Isolation of high-purity anthocyanin mixtures and monomers from blueberries using combined chromatographic techniques

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ABSTRACT

Research on the isolation and preparation of anthocyanins has intensified in recent years because of the requirements of quantitative and bioactive analyses. However, simple and effective methods for the scale purification of pure anthocyanins from natural products are rarely reported. In this study, high-purity anthocyanin mixtures and monomers were successfully isolated from wild blueberries using a combination of column chromatography and semi-preparative HPLC. We established an effective elution system to separate high-purity anthocyanin mixtures with aqueous ethanol containing 0.01% HCl first in an Amberlite XAD-7HP column (ethanol/H2O = 35:65) and then in a Sephadex LH-20 column (ethanol/H2O = 25:75). Crude anthocyanin extracts were isolated using the Amberlite column, and a purity of 32% was obtained based on UV–vis analysis. Three fractions of anthocyanin mixtures were isolated from the crude extracts using the Sephadex column with purities ranging from 59% to 68%. Three pure monomeric anthocyanins of malvidin-3-O-glucoside, pelargonidin-3-O-glucoside, and delphinidin-3-O-glucoside were also isolated by semi-preparative HPLC and identified by HPLC-DAD-ESI–MS/MS. The purities of these anthocyanins were determined by analytical HPLC and estimated to be 97.7%, 99.3%, and 95.4%, respectively. The results of this study may help promote the purification of anthocyanins from most blueberry varieties as well as from other plant materials.

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1. Introduction

Anthocyanins are naturally occurring phenolic secondary metabolites that belong to the flavonoid family. As water-soluble natural pigments, anthocyanins are promising alternatives to synthetic food colorings [1]. Besides their colorant properties, increasing evidence shows that anthocyanins exhibit potential health benefits [2–4]. Unfortunately, at present, low extraction percentages, instability, and difficulties in obtaining expensive standards hamper further bioactivity research on anthocyanins. As such, most of the research on anthocyanins is limited to the use of crude anthocyanin extracts from vegetables or fruits. However, the presence of non-anthocyanin phenolic compounds and other impurities inevitably interferes with the evaluation of the biological activities of crude anthocyanin extracts [1,5]. Considering these issues, isolation and preparation of pure anthocyanin standards from plant sources are needed for accurate quantification purposes.

Extensive research suggests that wild blueberries have a higher antioxidant capacity than cranberries, strawberries, plums, raspberries, and cultivated blueberries [6]. Wild blueberries have also been reported to be beneficial in maintaining memory function [7], inhibiting cancer growth [8], preventing atherosclerosis [9], and promoting gastrointestinal and digestive health [10]. Anthocyanins contribute to the beneficial properties of wild blueberries [7,11,12]. Despite their significant potential value, however, wild blueberries have not been widely used by the food industry because they are often consumed directly or used to produce juices and fruit wines. High value-added anthocyanin products (e.g., high-purity anthocyanin extracts) are still unavailable in the market. Thus, while technologically challenging, the preparation of pure anthocyanins from wild blueberries is a very promising endeavor [13,14].

The separation of anthocyanins from plant materials has been carefully studied using techniques such as solid-phase extraction (SPE) [15], high-speed counter-current chromatography (HSCCC) [16], column chromatography (CC) [17,18], and preparative high-performance liquid chromatography (HPLC) [19,20]. Despite the popularity of SPE and HSCCC, the residuals of immiscible organic solvents obtained via these techniques are difficult to remove, which is detrimental to further bioactivity experiments.
Fig. 1. An effective process for the preparation of high-purity anthocyanin mixtures (step 1) and anthocyanin monomers (step 2) from wild blueberry fruits.

As an important purification technology, CC has been widely used to isolate flavonoids, tannins, and monomeric anthocyanins, especially with the extensive application of Amberlite XAD-7 and Sephadex LH-20 columns [17,21,22]. However, most of the extraction procedures developed thus far involve toxic organic solvents, including methanol, acetone, formic acid, acetic acid, trifluoroacetic acid (TFA), and acetonitrile. Although the anthocyanin content of Chinese wild blueberries has been previously determined [23,24], data on the feasibility and systematic isolation of high-purity anthocyanins are insufficient. The present study was conducted in response to recent research issues concerning the nutritional and health benefits of blueberries, especially the wild blueberry variety. The aims of this study are: (1) to develop a natural and low-cost method for the purification of anthocyanin mixtures using combined CC techniques and (2) to explore a semi-preparative HPLC technology for obtaining pure monomeric anthocyanins from Chinese wild blueberry fruits.

2. Materials and methods

2.1. Reagents and standards

HPLC-grade formic acid and methanol were purchased from Merk (Darmstadt, Germany). Ethanol, ethyl acetate (EtOAc) and hydrochloric acid (HCl) of analytical grade were purchased from Beijing Chemistry Factory (Beijing, China). Deionised water was obtained from a Milli-Q Element water purification system (Millipore Co., Billerica, MA, USA). Standard of cyanidin-3-O-glucoside was purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA).

2.2. Plant materials

Fresh, ripe samples of lowbush wild blueberries fruits of the *Vaccinium uliginosum* L. species were collected from unmanaged,
Fig. 2. HPLC-DAD chromatograms of (A) crude anthocyanin extracts and (B–E) purified anthocyanin fractions from wild Chinese blueberry species. Anthocyanins were detected at 530 nm. Refer to Table 1 for the assignment of numbered peaks (1–14). Abbreviations: XAD-7, Amberlite XAD-7HP; LH-20, Sephadex LH-20.
naturally occurring woodlands situated in Changbai Mountain zones (41°41’ N, 127°42’–128°16’ E, Jilin province, China). Fruits were harvested between August and September of 2012, and then washed, stored in a freezer, at −20 °C until extracted.

2.3. Extraction and purification by combined column chromatography

A schematic of the proposed method for obtaining high-purity anthocyanin mixtures is illustrated in Fig. 1. First, 100 g of wild blueberry fruits was homogenized in a blender and extracted with 1 L of 0.1% HCl acidified 70% (v/v) ethanol for 24 h in the dark. After the extracts were filtered, the residue was dissolved with 1 L of 0.1% HCl acidified 70% (v/v) ethanol and extracted for 24 h using the same conditions previously described. The clear liquid from two extractions was combined and concentrated to a volume of 50 mL using a rotary evaporator at temperatures not exceeding 40 °C, which lasted for about 1 h. The resulting aqueous solution (50 mL) was then partitioned sequentially with ethyl acetate (EtOAc, 50 mL) four times. Due to the slight solubility of water and EtOAc, the volume of water phase would decrease in each step, thus a total of about 25 mL aqueous solution was collected and then kept at 4 °C in the dark. The aqueous phase was loaded onto a column (2.6 cm × 50 cm) of cation-exchange resin (Amberlite XAD-7HP; particle size: 20–60 mesh, wet, Sigma-Aldrich). The column was washed with 2 L of deionized water (0.01% HCl) at a flow rate of 1 mL/min to remove the majority of sugars, organic acids, proteins and ions, and then elution of anthocyanins was done using 1 L of 35% ethanol (0.01% HCl) at 1.5 mL/min. The eluate (around 500 mL) was collected based on the color band and UV–vis detection at 520 nm on the column. Finally, the eluate was concentrated using a rotary evaporator at temperatures not exceeding 40 °C, and the resulting solution was freeze-dried. To completely elute residual weak polar anthocyanins and other phenolic compounds, the column was eluted once more with 300 mL of 60–80% ethanol (0.01% HCl) at 1.5 mL/min, and the eluted solution was collected and concentrated for later use. One hundred milligrams of the 35% ethanol eluate was dissolved in 20 mL of deionized water and loaded onto a Sephadex LH-20 chromatographic column (1.0 cm × 60 cm; Sigma Chemical Co., St. Louis, MO, USA). The column was eluted with 1 L of 25% ethanol (0.01% HCl) at 1.2 mL/min, and the fractions were collected in test tubes of approximately 10 mL. A 100 μL aliquot of each fraction was subjected to anthocyanin content determination at 520 nm. Fractions with the same UV peaks were combined, freeze-dried, and stored at 4 °C.

2.4. Semi-preparative HPLC purification of pure monomeric anthocyanins

To obtain the major monomeric anthocyanins with high purity from the column fractions, semi-preparative scale HPLC (LC-6AD, Shimadzu, Japan) was performed (Fig. 1) using a semi-preparative column [Shim-pack PREP-ODS (H) kit, 20.0 mm × 250 mm, 5 μm]. The sample was injected into the column using a Rheodyne manual sample injector (7725i) with a 2 mL loop (Rohnert Park, CA, USA). Optimized conditions of the semi-preparative HPLC were obtained based on the conditions of analytical HPLC. The mobile phase consisted of methanol (solvent A) and 3% formic acid (solvent B), and the initial gradient composition was 15% solvent A and 85% solvent B. The elution conditions were as follows: solvent B: 0 min, 85%; 3 min, 80%; 7 min, 75%; 10 min, 75%; 55 min, 30%; 60 min, 30%; 65 min, followed by washing and re-equilibration of the column for 15 min. Other elution conditions were as follows: flow rate, 2 mL/min; column temperature, 25 °C; monitoring wavelength, 530 nm. Ten milligrams of the powdered anthocyanin fractions obtained by the Sephadex LH-20 column was dissolved.
in 3% formic acid to obtain a final concentration of 1 mg/mL. Samples were filtered using 0.45 μm syringe filters, and the injection volume ranged from 0.5 mL to 2 mL.

2.5. Total monomeric anthocyanin content

The total anthocyanin content (TAC) in fresh fruits and anthocyanin extracts was measured using the pH differential method [25]. The absorbance of anthocyanin was measured with a spectrophotometer (UV-2550, Shimadzu) at 510 nm in pH 1.0 buffer and at 700 nm in pH 4.5 buffer using \( A = (A_{510} - A_{700})_{pH\,1.0} - (A_{510} - A_{700})_{pH\,4.5} \) (molar extinction coefficient of cyanidin-3-O-glucoside, 26,900). The anthocyanin content was expressed in terms of mg/100 g fresh weight. Analysis was carried out in triplicate.

2.6. Identification of anthocyanins by HPLC-DAD-ESI–MS/MS

The anthocyanin composition in both fresh fruits and purified samples was analyzed by HPLC-DAD-ESI–MS/MS. Each of the samples was filtered through a 0.45 μm filter membrane and then analyzed by HPLC using a 1100 Series liquid chromatography system (Agilent Technologies Inc., USA) equipped with a diode array detector (DAD) and Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm, Agilent). Binary gradient elution was used in this study. The mobile phase was composed of two solvents, 100% methanol (solvent A) and 3% formic acid (solvent B). The linear gradient was achieved as follows: 85% B for 5 min, 85–80% B for 5 min, 80–75% B for 15 min, 75% B for 5 min, 75–30% B for 6 min, 30% B for 6 min, 30–85% B for 5 min, and remained at 85% for 6 min. The mobile phase was pumped through the system at a rate of 0.5 mL/min. The injection volume was 10 μL, and the oven temperature was set to 25 °C. Detection of anthocyanins was performed at 530 nm.

The mass detector used was an ABI Q-Trap mass spectrometer (Applied Biosystems Sciex, Foster City, USA) equipped with an electrospray ionization (ESI) source and then interfaced to a computer running Applied Biosystems Analyst version 1.4 software. Optimized instrument parameters used in the HPLC-DAD–MS/MS system were determined from a test using cyanidin-3-O-glucoside as a standard prior to sample detection. The MS/MS parameters were as follows: ESI source, positive mode; drying and nebulizing gas, nitrogen; nebulizer pressure, 30 psi; dry gas flow, 12 L/min; temperature, 400 °C; capillary voltage, 4.5 kV; scan range, from m/z 200 to 1000.

2.7. Statistical analysis

Determinations were carried out in triplicate. Data were collected and processed using OriginPro 8.5 (OriginLab Co., Northampton, MA, USA) and SPSS 16.0 (Chicago, IL, USA). Significant differences between means were determined by T-tests with \( P < 0.05 \) considered statistically significant difference.

3. Results and discussion

3.1. Anthocyanin composition and total anthocyanin content in fresh fruits

An HPLC chromatogram of the anthocyanin profile is presented in Fig. 2A, and the anthocyanin composition is given in Table 1. Assigned peaks with area percentages less than 0.4% were omitted from the chromatogram. While not all peaks could be detected because of their weak UV–vis and HPLC–MS spectra, at least 14 major anthocyanin peaks (Table 1) were identified by comparison of the retention times of the standards, the \( m/z \) of each anthocyanin molecule, and fragmentation patterns with the available values in the literature [26,27]. Five types of anthocyanin fragmentation ions [delphinidin (De) at \( m/z \) 303, cyanidin (Cy) at \( m/z \) 287, petunidin (Pet) at \( m/z \) 317, peonidin (Pee) at \( m/z \) 301, and malvidin (Mal) at \( m/z \) 331] and four types of glycosides [galactoside (Gal), glucoside (Glu), arabinoside (Ara), and xyloside (Xyl)] were detected in the present study. The most abundant compounds in

![Fig. 3. UV–vis spectra of (A) original crude extract solution in the range of 250–650 nm, (B) water phase after partitioning, (C) EtOAc phase after partitioning once, and (D) EtOAc phase after partitioning four times.](image-url)
wild Chinese blueberries were De derivatives (De, Pet and Mal), which account for 89.1% of the total anthocyanins. In contrast, the least abundant compounds were Cy derivatives (Cy and PEO), which account for only 9.6% of the total anthocyanins. The three major monomeric anthocyanins (peaks 2, 8, and 11 in Fig. 2) represented approximately 69.6% of the total peak area, and the most abundant monomeric anthocyanin was determined to be Mal-3-glu (31.9%). In this study, two anthocyanin-3-O-xilosides (peaks 13 and 14 in Fig. 2) were tentatively identified for the first time in Chinese wild blueberries.

Using the pH differential method, the average TAC in wild blueberry fruits was determined to be 144.7 ± 4.49 mg/100 g fresh fruit, which is highly similar to the data reported in studies on various wild blueberry species in China (ranging from 90 mg/100 g to 180 mg/100 g fresh fruit) [23,24] but slightly lower than those reported for various species of wild blueberries from other countries (ranging from 250 mg/100 g to 300 mg/100 g fresh fruit) [28]. Although the TAC in wild Chinese blueberries is not the highest among blueberry species, a prominent feature of the anthocyanin composition of this species is that all 14 anthocyanins found are monoglycosylated derivatives, which are highly polar and watersoluble; these monoglycosylated anthocyanins also exhibit more effective biological activities than diglycosylated or acetylated forms of anthocyanins [29–31]. Data on the anthocyanin composition of wild blueberries provide novel insights into their efficient purification and promote their potential use in food colorants, functional foods, and pharmacology.

3.2. Extraction and partitioning procedure

HPLC-DAD analysis results of the anthocyanin composition in fresh wild blueberry fruits (Table 1) indicate that the three anthocyanins of Mal-3-0-gluc (31.9%), Pet-3-0-gluc (21.4%), and De-3-0-gluc (16.2%) are key targets for separation and purification. The polarity of anthocyanins makes them soluble in several types of polar solvents, such as methanol, ethanol, acetonitrile, acetone, and water. The proper use of extraction solvents is crucial in the efficient release of anthocyanins from fruits. The most popular extraction solvents include methanol, acetone, and acetonitrile, as well as slightly acidified solvents, such as formic acid, acetic acid, TFA, and phosphoric acid [32,33]. However, while the extraction efficiency of these solvents is clearly excellent, the presence of toxic solvent residuals in the extracts is unacceptable because of the potential application of these purified extracts in the food and pharmaceutical industries. Considering the need for maximized release of anthocyanins and safety requirements, 70% (v/v) ethanol containing 0.1% (v/v) HCl was utilized to extract anthocyanins from wild blueberry fruits.

One of the key problems in the purification of anthocyanins is the presence of large amounts of sugar and other phenolic compounds in fruits. EtOAc could be used for the removal of sugars, apectins, and liposoluble phenolic compounds [34]. In our work, after extraction and concentration of the anthocyanins, the aqueous extract solutions obtained were partitioned against EtOAc. Enhanced isolation of anthocyanins (Fig. 3B) from the crude extract solutions (Fig. 3A) was observed because of the removal of non-anthocyanin phenolic compounds (Fig. 3C). After the aqueous crude extracts had been partitioned four times, the UV–vis spectra of the EtOAc phase (Fig. 3D) showed that large amounts of phenolics (the obvious absorption peak at around 360 nm) were dissolved in EtOAc. This finding reveals that EtOAc is an effective reagent for removing phenolics from extracts to obtain high anthocyanin purity. The major difference in the anthocyanin profiles of cultivated and wild varieties is the presence of acylated anthocyanins, which are completely absent in wild blueberries [35,36]. Therefore, EtOAc is not applicable in the purification of acylated anthocyanins from most cultivated blueberry fruits, as both acylated anthocyanins and phenolic compounds are soluble in EtOAc [37].

3.3. Separation of monomeric anthocyanins by combined column chromatography

The partitioned anthocyanin extracts were subjected to combined CC. Amberlite XAD-7HP, as a nonionic, moderately polar, acrylic resin, can be utilized to separate anthocyanins through non-polar and polar interactions [17]. After loading of the anthocyanin extracts, the column was washed with H2O containing 0.01% HCl and then eluted with aqueous ethanol containing 0.01% HCl (ethanol/H2O = 35:65). The anthocyanin-rich eluate was evaporated in vacuo at 40°C and then freeze-dried. HPLC chromatograms of the crude extract solutions (Fig. 2A) and the 35% ethanol crude powder (Fig. 2B) obtained at 530 nm were compared, and results indicate that nearly all 14 monomeric anthocyanins were eluted successfully, despite slight changes in the ratios of peak areas of each anthocyanin (Table 1). To track anthocyanin residuals on the sorbent, 60–80% ethanol was employed to elute anthocyanin residuals. HPLC analysis showed that only 5–10% anthocyanins with weak polarity could be detected in the ethanol eluate. These results reveal that 35% ethanol can elute the majority of the anthocyanins from the Amberlite XAD-7HP column without dramatic changes in the anthocyanin profile. The TAC in the dried anthocyanin crude powder amounted to 32% (Fig. 4), which indicates that
the TAC is strongly enhanced after the removal of sugar, pectins, non-anthocyanin phenolics, and other impurities.

The hydroxypropylated dextran-based Sephadex LH-20 resin is a beaded and cross-linked gel with a narrow molecular weight range [34]. In the present study, systematic investigations of the Sephadex LH-20 column using ethanol and HCl showed that elution with 25% ethanol containing 0.01% HCl is a very simple and effective method of fractionating anthocyanins. In this stage, a total of 50 test tubes (10 mL/tube) were collected. And then a 100 µL aliquot of each fraction was subjected to absorbance spectroscopy at 520 nm in order to plot an analytical curve (Fig. 4A). The eluate in the same absorption peak (Fig. 4A) was combined, thus three major fractions were obtained. The anthocyanin profiles in these three fractions were determined by HPLC-DAD–MS/MS (Fig. 2C–E, Table 1). Fraction I mainly consisted of Mal-3-O-glu, representing 86.8% of the total peak area (Fig. 2C), Fraction II included Pet-3-O-glu (47.3%) and residual amounts of Mal-3-O-glu (24.1%) (Fig. 2D), and Fraction III consisted of a mix of different anthocyanins, including De-3-O-glu (51.6%), Cy-3-O-glu (12.9%), and residual Pet-3-O-glu at low proportions (Fig. 2E). The Sephadex LH-20 column effectively separated various forms of anthocyanins, which indicates its applicability in systems designed to scale up the preparation of high-purity anthocyanin extracts.

After separation of the 35% ethanol crude powder into three fractions by the Sephadex LH-20 column and then freeze-drying, the TAC in different fractions was determined by the pH differential method (Fig. 4). The TAC in the three fractions ranged from 59% to 68%, with the highest values found in Fraction II. The high purity of the three fractions indicates that extracts obtained from Sephadex LH-20 size exclusion chromatography have 2-fold higher TAC (p < 0.01, Fig. 4) than crude extracts (purity: 32%) and about 400-fold higher TAC than fresh wild blueberry fruits (144 mg/100 g). While the TAC in Fraction I (59.5%) was not higher than that in Fraction II (68.0%), the Mal-3-O-glu in Fraction I accounted for 86.8% of the total peak area, which that illustrates over 51% of the total weight in Fraction I can be attributed to Mal-3-O-glu. Similarly, Pet-3-O-glu and De-3-O-glu appear to be the major monomeric anthocyanins in Fractions II and II, respectively. Mary [38] and Edith [39] employed a similar CC technology to separate anthocyanins from wild blueberries of North America and wild blackberries from Mexico, respectively, with results showing that total anthocyanins account for 59.5–75% of the

Fig. 5. Semi-preparative HPLC chromatograms of three fractions obtained from the Sephadex LH-20 column. Anthocyanins were detected at 530 nm. The anthocyanin monomers were purified by semi-preparative HPLC (A–C) once and (E, F) twice. Abbreviations: Mal, malvidin; Pet, petunidin; De, delphinidin; Cy, cyanidin; Glu, glucoside.
anthocyanin-enriched fractions purified by Amberlite XAD-7 and Sephadex LH-20 columns; in these studies, methanol and acetone were used as the major elution solvents. In the present study, the low concentrations of ethanol used in the Amberlite XAD-7HP (ethanol/H2O = 35:65) and Sephadex LH-20 (ethanol/H2O = 25:75) columns offer a more cost-effective option for the preparation of anthocyanin products. Our proposed method, which combines two chromatographic processes using natural solvents (ethanol and HCl), achieves ideal results without the excessive use of toxic organic solvents; thus, it may be applied in the scale production of high-purity anthocyanin extracts.

3.4. Semi-preparative HPLC of monomeric anthocyanins

Semi-preparative HPLC is an efficient and gentle means of fractionating different types of anthocyanins from crude plant extracts [40–42]. Considering the presence of overlapping peaks of anthocyanins in the chromatograms of crude anthocyanin extracts prepared by the Amberlite XAD-7HP column, many of the monomeric anthocyanins (e.g., peaks 2 and 8 in Fig. 2B) are obviously not purified simply through semi-preparative HPLC. Therefore, the three fractions collected from the Sephadex LH-20 column were subjected to semi-preparative HPLC. Based on the semi-preparative HPLC chromatogram of the three fractions (Fig. 5A–C), the eluates of the major anthocyanins in each fraction can be selected and collected. In order to get the utmost possible amount of pure anthocyanins, increasing the injection volume or concentration of the samples is very necessary. However, the above operation may lead to the overload of column and detector, therefore, most of the peaks in Fig. 5A–C were flat topped peaks. The eluates of the major peaks were re-injected to significantly improve the purity of anthocyanin monomers. In our study, three major peaks were collected (Fig. 5D–F), and the eluates of each peak were concentrated with a rotary evaporator and freeze-dried. The peaks in Fig. 5D–E are more or less symmetrical, while the peak shape in Fig. 5F is slightly asymmetrical. This special phenomenon indicated that there were still some impurities in De-3-O-glu solution. In order to collect the De-3-O-glu eluate as pure as possible, every time we did not start to collect De-3-O-glu eluate until most of the impurities had been eluted in the column. The eluate in the middle part of the peak would be collected selectively. Based on these skills, the purity of anthocyanin monomers had been improved largely (Fig. 6A–C).

The chemical structures of each of the monomeric anthocyanins were further determined by HPLC-DAD–MS/MS (Fig. 6A–C) using Cy-3-O-glu as a standard (Fig. 6D). Through comparison between the HPLC profiles of the crude extract solution (Fig. 2A) and the MS/MS fragmentation ions (Fig. 7A–F), the three HPLC peaks corresponded to Mal-3-O-glu ([M+H] (m/z) 493, MS/MS (m/z) 331 in Fig. 7A and B), Pet-3-O-glu ([M+H] (m/z) 305 in Fig. 6B, [M+H] (m/z) 479, MS/MS (m/z) 317 in Fig. 7C and D), and De-3-O-glu ([M+H] (m/z) 20.2 in Fig. 6C, [M+H] (m/z) 465, MS/MS (m/z) 303 in Fig. 7E and F). The overall purity of each anthocyanin was assayed by analytical HPLC and calculated as follows: 97.7% for Mal-3-O-glu, 99.3% for Pet-3-O-glu, and 95.4% for De-3-O-glu. The purity of De-3-O-glu was slightly lower than
Pet-3-O-glu and Mal-3-O-glu, which indicated the existence of some impurities whose polarity was similar to De-3-O-glu, especially the interference of De-3-O-gal. Further work needs to be done to improve its purity. Ana et al. [43] isolated two anthocyanin fractions from a Camarosa strawberry variety using Amberlite XAD-7 and countercurrent chromatography and obtained pelargonidin-3-O-glucoside and pelargonidin-3-O-rutinoside purities of 90% and 92%, respectively. Hitoshi et al. [4] obtained four pure anthocyanins (i.e., delphinidin-3-O-β-rutinoside, cyanidin-3-O-β-rutinoside, delphinidin-3-O-β-glucoside, and cyanidin-3-O-β-glucoside) with >99.5% purity from black currant using enzyme treatment, Amberlite XAD-7HP chromatography, and preparative HPLC. The combined CC and semi-preparative HPLC method used in this study was not only effective in separating pure monomeric anthocyanins from wild blueberries but also showed additional advantages of simplicity, stability, and acceptability for industrial purposes.

4. Conclusion

In this work, we developed an effective strategy for the scale preparation of high-purity anthocyanin mixtures. Ethanol, EtOAc, and HCl, which can serve as good substitutes for toxic methanol, acetone, and TFA, were highly suitable for the extraction and purification of anthocyanin mixtures. We also established an innovative semi-preparative HPLC procedure that effectively separates pure anthocyanin monomers from blueberry samples. Pure anthocyanins are useful not only as standards for quantitative measurement but also as benchmarks for evaluations of the biological capacities of plant extracts. To expand the scope of application...

Fig. 7. ESI-MS and ESI-MS² spectra of three monomeric anthocyanins. The MS was operated in positive mode. Abbreviations: Mal, malvidin; Pet, petunidin; De, delphinidin; Cy, cyanidin; Glu, glucoside.
of our proposed method and obtain high-purity extracts from other plant materials, certain improvements may be made. Overall, considering relevant regulations on the safety and nutritional requirements of natural products, this study presents a practical and valuable strategy for preparing high-purity anthocyanin mixtures and monomers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.12.070.

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