Anthocyanin Determination in Black Raspberry (*Rubus occidentalis*) and Biological Specimens Using Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry

QINGGUO TIAN, ROBEENA M. AZIZ, GARY D. STONER, AND STEVEN J. SCHWARTZ

ABSTRACT: The capabilities of mass spectrometry for microscale determination of anthocyanins were investigated using high-performance liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) and tandem mass spectrometry (MS-MS). Four anthocyanins [cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-(2^G-xylosylrutinoside) and cyanidin 3-rutinoside] were characterized in black raspberry samples by LC-ESI/MS-MS using both positive and negative ion analyses. Quantification of anthocyanins was conducted using ESI/MS-MS with selected reaction monitoring (SRM). Linear responses of several anthocyanins were determined during MS-MS analyses. Detection limits as low as 1 femtomol for most anthocyanins were obtained during ESI/MS-MS. Compared with other quantitative procedures such as high-performance liquid chromatography (HPLC) and ultraviolet/visible spectrophotometry, the current method provides an improved sensitive, specific technique for direct determination of intact anthocyanins. The developed methodology was successfully applied to analysis of trace levels of anthocyanins in human plasma and epithelial cells.

Keywords: anthocyanins, human plasma, cells, quantification, LC-ESI/MS, MS-MS

Introduction

Anthocyanins represent an important group of secondary plant metabolites responsible for the red, purple, and blue colors of many fruits, vegetables, and flowers. Concentrations of anthocyanins in berries are particularly high (Timberlake and Henry 1988). Freeze-dried black raspberries were reported to contain as high as 1.7% of anthocyanins (Xue and others 2001). More than 500 types of anthocyanins have been identified and structurally elucidated from nature (Harborne and others 1999). The positive therapeutic functions of anthocyanins such as anti-inflammatory (Lietti et al. 1976), radiation-protective (Minkova and others 1990), and antineoplastic effects (Kamei and others 1995), supported by epidemiological studies (Block and others 1992; Ames and others 1993; Willett 1994; Willett 2000), have promoted interest in the determination of these phytochemicals in plants, foods, blood, and tissue samples. Conventionally, ultraviolet/visible (UV-vis) spectrophotometry and high-performance liquid chromatography (HPLC) have been regarded as standard methods for anthocyanin characterization and quantification (Fuleki and Francis 1968a, 1968b, 1968c; Francis 1982; Hong and Wrolstad 1990b). Photodiode-array detection (PDA) provides additional spectral information, facilitating the identification of anthocyanins (Hong and Wrolstad 1990b). However, similarities in UV-vis spectra and co-elution have often made the identification and quantification of individual anthocyanins by HPLC and spectrophotometry difficult. In addition, the determination of anthocyanins at very low concentrations or in small samples, such as cell extracts and tissue biopsies, requires more sensitive analytical techniques for quantitative analyses.

Mass spectrometry and tandem mass spectrometry offer an alternative technique for rapid identification of anthocyanins by providing not only molecular weight but also specific molecular fragmentation information helpful for identifying these compounds. Previous studies have demonstrated the application of mass spectrometry using atmospheric pressure chemical ionization (APCI) (Mullen and others 2002), electrospray ionization (ESI) (Giusti and others 1999; Oliveira and others 2001; Lopes-Da-Silva and others 2002), and matrix-assisted laser desorption/ionization (MALDI) (Sugui and others 1998; Sugui and others 1999; Wang and Sporns 1999) for detection and identification of anthocyanins in plants and foods. However, the quantitative analysis of anthocyanins by mass spectrometry is seldom reported because of the prominent UV-Vis absorption spectra of these compounds allowing for selective detection in the visible light range. Anthocyanin determination in botanical supplements was recently conducted using HPLC in combination with liquid chromatography mass spectrometry (LC-MS) for identification (Chandra and others 2001). MALDI-MS has also been reported to be an advantageous alternative to quantify anthocyanins in red wine and fruit juice because the absorbance of anthocyanins is pH dependent and solvent dependent, which may render variable results in HPLC analysis (Wang and Sporns 1999).

The present study describes the development of a liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS) method for qualitative and quantitative determination of anthocyanins from black raspberry. The linearity of re-

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sponse and detection limit of each anthocyanin were determined during LC-ESI/MS-MS analyses. The developed methodology was applied to the microscale analysis of trace levels of anthocyanins in human plasma and epithelial cell extracts.

Materials and Methods

Chemicals

American Chemical Society (ACS)-grade formic acid, HPLC-grade dichloromethane, methanol, and water were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Anthocyanins including cyanidin 3-arabinoside, cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, and cyaniding 3,5-diglucoside were purchased from Polyphenols Laboratories (Hanaveien, Norway).

Black raspberry extract

Sample preparation and extraction were conducted as described previously (Huang and others 2002). In brief, freeze-dried black raspberries were extracted with 3 volumes of methanol overnight and repeated 3 times. The extract was dried under reduced pressure (< 40 °C) using a rotavapor. An aliquot of the extract was further purified using a silica-gel column and eluted with dichloromethane-methanol (1:1, v/v) and then methanol. The methanol fraction was dried under reduced pressure to remove methanol. The total anthocyanin content in the fraction was 35.58% (w/w) (cyanidin 3-glucoside:cyanidin 3-sambubioside:cyanidin 3-(2^{G}-xylosylrutinoside):cyanidin 3-rutinoside = 9.8:5.9:28.1:56.2).

Human plasma extract

Human plasma was obtained from 3 subjects after consumption of freeze-dried black raspberries at 32 to 40 g/d (equivalent to 0.56 to 0.708 g of anthocyanins/d) for 2 wk in a separate study. The clinical study conducted at Ohio State Univ. is in compliance with the protocol, the Institutional Review Board (IRB), the code of Federal Regulations, and The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use and Good Clinical (ICH/GCP) Guidelines. The anthocyanin composition in the freeze-dried black raspberry was present in a ratio of 15.6:5.7:23.9:54.8 [cyanidin 3-glucoside:cyanidin 3-sambubioside:cyanidin 3-(2^{G}-xylosylrutinoside):cyanidin 3-rutinoside]. To 1 mL of plasma was added 5 mL of methanol and about 4 g of sodium sulfate. The mixture was vortexed for 1 min and centrifuged, and the supernatant was filtered though a 0.45-μm nylon filter (Waters Co., Milford, Mass., U.S.A.). The solution was dried under nitrogen and dissolved in 1 mL of methanol containing 0.1% formic acid for analysis.

Cell extract

Human squamous epithelial cells (ATCC, Manassas, Va., U.S.A.) were cultured for 48 h in a medium consisting of one half Dulbecco's Modified Eagle Medium (DMEM) and one half Ham's F-12 Medium, supplemented with 15 m*M* N-[2-hydroxyethyl] piperazine-N´-[2-ethanesulfonic acid] (HEPES) buffer, 2 m*M* L-glutamine and 10% fetal bovine serum. The cells were then treated for 24 h with 100 μg/mL of methanol fraction of black raspberry extract in the cultured medium for a period of 24 h. After treatment with berry extract, the cells were trypsinized and centrifuged in the culture medium. The medium was decanted and the cells were washed twice with phosphate buffered saline (PBS). Cells were then reconstituted in 1 mL of 100% methanol and freeze-thawed until ruptured. The methanol solution was dried under nitrogen and then dissolved in deionized water and loaded on a preactivated Sep-Pak C_{18} cartridge (Waters Co.). After washing with 6 mL acidified deionized water, anthocyanins were eluted with 6 mL of methanol containing 0.1% formic acid. The methanol eluate was dried under nitrogen and dissolved in 400 μL of methanol containing 0.1% formic acid for subsequent LC-MS analyses.

LC-MS-MS

Black raspberry extracts were dissolved in 90% solvent A (water containing 1% formic acid) and 10% solvent B (acetonitrile containing 1% formic acid). The solution was sonicated for 10 s and filtered through a 0.2-μm nylon filter (Waters Co.). HPLC analysis was performed using a Waters 2695 gradient HPLC separation module equipped with an autoinjector and a 996 PDA UV/visible absorbance detector. Separation was carried out on a Symmetry C_{18} 4.6 × 75 mm (3.5 μm) reversed-phase column (Waters Co.). The solvent system consisted of a step gradient from 90% A to 50% B in 15 min, to 90% A in 5 min, then holding for 5 min to equilibrate the column. Absorption spectra of anthocyanins were recorded from 200 to 800 nm with the in-line PDA detector. The injection volume was 10 μL.

Mass spectrometry was performed on a quadrupole ion tunnel mass spectrometer (Quattro Ultima, Micromass Limited, Manchester, U.K.) equipped with a Z-spray ESI source. Calibration of the mass spectrometer was performed using sodium iodide and caesium iodide. Instrument control and data analysis were accomplished with Masslynx V3.5 software. The eluate from the HPLC column at a flow rate of 0.7 mL/min was directly introduced to the mass spectrometer without solvent splitting. Selected-ion monitoring (SIM) was used to record the abundance of anthocyanin molecular cations. Positive ion ESI conditions for anthocyanin analysis included a capillary voltage of 3.0 kV, cone voltage of 45 V, radio frequency (RF) lens voltage 1 (RF-1) of 50 V, source temperature of 120 °C, and desolvation gas temperature of 500 °C flowing at 16.3 L/min. During collisionally activated dissociation (CAD) experiments, argon at a pressure of 50000 Pascal was used as the collision gas. Collision energy was adjusted so that the abundance of the selected precur-

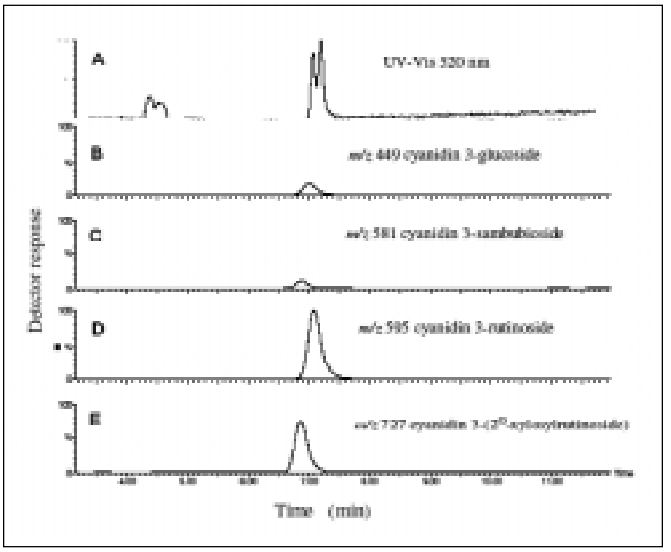

Figure 1—Positive ion electrospray ionization liquid chromatography mass spectrometry (LC-MS) of anthocyanins in a black raspberry extract using selected-ion monitoring (SIM). (a) High-performance liquid chromatography (HPLC) chromatogram (520 nm); (b) cyanidin 3-glucoside; (c) cyanidin 3-sambubioside; (d) cyanidin 3-rutinoside; (e) cyanidin 3-(2^{G}-xylosylrutinoside).

sor ion (molecular cation) was attenuated approximately 50%. Negative ion analysis was conducted at a capillary voltage of –3.0 kV and a cone voltage of –45 V; other parameters were identical to positive ion analysis.

Quantification was carried out using positive ion LC-ESI/MS-MS with selected reaction monitoring (SRM) by measurement of the transition of cyanidin 3-glucoside (*m/z* 449→287), cyanidin 3-sambubioside (*m/z* 581→287), cyanidin 3-rutinoside (*m/z* 595→287), and cyanidin 3-(2^G-xylosylrutinoside) (*m/z* 727→287). Cyanidin 3-arabinoside (*m/z* 419→287) was used as an internal standard during extraction and analysis and cyanidin 3-(2^G-xylosylrutinoside) was expressed as cyanidin 3-glucoside equivalent because an authentic standard of this compound is not commercially available.

Results and Discussion

Anthocyanins in black raspberry

During positive ion LC-ESI/MS, 4 anthocyanins [cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-(2^G-xylosylrutinoside) and cyanidin 3-rutinoside] were detected in black raspberry extracts. Figure 1 shows an example of LC-ESI/MS analysis of a black raspberry extract using selected ion monitoring (SIM). Although only 2 unresolved peaks were observed in the HPLC chromatogram (520 nm) due to the co-elution of anthocyanins (Figure 1a), mass spectrometry analysis indicated the presence of 4 anthocyanins based on the detected molecular cation of each anthocyanin (Figure 1b through 1e). Cyanidin 3-glucoside and cyanidin 3-(2^G-xylosylrutinoside) are reported difficult to separate by HPLC (Hong and Wrolstad 1990a); however, the resolution of all 4 anthocyanins was achieved by mass spectrometry. The identities of the 4 anthocyanins were further confirmed by MS-MS analysis using a fragment ion scan procedure. Compared with LC-MS analysis with SIM that only detects the molecular cations, MS-MS using fragment ion scan provides both molecular weight and fragment ion information (Giusti and others 1999). Abundant aglycone moiety (cyanidin) corresponding to each anthocyanin was produced during MS-MS analyses (Figure 2). In addition, the molecular ion and some structurally important fragment ions produced by loss of 1 sugar from diglycoside and triglycoside anthocyanins were also observed in the current study. Interestingly, the aglycone fragment ion (cyanidin cation) was always observed in a higher abundance than the fragment ions resulted from loss of rhamnose within the spectra of cyanidin 3-rutinoside and cyanidin 3-(2^G-xylosylrutinoside) at different collision energies (10, 15, 20 eV), indicating that the glycosidic bond between cyanidin and glucose was readily fragmented in comparison to that between rhamnose and glucose (Figure 2c, 2d). However, the fragment ion corresponding to loss of xylose did not exist in the CAD spectra of cyanidin 3-sambubioside and cyanidin 3-(2^G-xylosylrutinoside) (Figure 2b, 2d), possibly due to the strong glycosidic bond between xylose and glucose within these 2 anthocyanins. The fragmentation behavior of these anthocyanins during LC-ESI/MS-MS is consistent with a previous report using direct infusion analysis (Giusti and others 1999), and these identified anthocyanins matched previously published data on anthocyanin composition of black raspberry (Torre and Barritt 1977).

For comparison, negative ion LC-ESI/MS and MS-MS were also conducted. Interestingly, loss of 2 protons from molecule $[M - 2H]^-$ corresponding to each anthocyanin was detected rather than the commonly observed deprotonated molecular $[M - H]^-$ or molecular anion (spectra not shown). Moreover, collisionally activated dissociation of $[M - 2H]^-$ of each anthocyanin produced a major fragment ion corresponding to [aglycone - $2H]^-$ at *m/z* 285. The lack of deprotonated molecular ion and molecular anion could be explained by the fact that cyanidin and the corresponding anthocyanins carry a positive charge; the deprotonated molecular or deprotonated aglycone molecular could not be detected because of the neutralization of the charge. However, the unique $[M - 2H]^-$ and [aglycone - $2H]^-$ observed in negative ion analysis may provide additional information for identification of these compounds.

Figure 3 shows the linear response of individual anthocyanins at different concentrations during LC-ESI/MS-MS analysis. Although monoglycoside anthocyanins have been reported to exhibit similar responses in MALDI-MS (Wang and Sporns 1999), cyanidin 3-glucoside was found to be considerable higher in response

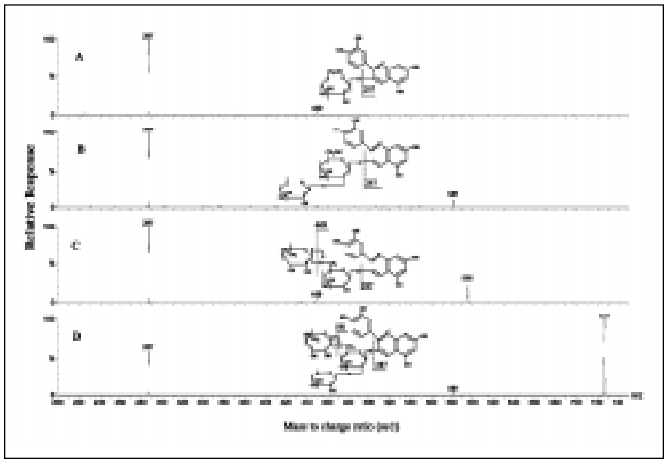

Figure 2—Positive ion liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS) of anthocyanins from black raspberry. (a) Cyanidin 3-glucoside; (b) cyanidin 3-sambubioside; (c) cyanidin 3-rutinoside; (d) cyanidin 3-(2^G-xylosylrutinoside).

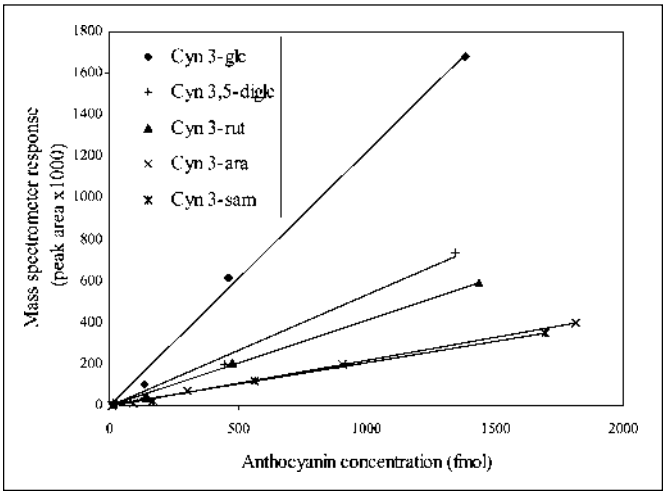

Figure 3—Linear responses of individual anthocyanins at different concentrations during liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS) analysis using selected reaction monitoring (SRM). Cyanidin 3-glucoside (◆), $Y = 1219.2\chi$ ($R^2 = 0.9958$); cyanidin 3,5-diglucoside (+), $Y = 535.18\chi$ ($R^2 = 0.9904$); cyanidin 3-rutinoside (▲), $Y = 411.42\chi$ ($R^2 = 0.99969$); cyanidin 3-arabinoside (×), $Y = 219.48\chi$ ($R^2 = 0.9995$); cyanidin 3-sambubioside (*), $Y = 207.46\chi$ ($R^2 = 0.9985$). *Y* represents peak area and χ represents quantity of anthocyanin (femtomol).

in comparison to the other anthocyanins, and cyanidin 3-arabinoside showed similar response as cyanidin 3-sambubioside during ESI/MS-MS analysis. In addition, the 3 diglycoside anthocyanins with different sugar substitutes also showed different responses. Cyanidin 3, 5-diglucoside was higher in response than cyanidin 3-rutinoside and cyanidin 3-sambubioside, indicating that both glycoside species and degree of substitution may affect the response of anthocyanins during ESI/MS-MS. Linear responses were obtained for most anthocyanins in the range of 8 to about 1500 fmol (femtomol) with a correlation coefficient (R) greater than 0.99. A detection limit (S/N > 3) of approximately 1 fmol (femtomol) was obtained for these anthocyanins during LC-ESI/MS-MS analyses, representing significant improvement over HPLC analysis using UV-Vis that only detects nanomolar levels of anthocyanins (Nielsen and others 2003a).

Human plasma

In contrast to some flavonoid glucosides such as quercetin glycosides absorbed in the aglycone form (Erlund and others 2000; Graefe and others 2001), all 4 anthocyanins were found intact in human plasma. This is consistent with previous reports that most of anthocyanins are found as the intact glycosides in human blood or urine (Wu and others 2002). However, our current study showed that cyanidin 3-rutinoside (9.51, range 6.2 to 12.1 pmol/mL plasma) (SD = 2.4; n = 3) was absorbed in higher proportions compared with their native compositions in black raspberry, indicating cyanidin 3-rutinoside was more readily absorbed than the other 3 anthocyanins. Similar findings were also recently reported in humans and rabbits (Nielsen and others 2003b). Moreover, cyanidin 3-sambubioside (1.6, range 1.31 to 1.97 pmol/mL plasma) (SD = 0.27; n = 3) was also absorbed in higher proportion than cyanidin 3-(2^G-xylosyrutinoside) (0.775, range 0.56 to 1.1 pmol/mL plasma) (SD = 0.22; n = 3) and cyanidin 3-glucoside (0.17, range 0.15 to 0.21 pmol/mL plasma) (SD = 0.025; n = 3). Given this observation, we speculate that diglycoside anthocyanins may be preferentially absorbed and circulated relative to monglycoside and triglycoside anthocyanins in human blood.

Cell extract

Similar to human plasma, all 4 anthocyanins were found in epithelial cells after incubation of a black raspberry extract (Figure 4). Cyanidin 3-rutinoside (1.1 pmol/5.0×10^7 cells) was detected as the predominant compound, followed by cyanidin 3-glucoside (0.225 pmol/5.0×10^7 cells) and cyanidin 3-sambubioside (0.07 pmol/5.0×10^7 cells). However, very low concentration of cyanidin 3-(2^G-xylosylrutinoside) was found in the cell extract although this anthocyanin is the 2nd most abundant compound in the methanol fraction. The higher proportions of cyanidin 3-rutinoside and cyanidin 3-glucoside absorbed in the cells indicated that selective absorption and discrimination of anthocyanins may be occurring based on the number and type of sugar substituents (McGhie and others 2003).

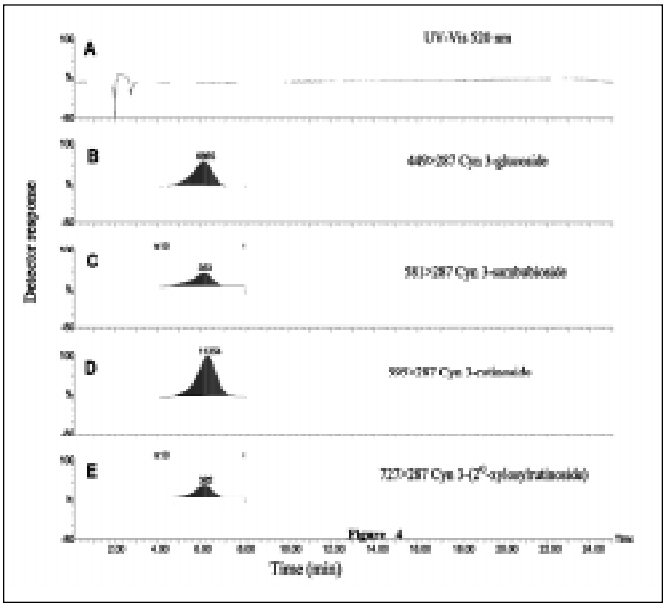

Figure 4—Positive ion liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS) determination of anthocyanins in human epithelial cells using selected reaction monitoring (SRM). (a) High-performance liquid chromatography (HPLC) chromatogram (520 nm); (b) cyanidin 3-glucoside (*m/z* 449→287); (b) cyanidin 3-sambubioside (*m/z* 581→287); (c) cyanidin 3-rutinoside (*m/z* 595→287); (d) cyanidin 3-(2^G-xylosylrutinoside) (*m/z* 727→287). The number on top of each peak represents the peak area.

Conclusions

In conclusion, electrospray ionization LC-ESI/MS-MS represents a useful tool for identification and quantification of anthocyanins in comparison to UV-Vis spectrophotometry and HPLC using absorption detection. The highly sensitive and selective LC-ESI/MS-MS technique allows for rapid quantification of individual anthocyanins in complex matrices. This technique was successfully applied to the determination of anthocyanins in cells and in human plasma of limited sample size and represents a useful tool for absorption and bioavailability studies of anthocyanins.

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