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Identification of Anthocyanin in Bayberry (*Myrica rubra* Sieb. et Zucc.) by HPLC-DAD-ESIMS and GC

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ABSTRACT

Bayberry (*Myrica rubra* Sieb. et Zucc.) anthocyanins were extracted with 0.15% HCl in methanol, removed from other polyphenols by partition with petroleum ether and ethyl acetate, and then purified in a C-18 solid phase cartridge. Pigments were analyzed by high-performance liquid chromatography (HPLC), photodiode array detection (DAD), electronic spray ion mass spectrometry (ESIMS) and gas chromatography (GC) without the standard. The major anthocyanin, which contained 97% of the total peak area, was identified as cyanidin 3-glucoside. This pigment may be taken as a marker for authenticity control of bayberry products and has potential as a reference compound of cyanidin 3-glucoside in anthocyanin analysis. When standard chemicals are not available, HPLC-DAD-ESIMS and GC are useful in anthocyanin identification if the anthocyanin profile is not overly complex in the studied materials.

Key words: bayberry, anthocyanin, HPLC-DAD-ESIMS, GC

INTRODUCTION

Bayberry (*Myrica rubra* Sieb. et Zucc.) is a tree growing wildly and under cultivated conditions in Southern China. Bayberry fruits have a special sweet, sour taste and exquisite flavor, and have been used for food and medicinal purpose for more than 2000 years[(1)]. However, the fruits of most cultivars ripe in the hot and raining season of May to July and can only be stored fresh with attractive dark red color for 3 days at 20-22°C and 9-12 days at 0-2°C[(2)]. Bayberry juice is an alternative product for longer consumption, in which *Biqi* cultivar has always been used as the major material because of its mass production and excellent quality. Color instability and haze formation during juice processing and storage are major concerns to food technologists[(3-5)].

Anthocyanins are responsible for most red, blue, and purple colors of flowers, fruits and other plant tissues[(6)]. They are also related to color degradation and haze formation during juice processing and storage[(7-8)]. Anthocyanins from bayberry fruits were analyzed by Ye *et al*.[(9)] using a paper chromatographic method. The pigments were found to consist of cyanidin 3-glucoside, pelargonidin 3-glucoside and delphinidin 3-glucoside. However, Ye's method was time consuming and the identification based on mobilities (R_f value) in the lack of standards is arguable because the R_f values can vary from worker to worker even under similar analytical conditions. Even if used with standards, identification based on R_f values is also questionable due to expected coelution.

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High performance liquid chromatography (HPLC) with a photodiode array detector (DAD) is powerful in determination of anthocyanins[(10)]. HPLC-DAD coupled with a mass spectrometer (MS) has been proven to be an effective method for anthocyanin identification[(11-13)]. Gas chromatography (GC) also has been successfully used in identification of the sugar moieties of the anthocyanins after hydrolysis and derivatization[(14)]. The HPLC method[(15)] and HPLC-DAD-ESIMS method[(16)] were applied in bayberry anthocyanin identification, with the use of standard anthocyanin of cyanidin 3-glucoside. However, standard chemicals are rare and expensive, so comparing these chemicals in every experiment is not practical in routine analysis. The aim of this investigation is to characterize the anthocyanins in partially purified extracts from bayberry fruits by HPLC-DAD-ESIMS and GC, which may be an alternative in anthocyanin identification when standard chemicals are unavailable.

MATERIALS AND METHODS

I. *Plant Material*

Mature bayberry fruits of *Biqi* cultivar were hand-harvested in June 23, 2005 in Cixi, Zhejiang Province, P. R. China. The fruits were transported to our laboratory in 10 hours by a refrigerator van at 5°C, de-stemmed, washed and individually quickly frozen (IQF). The IQF fruits were packed in 500 g polyethylene bags and stored at -20°C until used. The total monomeric anthocyanin content was 125.2 mg/100 g of fresh weight (FW), deter-

mined by the pH differential method as cyanidin 3-glucoside[17]. The fruits had a pH of 3.15, soluble solids of 9.5, and titratable acidity of 0.86 g /100 g FW (expressed as citric acid).

II. *Pigment Extraction*

Extraction was done following the modified procedure described by Pazmiño-Durán *et al*.[18]. Twenty grams of fruit flesh were cut from 10 randomly selected thawed bayberry fruits. The flesh was blended with 100 mL of 0.15% HCl in methanol (v/v) and stored overnight at 5°C. The mixtures were filtered on a Büchner funnel and the filter cake residue was re-extracted with 0.15% HCl in methanol until a clear solution was obtained. Filtrates were combined and concentrated to ca. 20 mL in a rotavapor at 35°C under reduced pressure. The aqueous extract was made up to 100 mL with distilled water.

III. *Anthocyanin Purification*

Five milliliters of extract were shaken in a separatory funnel with 10 mL of petroleum ether (b.p. 40-60°C) and then 10 mL of ethyl acetate to remove non-polar impurities and other flavonoids[14]. The aqueous portion was collected. One milliliter of aqueous extract was adsorbed onto a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA, USA) previously activated with methanol followed by 0.01% aqueous HCl[10]. Sugars, acids and other water-soluble compounds were eluted with 2 mL of 0.01% aqueous HCl and anthocyanins were subsequently recovered with 2 mL of methanol containing 0.01% HCl. The methanolic extract was concentrated to dryness using a rotavapor at 35°C and the pigments were re-dissolved in 1 mL of deioned water containing 0.01% HCl.

IV. *Acid Hydrolysis*

Fifteen milliliters of 2 M HCl were added to 1 mL of purified pigment extract in a screw-cap test tube, flushed with nitrogen and capped[10]. The pigment was hydrolyzed for 45 min at 100°C, and then cooled in anice bath. The hydrolysis solution of 2 mL was adsorbed onto a C-18 Sep-Pak cartridge. Sugars were eluted from the minicolumn with 4 mL of 0.01% aqueous HCl and collected for identification.

V. *Identification of Sugars*

The sugar solution from the hydrolysis was concentrated to approx. 2 mL with a rotavapor, dried under 45°C, and stored overnight in a desiccator under reduced pressure. Ten milligram of hydrochloric hydroxylamine and 0.5 mL of pyridine were added to the dried sugar in a vial, shaken and then kept at 90°C for 30 min. The solution was cooled and 0.5 mL of acetic anhydride was added. The vial was shaken again and kept at 90°C for another 30 min, after which it was cooled for gas chromatography.

Analyses of the sugar derivatives were carried out on a Shimadzu 14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a chromatographic workstation software. A DB-1701 quartz capillary column (30 m × 0.53 mm I. D., 1.0 μm; Agilent Corp., USA) was used. The injection volume was 1 μL and the split ratio was 30:1. The flow rates of the carrier gas (N_2), flame gas (H_2), combustion-supporting gas (air), and make up gas (N_2) was 3, 45, 400 and 30 mL/min, respectively. The injector and detector temperature was set at 260°C. The oven was held at 185°C for 3 min, programmed from 185-240°C at 3°C/min and kept at the ultimate temperature for 20 min.

Rhamnose, arabinose, xylose, mannose, glucose, and galactose were used as standard sugars.

VI. *HPLC-DAD-ESIMS Analysis*

HPLC-DAD-ESIMS analysis was done on a Waters platform ZMD 4000 system composed of a Micromass ZMD mass spectrometer and a Waters 2690 HPLC equipped with a Waters 996 photodiode array detector (Waters corp. Milford. MA, USA). Data were collected and processed via a personal computer running MassLynx software version 4.0 (Micromass, a diversion of Waters Corp., MA, USA). Anthocyanins in 5 μL aliquot of purified bayberry extract were separated by an ODS-Hypersil column (250 × 4 mm I.D., 5 μm particle size; Agilent Corp., USA). Solvent A was 5% formic acid and 95% water (v/v), whereas solvent B was 5% formic acid, 45% water and 50% methanol (v/v/v). The elution profile consisted of a linear gradient from 0 to 100% B for 40 min, from 100% to 0 B for 10 min and washing (100% methanol) and re-equilibration of the column for 10 min with the flow rate of 0.3 mL/min. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter (Millipore Corp., Bedford, MA, USA).

UV-visible absorption spectra were recorded on-line during HPLC analysis. Spectral measurements were made over the range of 200-600 nm with 520 nm as detection wavelength. Relative amounts of each anthocyanin were reported as percentages of total peak area.

Mass spectra were achieved by electrospray ionization in positive mode (ESI^+). The following ion optics was used: Capillary 3.87 kV, cone 30 V, and extractor 7 V. The source block temperature was 120°C and the desolvation temperature was 300°C. The electrospray probe-flow was adjusted to 70 mL/min. Continuous mass spectra were recorded over the range m/z 150-950 with scan time of 1 sec and interscan delay of 0.1 sec.

RESULTS AND DISCUSSION

According to the report of Ye *et al*[9], cyanidin 3-glucoside was about 95% of the total anthocyanin spot

area based on the paper chromatographic method. Pelargonidin 3-glucoside and delphinidin 3-glucoside might also exist in bayberry fruits. However, the partially purified methanolic extracts of *Biqi* bayberry fruits showed only one major anthocyanin in the HPLC chromatogram detected at 520 nm (Figure 1). This peak eluted at 23.78 min under the analytical conditions and contained 97% of the total peak area. The discrepancy between our results and those of Ye *et al*[9] might be caused by the different analytical methodologies. Furthermore, the clean up procedures was performed in our study while Ye *et al*[9] analyzed the crude acid methanol extractions without any pretreatment. The low concentrations of other anthocyanins could become even lower after sample preparation including petroleum ether and ethyl acetate partition and C-18 minicolumn purification. Other anthocyanins might also be detected in the HPLC chromatogram (Figure 1), but each represented less than 1% of the total peak area and the concentration was too low to be identified. The total ion chromatogram of the anthocyanins on mass spectrometry confirmed the one major anthocyanin in the bayberry fruits (Figure 1).

UV-visible spectrum of this compound showed the visible λ_{max} at 522 nm (Figure 2), which was in the range of the visible λ_{max} of cyanidin 3-glucoside (520-525 nm) in acidic methanol solutions, according to the report of Hong and Wrolstad[10]. The spectra of anthocyanin peaks can also provide information about the presence of acylating groups. The ratio of absorbance at the acyl maximum (310-340 nm) to absorbance at the anthocyanin (Acy) maximum wavelength (522 nm), λ_{max}acyl/λ_{max}Acy, is a measure of the molar relation of the cinnamic acid to the anthocyanin[19]. In acidified methanolic solutions, a ratio of 48% to 71% is indicative of a 1/1 molar ratio, while a ratio of 83% to 107% is characteristic of a 2/1 molar ratio of cinnamic acid to the anthocyanin. After calculation, the ratio of λ_{max}acyl/λ_{max}Acy was only 18%, suggesting the absence of cinnamic acid acylation of the bayberry anthocyanin. The ratio of $Abs_{440}/Abs\lambda_{max}$ was 29%, indicating glycosidic substitution at position 3 or 7 of the flavylium, and high probability at position 3[10,20]. The UV-visible information suggested that the bayberry anthocyanin is glycosidicly substituted at position 3 and no hydroxylated aromatic organic acid acylated.

The mass spectral fragmentation pattern is shown in Figure 3. The bayberry anthocyanin had a molecular ion (M^+) at m/z 449.2 and a fragment ion at m/z 287.1 (M-162, loss of a hexose unit), which corresponded with cyanidin 3-glucoside or cyanidin 3-galactoside and cyanidin[12,21]. After acid hydrolysis and derivatization, only glucose was detected by GC (Figure 4), which suggested glucose was substituted at the bayberry anthocyanidin. Combined with the UV-visible information, ESI^+-MS spectral fragmentation pattern and GC analysis, the *Biqi* bayberry anthocyanin is thus unambiguously identified as cyanidin 3-glucoside. By comparing with the standard, the HPLC method[15] and the HPLC-DAD-ESIMS[16] method also demonstrated that cyanidin 3-glucoside was the only major anthocyanin in bayberry fruits.

The HPLC method with ESIMS and DAD detection had been used extensively and effectively in anthocyanin identification[11-13]. This method was even used to detect trace levels of anthocyanins in plasma with the limit of detection range of 19-54 nM[22]. The use of a photodiode array detector allows for the analysis of spectral characteristics that give information about the aglycon, acylation and glycosylation patterns[10,19,20]. ESIMS produces primarily intact molecular ions, and sometimes fragmentation can be accomplished by varying ionization conditions[21]. Under the conditions applied in this experiment, clear profiles were obtained, with the presence of intact molecular ion and the aglycon fragmentation of cyanidin 3-hexoside (bottom of Figure 1, and Figure 3). The sugar moiety of bayberry anthocyanin was confirmed as glucose by gas chromatography.

It is possible to find plants in nature with one main

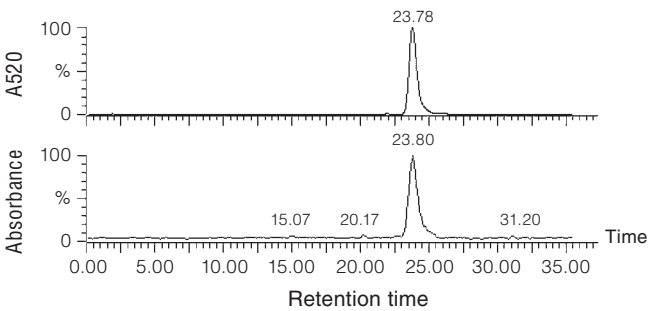

Figure 1. HPLC chromatogram detected at 520 nm (top) and total ion chromatogram in positive model (bottom) of bayberry anthocyanin.

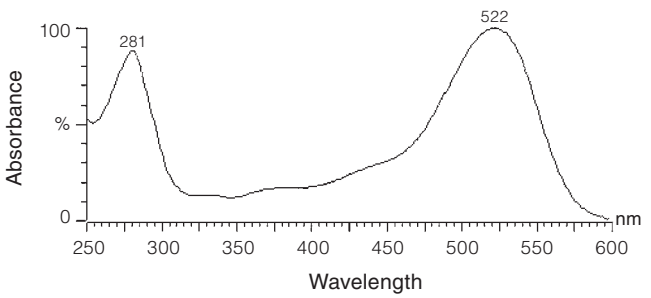

Figure 2. UV-visible absorption spectrum of bayberry anthocyanin.

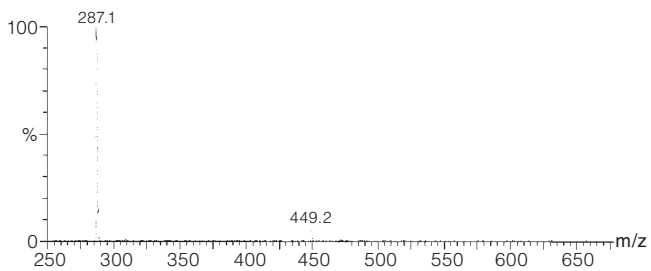

Figure 3. ESI^+-MS fragmentation pattern of cyanidin 3-glucoside from bayberry fruits.

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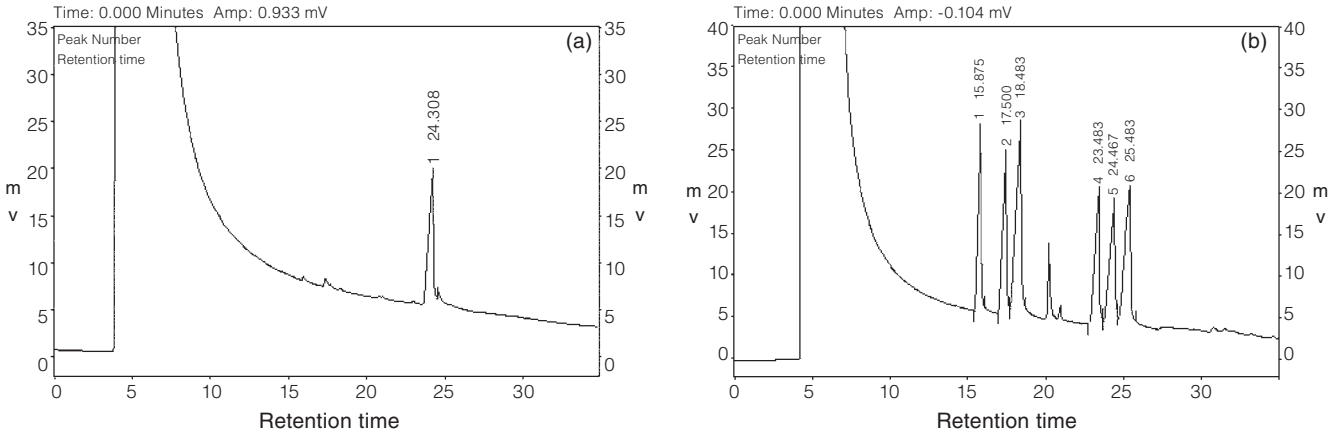

Figure 4. Gas chromatograph of sugar moiety of bayberry anthocyanin (a) and the standard sugars of rhamnose, arabinose, xylose, mannose, glucose, and galactose (b, peak 1-6 ordinally). Peak 1 in Figure (a) was thus identified as glucose.

type of anthocyanin, such as pelargonidin 3-glucoside in *panax ginseng* fruits and cyanidin 3-glucoside in *camellia japonica* flowers, whereas most other plants have mixtures[23]. However, to our knowledge, the percentage of one type of anthocyanin accounted for 97% of the total anthocyanin pigments in any plant has rarely been reported. Thus, the anthocyanin from bayberry fruits, cyanidin 3-glucoside, may be taken as a marker for authenticity control of bayberry products, because adulteration of any other anthocyanin containing materials such as grape juice or plum juice may lead to more complex peaks in the HPLC chromatogram. Moreover, the high simplicity of this pigment in bayberry has a potential as reference compound of cyanidin 3-glucoside in anthocyanin analysis in other plant materials.

When standard chemicals are unavailable, combination of HPLC-DAD-ESIMS and GC is an alternative method for anthocyanin identification. In the present study, the method was successful and effective in bayberry anthocyanin qualitative determination. However, this method has its limitation. For example, the method can identify whether anthocyanins are acylated with cinnamic acids and sometimes the molecular weights of them, but cannot confirm their actual structures because isomeric compounds may exist. In addition, when the sugar moiety of the anthocyanin consisting of disaccharides (e.g. rutinose) or more than two types of monosaccharide substituted at different positions of the flavylium (e.g. 3 and 7 position), the difficulties of identification might also increase. In such case, pure chemicals or other analytical method such as NMR is needed.

CONCLUSIONS

Anthocyanin pigments from bayberry (*Myrica rubra* Sieb. et Zucc., cv. *Biqi*) were investigated by the method of HPLC-DAD-ESIMS and GC. Cyanidin 3-glucoside was the only one major anthocyanin in bayberry fruit. This pigment may be used as a marker for authenticity control of bayberry products and as a reference compound of cyanidin 3-glucoside in anthocyanin analysis. When the standard chemicals are not available, this method is an alternative in identification of anthocyanins if the anthocyanin profile is not highly complex in the studied materials.

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