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Determination of Anthocyanins and Total Polyphenols in a Variety of Elderberry Juices by UPLC-MS/MS and Other Methods

H. Wu[1,2,5], **M.C. Johnson**[1,2], **C.-H. Lu**[2,3], **K.L. Fritsche**[2,3], **A.L. Thomas**[2,4], **Z. Cai**[5], and **C.M. Greenlief**[1,2]

[1] Department of Chemistry, University of Missouri, Columbia, MO, USA

[2] Center for Botanical Interaction Studies, University of Missouri, Columbia, MO, USA

[3] Division of Animal Sciences, University of Missouri, Columbia, MO, USA

[4] Southwest Research Center, University of Missouri, Columbia, MO, USA

[5] Department of Chemistry, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China

Abstract

Elderberry (*Sambucus* spp.) juice contains a variety of polyphenols, mostly anthocyanins. In order to understand the variation of polyphenol levels by genotype, various elderberry juice samples were analyzed for total phenolics (TP), total monomeric anthocyanins (TMA) and individual anthocyanin content. The Folin-Ciocalteu total phenolic method and pH differential method were used to measure the TP and TMA content, respectively. The TP and TMA concentrations of elderberry were found to vary greatly among different genotypes. TMA content varied from 2.1% for ‘Sperandio’ to 60.6% for the ‘Bob Gordon’ cultivar. In addition, ultra-performance liquid chromatography with triple quadrupole mass spectrometry was used to separate and detect individual anthocyanins from samples prepared by solid phase extraction. Multiple-reaction-monitoring was used to process data for the reduction of false positives, maximizing selectivity, and reliable quantification. The quantitative performance of the method was validated, and a detection limit of 0.3 ng·ml^{-1} for cyanidin 3-*O*-glucoside was determined. This newly developed method may serve to characterize and profile various anthocyanins in elderberry juices for quality control, assessment of dietary intake, and anthocyanin-based biomedical studies.

Keywords

anthocyanidins; electrospray; solid phase extraction; Folin and Ciocalteu

INTRODUCTION

Elderberry (*Sambucus* spp.) is a widespread genus that is native or naturalized in many parts of Europe, Asia, North Africa, and North America. Elderberry products are consumed as dietary supplements for their potential health benefits (Netzel et al., 2005). Researchers have linked elderberry products to anti-influenza (Roschek et al., 2009), anti-oxidant, anti-carcinogenic, anti-viral, and antibacterial activities (Werlein et al., 2005; Milbury et al., 2002; Tarascou et al., 2011). Elderberry products are also suggested to manifest an array of health promoting benefits such as oxidative stress protection, anti-inflammatory effects and

inhibition of some human tumor cells (Rodrigo et al., 2011; Kong et al., 2003; Elisia et al., 2007; Bagchi et al., 2006; Dreiseitel et al., 2008). Anthocyanins, among other polyphenols, have been hypothesized to be responsible for these effects based on their ability to quench free radicals. They are also responsible for the rich colors observed in plants and fruits (Zhang et al., 2005).

Recent effort in elderberry cultivation research has led to the development of promising new genotypes and cultivars. Quantification methods for total phenolics (TP) and anthocyanin concentrations from elderberry have been utilized to analyze the quality of different types of fruit. Elderberry juice characteristics have been shown to vary with genotype, production site, environmental conditions, and cultural practices (Finn et al., 2008; Özgen et al., 2010; Thomas et al., 2013). However, the underlying causes of the variance in anthocyanin content are still unknown. In this study we utilized high-throughput UV-Vis spectrophotometric methods to understand the effects of genotype and year-to-year environmental variation on TP and total monomeric anthocyanins (TMA) concentration of elderberry juice.

Another goal of this research was to develop a sensitive method to analyze individual anthocyanins in a complex matrix. Recently solid phase extraction (SPE) methods have gained popularity for their efficiency (Vallas et al., 2009; Hurtado-Fernandez et al., 2010; Diaz-Garcia et al., 2013) including cation-exchange, C18, HLB, and LH-20 (Lin and Tang, 2008; He and Giusti, 2011; Cavalcanti et al., 2011; Wu et al., 2006). Therefore we combined a mixed-mode cation exchange SPE column method with an ultra-performance liquid chromatography electrospray ionization mass spectrometry (UPLC-ESI-MS) method to advance the current analytical capacity. In this study we developed, optimized and verified a UPLC-MS/MS method for anthocyanin profiling and quantification for future biomedical studies.

MATERIALS AND METHODS

Elderberry samples were harvested from two Missouri (USA) growing locations (Mt. Vernon and Jefferson City) during 2009-2011. Nine elderberry genotypes were included: ‘Bob Gordon’, ‘Dallas’, ‘Marge’, ‘Ocoee’, ‘Ozark’, ‘Ozone’, ‘Sperandio’, ‘Wyldewood’, and ‘York’. All genotypes are American elderberry [*Sambucus nigra* L. subsp. *canadensis* (L.) Bolli] except for ‘Marge’, which is of European origin (*Sambucus nigra* L. subsp. *nigra*). Each site included four randomized field replications (plots) of each genotype. Details on genotypes used and field production methods (sites, soils, climate, planting, management, harvesting, etc.) can be found in Thomas et al. (2015a,b). Immediately upon harvest, fruit was placed into zippered plastic freezer bags and frozen (−20°C). Samples were later de-stemmed, thawed, juiced, filtered through a 0.45 μm nylon filter, and re-frozen (−20°C) before analysis. TP and TMA content of the juice were measured in March 2013 and May 2013, respectively.

Juice samples for TP and TMA were analyzed from either 3 or 4 replicated field plots per genotype, with each sample quantified in triplicate (n = 9 or 12). A two-variable t-test, at the 95% confidence level, was performed on the data.

Chemicals

Methanol, formic acid, ammonium hydroxide, sodium carbonate, potassium chloride, acetonitrile, and water were purchased from Fisher Scientific (HPLC grade, Fair Lawn, NJ, USA). The cyanidin 3-*O*-glucoside, gallic acid, Folin-Ciocalteu phenol reagent (FCR), and sodium acetate trihydrate were purchased from Sigma (Saint Louis, MO, USA). Oasis (MCX) mixed-mode reversed-phase/strong cation exchange SPE cartridges (3 cc/60 mg) were purchased from Waters (Milford, MA, USA).

Total Phenolic Testing

The Folin-Ciocalteu TP assay was followed with slight modifications (Singleton and Rossi, 1965). Samples were equilibrated for two hours at room temperature as opposed to heating. Gallic acid standards were prepared and treated in the same manner as the samples. Absorbance values were measured in triplicate at λ of 760 nm using a PerkinElmer Enspire 2300 multimode plate reader. TP concentration is expressed as gallic acid equivalents (GAE $mg{\cdot}ml^{-1}$).

Total Monomeric Anthocyanin

The pH differential method (Giusti and Wrolstad, 2001) was used to estimate the TMA content of elderberry juice. Absorbance values were measured in triplicate at λ of 520 nm and 700 nm. Cyanidin 3-*O*-glucoside standards were prepared and treated in the same manner as the samples. Total monomeric anthocyanin values are represented as cyanidin 3-*O*-glucoside (C3G) equivalents (C3GE $mg{\cdot}ml^{-1}$).

Solid Phase Extraction (SPE)

A MCX cation-exchange SPE separation method (He and Giusti, 2011) was used for anthocyanin separation from other matrix compounds. The cartridge was first washed with 6 ml of acidified water (0.1% formic acid), after 100 μl of elderberry juice was added to the SPE cartridge. The fraction of other phenols was collected by elution with 6 ml of acidified methanol (0.1% formic acid). Subsequently, the anthocyanins were eluted with 2 ml of methanol and 2 ml of water/methanol (40:60, v/v), both containing 1% NH_4OH. Immediately, 250 μl of formic acid was added to the combined alkaline eluent. Samples were dried in a Buchi rotary evaporator (35°C) and the fractions were re-dissolved in acidified water.

UPLC-MS/MS analysis

Separation of anthocyanins was achieved on a C18 column (Acquity BEH, 1.7 μm, 50 × 2.1 mm, Waters, Milford, MA, USA) at room temperature (~20°C) using a Waters Xevo TQ-S UPLC-MS/MS system. The mobile phase included 4.5% formic acid in LCMS grade water (mobile phase A) and 0.1% formic acid in HPLC grade acetonitrile (mobile phase B). The flow rate was 400 $\mu l{\cdot}min^{-1}$. The gradient started at 95% mobile phase A and was lowered to 5% mobile phase A over 4 min, followed by a 30 s isocratic step and a 30 s re-equilibration in 95% mobile phase A.

The following conditions were used for the electrospray ionization (ESI) source: source temperature 150°C, desolvation temperature 350°C, capillary voltage 2.0 kV, cone voltage 12 V, and nebulizer gas 500 $L \cdot h^{-1}$ N_2. Argon was used as the collision gas. The collision energies were optimized and ranged from 10 to 40 eV for individual analytes. The ESI source was operated in the positive ion mode. Instrument control and data processing were performed by using MassLynx software (version 4.1, Waters, Milford, MA, USA). Cyanidin 3-*O*-glucoside standard solutions were prepared with concentrations ranging from 1 $ng \cdot ml^{-1}$ to 1 $\mu g \cdot ml^{-1}$ in methanol. Analyte identity was determined based on retention time and mass spectra, MS/MS spectra, and quantitation was based on analyte to C3G area ratios.

RESULTS AND DISCUSSION

Total Phenolics and Total Monomeric Anthocyanins

Phosphomolybdenum complexes in a 5+ oxidation state are present in the FCR stock solution. These complexes are reduced to a 4+ oxidation state in the presence of polyphenols. This oxidation state has a maximum absorption wavelength of 760 nm; at this wavelength few optical interferences exist. Therefore, by adding the FCR in excess to elderberry juice, one can estimate the TP content.

Elderberry juice has been shown to contain a variety of anthocyanins. Although the molecules vary based on the number and type of sugar moieties attached, their absorption spectra are very similar. Most anthocyanins exhibit a maximum wavelength of absorption ranging from 500-530 nm (Giusti and Wrolstad, 2001); and deviations among samples in this range are negligible due to their broad maximum peak. The TMA content of juice samples can be estimated by taking absorbance measurements of juice samples at 520 nm, which is the maximum wavelength of absorption of the cyanidin 3-*O*-glucoside standard in the solvent used.

Table 1 shows that the TP content of elderberry juice varies highly among genotypes grown at the same location and growing season (Jefferson City, MO, 2011, in this case). The juice of the ‘Wyldewood’ cultivar contained the highest TP content (8.59 $mg \cdot ml^{-1}$ GAE), and the juice of ‘Ocoee’ the lowest (2.16 $mg \cdot ml^{-1}$ GAE). Five of the nine genotypes evaluated (‘Wyldewood’, ‘Bob Gordon’, ‘Ozark’, ‘Ozone’, and ‘Ocoee’) had TP levels (in descending order) that were statistically different from each other, underscoring the influence of genetics on these polyphenol levels.

TMA content of elderberry also varied highly among samples. The ‘Ozark’ and ‘Sperandio’ genotypes displayed the highest and lowest concentrations with 5.25 and 0.09 $mg \cdot ml^{-1}$ C3GE, respectively (Table 1). For the TMA content, the genotypes can be divided into four statistically equivalent groups, in descending order: (1) ‘Ozark’, ‘Wyldewood’, and ‘Bob Gordon’; (2) ‘Marge’ and ‘Ozone’; (3) ‘Dallas’, ‘Ocoee’, and ‘York’; and (4) ‘Sperandio’. Each group is statistically different from the other groups. It is interesting that the polyphenol content does not necessarily correlate with anthocyanin content. Certain genotypes possess copious amounts of anthocyanins, while others have other polyphenols largely contributing to their TP profile.

Growing locations and growing seasons were examined for their impact on TP and TMA content of elderberry juice (Table 2). Our preliminary data suggest that the TP and TMA content of juice of the same genotype can vary significantly from year-to-year and site-to-site, likely due to variations in environmental factors. A larger study involving different genotypes, growing location and season is currently underway utilizing the methods described here.

UPLC Optimization

The UPLC conditions were optimized to separate individual anthocyanins. Optimal separation was achieved with methanol as an organic phase and 10 mM formic acid as an aqueous phase. Formic acid also helped to minimize chromatographic peak tailing. However, the ionization efficiency and limits of detection were slightly reduced with its presence (1-2% of a given ion signal). The combination of methanol and water balanced with formic acid has previously been used to analyze anthocyanin (He and Giusti, 2011). Anthocyanin separation is optimum in an acidic environment at a pH of less than 2.

Profiling and Quantification of Anthocyanins

The total ion response of individual anthocyanin mass pairs is related to the concentration of that anthocyanin present in an elderberry juice sample according to our observations using C3G. In addition, the use of multiple reaction monitoring increases the robustness of this model because up to thirty parent-daughter mass pairs can be analyzed simultaneously.

Ion chromatograms for a number of MS/MS transitions are shown in Figure 1. From the ion chromatograms, we were able to quantify several anthocyanins and anthocyanidins from an elderberry juice sample (Table 3). The column labeled $[M\text{-}H]^+$ in Table 3 corresponds to the mass number on the right side of Figure 1 for its ion chromatogram. The ion chromatograms are presented in the same order as the anthocyanins listed in Table 3. Based on the 18 individual anthocyanins we screened, we found the cyanidin based anthocyanins had the highest concentrations, specifically cyanidin 3-*O*-sambubioside, cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside, cyanidin 3-*O*-sambubioside, and cyanidin 3-*O*-coumaroyl-sambubioside. This is consistent with previous elderberry anthocyanin studies analyzed by other methods (Lee and Finn, 2007). Also, delphinidin, peonidin, and pelargonidin-based anthocyanins were detected, while malvidin and petunidin-based anthocyanins were not. Previous studies have reported that the majority of anthocyanins in American and European elderberry are cyanidin-based anthocyanin, with traces of delphinidin-based anthocyanin, peonidin-based anthocyanin, and pelargonidin-based anthocyanin (Lee and Finn, 2007; Veberic et al., 2009). Our results are consistent with these previous studies and demonstrate that this method could be used in a broader study examining a number of genotypes, growing seasons, and location.

CONCLUSION

The TP and TMA contents of elderberry juice were shown to vary based on genotype, growing location, and growing season, consistent with previous literature (Finn et al., 2008; Özgen et al., 2010; Thomas et al., 2013). Solid phase extraction is an effective method to

adequately separate anthocyanin compounds. UPLC-MS/MS detection is an extremely robust and efficient method for quantifying individual anthocyanin compounds in elderberry juice. Cyanidin was determined to be the most abundant anthocyanin in several elderberry juice samples. The method we developed could be used in various future studies including genotype authenticity, environmental impacts, and biomedical research.

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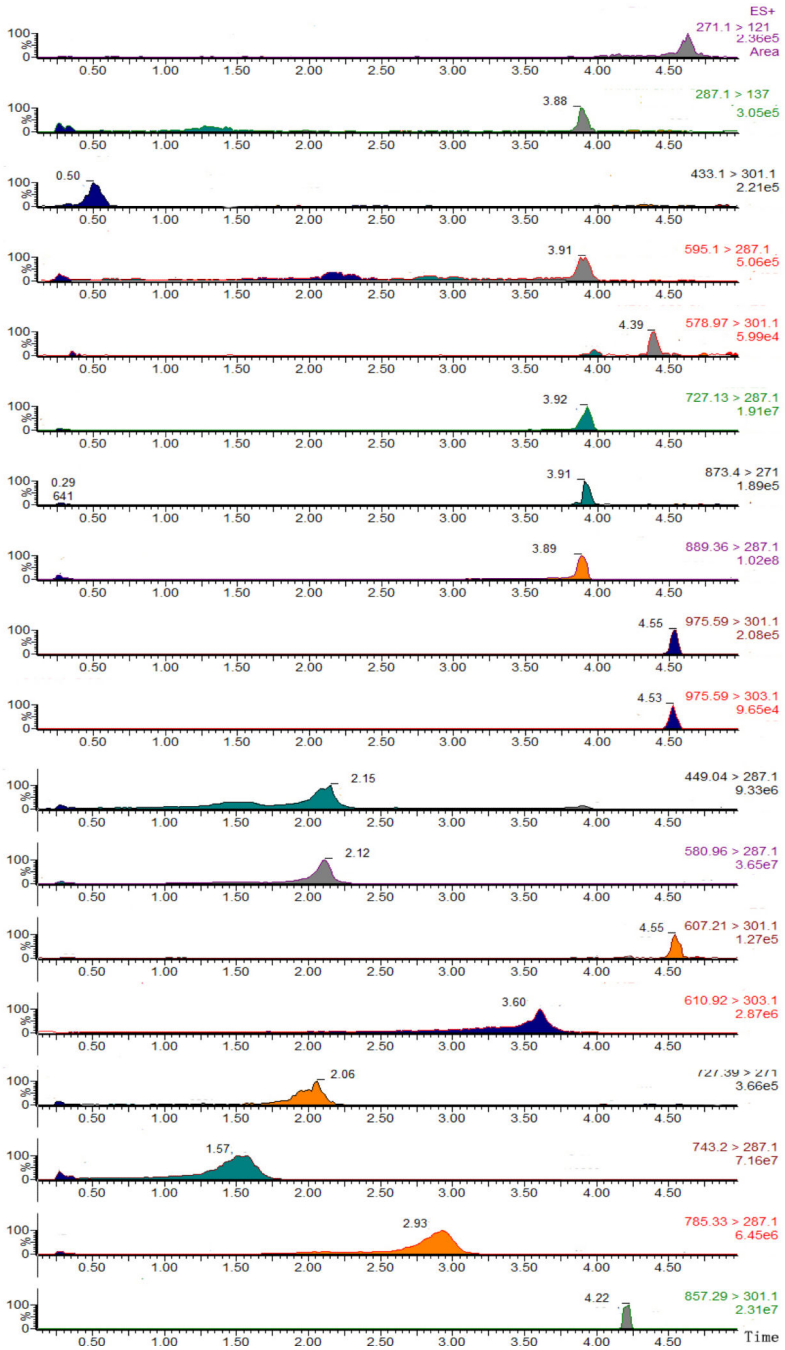

Fig. 1.

Several individual anthocyanin chromatograms observed in elderberry juice utilizing multiple reaction monitoring. Table 3 contains the names of the anthocyanins for a given $[M\text{-}H]^+ \rightarrow MS^n$ transition. The names/transitions are listed in Table 3 in the order of top to bottom within the figure.

Table 1

Total phenolics (TP) and total monomeric anthocyanins (TMA) content of elderberry juice samples of different genotypes harvested from the same growing location and year (Jefferson City, MO, 2011).

Genotype	*n*	TP (mg·ml^{-1} GAE ± SE)	TMA (mg·ml^{-1} C3GE ± SE)	Percent anthocyanin
Bob Gordon	12	7.21 ± 0.11	4.37 ± 0.56	60.6
Dallas	12	4.89 ± 0.28	0.75 ± 0.07	15.3
Marge	12	3.14 ± 0.55	1.31 ± 0.14	41.7
Ocoee	12	2.16 ± 0.23	0.68 ± 0.11	31.5
Ozark	12	6.10 ± 0.05	5.25 ± 0.70	86.1
Ozone	9	5.62 ± 0.06	1.15 ± 0.04	20.5
Sperandio	9	4.30 ± 0.79	0.09 ± 0.01	2.1
Wyldewood	12	8.59 ± 1.02	4.67 ± 0.44	54.4
York	12	3.98 ± 1.19	0.64 ± 0.06	16.1

Abbreviations: GAE = gallic acid equivalents; C3GE = cyanidin 3-*O*-glucoside equivalents; SE = standard error.

Table 2

Total phenolics (TP) and total monomeric anthocyanins (TMA) content of elderberry juice of the 'Bob Gordon' genotype at different locations and during different seasons.

Fruit harvest year and location	*n*	TP (mg·ml^{-1} GAE ± SE)	TMA (mg·ml^{-1} C3GE ± SE)	Percent anthocyanin
2009 Mt. Vernon	12	2.57 ± 0.41	0.19 ± 0.03	7.4
2010 Mt. Vernon	12	7.13 ± 0.14	3.40 ± 0.49	47.6
2010 Jeff. City	9	4.31 ± 0.52	2.04 ± 0.31	47.3

Abbreviations: GAE = gallic acid equivalents; C3GE = cyanidin 3-*O*-glucoside equivalents; SE = standard error.

Table 3

Parent-daughter ion masses, retention times and concentrations of individual anthocyanins and anthocyanidins present in a sample of 'Wyldewood' elderberry harvested in 2011.

Anthocyanin	$[M-H]^+$ (Da)	MS^n (Da)	Retention time (min)	Collision energy (eV)	Concentration ($ng \cdot ml^{-1}$ C3GE)
Delphinidin	271.1	121.0	4.65	30	ND
Cyanidin	287.1	137.1	3.88	20	28.8
Peonidin 3-*O*-arabinoside	433.1	301.1	0.50	20	16.2
Cyanidin based anthocyanin-1	595.1	287.1	3.91	30	ND
Peonidin based anthocyanin-1	579.0	301.1	4.39	40	ND
Cyanidin 3-*O*-coumaroyl-sambubioside	727.4	287.1	3.92	40	1190.2
Pelargonidin based anthocyanin-1	873.4	271.0	3.91	35	ND
Cyanidin 3-*O*-coumaroyl-sambubioside-5-glucoside	889.4	287.1	3.92	35	3649.2
Peonidin based anthocyanin-2	975.6	301.1	4.55	35	ND
Delphinidin based anthocyanin-1	975.6	303.1	4.53	40	ND
Cyanidin 3-*O*-glucoside	449.1	287.1	2.15	20	107.0
Cyanidin 3-*O*-sambubioside	581.0	287.1	2.12	30	2509.2
Peonidin based anthocyanin-3	607.2	301.1	4.55	40	ND
Delphinidin 3-*O*-rutinoside	610.9	303.1	3.60	40	651.6
Pelargondin based anthocyanin-2	727.4	271.0	2.06	35	13.9
Cyanidin 3-sambubioside-5-glucoside	743.2	287.1	1.57	20	8192.7
Cyanidin based anthocyanin-2	785.3	287.1	2.93	35	651.6
Peonidin based anthocyanin-2	857.3	301.1	4.22	35	411.2

Abbreviations: $[M-H]^+$ = parent ion mass; MS^n (Da) = daughter ion mass; ND = not detected.