]^+$ (301.3)	fruits and pomace
malvidin 3-galactoside	$[M + H]^+$ (493.2), $[M + H - Gal]^+$ (331.4)	fruits
malvidin 3-glucoside	$[M + H]^+$ (493.2), $[M + H - Glu]^+$ (331.3)	fruits
malvidin 3-arabinose	$[M + H]^+$ (463.2), $[M + H - Ara]^+$ (331.3)	fruits and pomace
flavanols		
quercetin 3-*O*-rutinoside (rutin)	$[M - H]^-$ (609.4)	leaves
quercetin 3-*O*-glucuronide	$[M - H]^-$ (477.2)	leaves
quercetin	$[M - H]^-$ (301.1)	leaves
phenolic acids		
5-caffeoylquinic acid (neochlorogenic acid)	$[M - H]^-$ (353.4), $[M - H - Cal]^-$ (191.4)	leaves
3-caffeoylquinic acid (chlorogenic acid)	$[M - H]^-$ (353.4), $[M - H - Cal]^-$ (191.3)	leaves
3,5-di-*O*-caffeoylquinic acid	$[M - H]^-$ (515.3)	leaves
4,5-di-*O*-caffeoylquinic acid	$[M - H]^-$ (515.2), $[M - H - Cal]^+$ (353.8)	leaves

existed.[7] In addition, chlorogenic acid and quercetin 3-*O*-rutinoside (rutin) were the major polyphenolic compounds in the leaves of rabbiteye blueberry cultivated in Japan.[30] Anthocyanins were not detected in green leaves in this study (data not shown). However, when leaves turned red, the synthesis of cyanidin glycosides was activated.[24]

The antioxidant capacity of polyphenols is determined by their structure, such as how easy a hydrogen atom from a hydroxyl group can be donated to a free radical and the ability of the compounds to support an unpaired electron.[4] In general, the number and position of hydrogens donated by the aromatic ring of the phenolic molecule directly determine its antioxidant capacity.[31] The glycosylation of a flavonoid reduces its antioxidant capacity as this process decreases the number of hydroxyl groups,[32] and the 3-OH group that is attached to the 2,3-double bond and adjacent to the 4-carbonyl in the C ring plays a very important role in scavenging free radicals.[31] Therefore, quercetin detected in the extract of BBLPs had higher antioxidant capacity than quercetin 3-*O*-glucuronide and the positive control rutin. This may explain why the extract of BBLPs had a higher antioxidant capacity than rutin. In addition, phenolics with an unsaturated C ring that allows electron delocalization across the molecule for stabilization of aryloxyl radicals always possess higher antioxidant capacity,[31] so the quercetin, its derivatives, and anthocyanins detected in the fruits and leaves had higher antioxidant capacity than procyanidins, e.g., catechin.

Previous studies demonstrated that the antioxidant capacity of phenolic compounds decreased in the following order: quercetin > anthocyanins (cyanidin glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, and peonidin 3-rutinoside) > rutin > chlorogenic acid.[33] Some antioxidants, such as ascorbic acid, tocopherol, and polyphenols, may have synergistic effects on antioxidant activity.[20] The higher antioxidant activities of the extracts of BBFPs, BBPPs, and BBLPs compared to that of rutin may result from the presence of polyphenols with higher antioxidant activity and the synergistic effects between different antioxidants.

The fruits, pomace, and leaves of rabbiteye blueberry in Nanjing were excellent sources of polyphenols and antioxidants. Polyphenols detected in the fruits, pomace, and leaves of blueberry have positive effects on human health, and therefore, they may have potential for use in the development of neutraceuticals or a functional food ingredient to benefit human

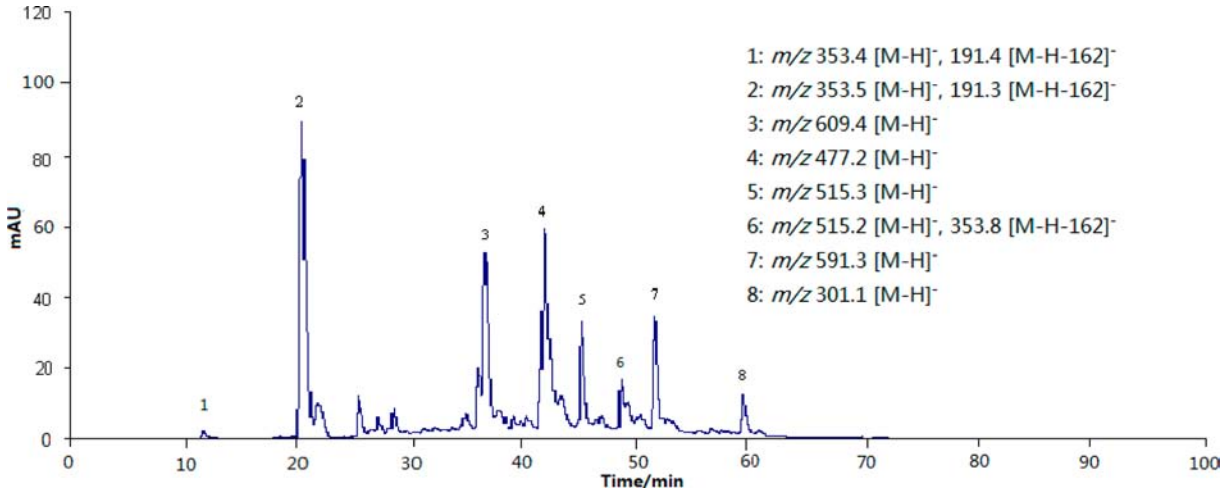

Figure 4. Chromatographic separation and UV detection (λ = 320 nm) of polyphenols in the extract of BBLPs. The following eight peaks were tentatively identified: peak 1, 5-caffeoylquinic acid (neochlorogenic acid); peak 2, 3-caffeoylquinic acid (chlorogenic acid); peak 3, quercetin 3-*O*-rutinoside (rutin); peak 4, quercetin 3-*O*-glucuronide; peak 5, 3,5-di-*O*-caffeoylquinic acid; peak 6, 4,5-di-*O*-caffeoylquinic acid; peak 7, unknown quercetin derivative; peak 8, quercetin.

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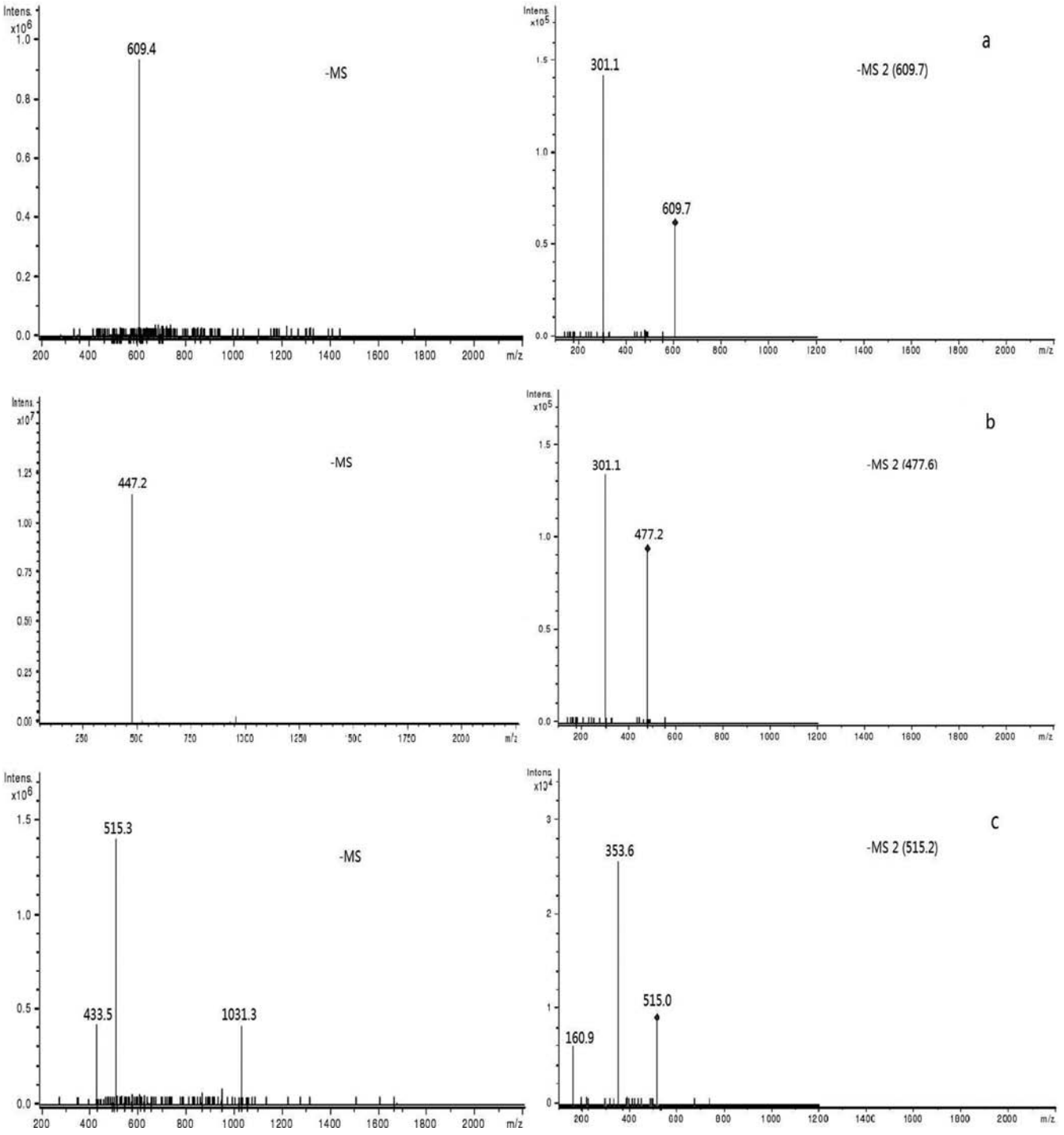

Figure 5. Negative ion MS and MS^2 spectra of polyphenols in the extract of BBLPs (not all shown): (a) peak 3, (b) peak 4, and (c) peak 5.

health. The consumption of fruits can provide a good source of antioxidants or nutritional properties. Blueberry pomace should be viewed as an excellent source of anthocyanins, and blueberry leaves, which possess a high level of flavonoids that were known to possess both antimicrobial and antioxidant activities, could be used in cosmetics and pharmaceuticals. These results may be helpful in the search for new effective antioxidants from blueberry fruits, pomace, and leaves in China and the identification of two byproducts (i.e., blueberry pomace and leaves) in the blueberry industry.

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Notes

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