Plant Science 262 (2017) 91–102

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Characterization and quantification of flavonoids and organic acids over fruit development in American cranberry (*Vaccinium macrocarpon*) cultivars using HPLC and APCI-MS/MS

Yifei Wang[a], Jennifer Johnson-Cicalese[b], Ajay P. Singh[a], Nicholi Vorsa[a,b,*]

[a] Department of Plant Biology, Rutgers University, New Brunswick, NJ, USA
[b] Philip E. Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers University, Chatsworth, NJ, USA

ARTICLE INFO

Keywords:
Cranberry
Anthocyanins
Flavonols
Proanthocyanidins
Organic acids
Fruit development

ABSTRACT

Cranberry flavonoids, including anthocyanins, flavonol glycosides and proanthocyanidins, and organic acids were characterized and quantified by HPLC and LC–MS/MS during fruit development and ripening in eight cranberry cultivars. Anthocyanin biosynthesis initiated at early fruit development and reached highest level in mature fruit, with significant differences between cultivars. Major flavonol glycosides, including the most abundant quercetin-3-galactoside and myricetin-3-galactoside, showed consistent concentrations during the season with moderate fluctuation, and were at similar levels in mature fruits of the eight cultivars. Proanthocyanidins declined during fruit development and then increased slightly in later maturation stages. Levels of various proanthocyanidin oligomers/polymers with different degree-of-polymerization were highly correlated within a cultivar during fruit development. Cultivars with coancestry exhibited similar levels (high/low) of anthocyanins or proanthocyanidins, indicating genetic effects on biosynthesis of such flavonoids. All cultivars showed similar levels of malic and citric acids, and declining levels of quinic acid during fruit development. Benzoic acid was extremely low early in the season and increased sharply during fruit ripening. Levels of quinic and citric acids were significantly different among cultivars in the mature fruit. Concentrations of proanthocyanidins, anthocyanins, quinic acid and benzoic acid have a strong developmental association in developing ovaries.

1. Introduction

American cranberry (*Vaccinium macrocarpon*) has long been recognized as one of the leading plant sources of bioactive secondary metabolites with important implications to human health. Among cranberry secondary metabolites, phenolic compounds have received most of the research interest due to their high content in cranberry and well-proven health benefits. Cranberry phenolics are comprised of flavonoids, including flavonols, anthocyanins and flavan-3-ols (proanthocyanidins), as well as phenolic acids [1]. Besides phenolics, cranberry also contains various non-phenolic organic acids such as quinic, citric and malic acids, and small amounts of benzoic and glucuronic acids [2].

Cranberry anthocyanins and flavonols mainly appear as monomeric glycosides conjugated to a number of different sugar moieties [3,4]. Cranberry proanthocyanidins (PACs), on the other hand, are oligomers or polymers of flavan-3-ols. While the most common linkage between PAC building blocks is C—C bond (B-type) [5], in cranberry and few other plant species, a C—O—C bond also occurs and forms a double linkage (A-type) with the C—C bond [6], making them structurally unique compared with B-type PACs found in blueberry, grape and cocoa. Both flavonols and PACs have shown various bioactivities, such as anti-oxidant, anti-cancer and anti-inflammatory activities, as well as cardiovascular health benefits [7–13].

Organic acids are important components in cranberry fruits and contribute to their characteristic flavor. Besides being employed to determine fruit quality such as maturity and flavor [14,15], they also have been widely used as food additives in juice and beverage products to improve nutrition and preservation stability [16]. Compositions of flavonoids and organic acids in cranberry fruits can be influenced by multiple factors such as cultivar, maturity and growth conditions. Although early studies revealed composition patterns of different phytochemicals in cranberry fruit and other species [15,17–19], diversity of plant materials and analyzed compounds were limited. Comprehensive studies with extensive sampling and analytical approaches are needed for a better understanding of how multiple factors, including genetic

* Corresponding author at: Department of Plant Biology, Rutgers University, New Brunswick, NJ, USA.
E-mail address: vorsa@aesop.rutgers.edu (N. Vorsa).

http://dx.doi.org/10.1016/j.plantsci.2017.06.004
Received 11 April 2017; Received in revised form 16 May 2017; Accepted 6 June 2017
Available online 08 June 2017
0168-9452/

background, growth stage, and biosynthetic pathway, can affect phytochemical profiles of cranberry.

In the present study, we investigated the concentrations of four major classes of cranberry phytochemicals – anthocyanins, flavonols, PACs and organic acids, during fruit development and ripening in eight cranberry cultivars representing diverse genetic backgrounds. HPLC and LC–MS/MS methods have been developed and optimized to identify individual cranberry phytochemicals and provide accurate quantitative analysis. The objectives were to determine: (1) whether flavonoid and organic acid constituents and their levels are a function of fruit development stages, (2) if there is evidence for genetic variation, and (3) which cultivars have higher levels of phytochemical constituents.

2. Materials and methods

2.1. Plant material

The eight cranberry cultivars used were: selections domesticated from endemic cranberry populations – Early Black (EB) selected in 1852, Howes (HO) selected in 1843, and Ben Lear (BL) selected in 1910; 1st breeding cycle cultivars Stevens (ST) and #35, developed in 1940s; and 2nd breeding cycle cultivars Crimson Queen (CQ), Demoranville (DM) and Mullica Queen (MQ), released post 2000. Their genetic relationship and coancestry is summarized in Fig. 1. The cultivar trial was established at Philip E. Marucci Center for Blueberry and Cranberry Research and Extension in May 2010; plots were in a randomized complete block design (RCBD) with 3 replications (blocks). Fruit samples were harvested from each plot at 7–17 day intervals over 8 dates in 2014, initiating at mid to late fruit set on Jul 21 through fruit maturity on Oct 6. Sampling dates were Jul 21 (Julian Day (JD) 202), 28 (JD 209); Aug 7 (JD 219), 18 (JD 230), 28 (JD 240); Sep 8 (JD 251), 19 (JD 262); and Oct 6 (JD 279). Samples were placed in a −20 °C freezer within 1 h of collection and remained there until analysis. For phytochemical analysis, samples of each replicate (block) were analyzed as a set (8 dates × 8 varieties = 64 samples/set). Note: replicate variation includes laboratory experimental variation of the set.

2.2. Reagents

All solvents were purchased from EMD Millipore (Billercia, MA). Acetic acid was purchased from Avantor Performance Materials (Center Valley, PA), formic acid was purchased from Mallinckrodt Baker (Phillipsburg, NJ) and phosphoric acid was purchased from Amresco (Solon, OH). Sephadex® LH-20 was obtained from GE Healthcare Bio-Science (Piscataway, NJ) and BAKERBOUND® Diol was obtained from Avantor Performance Materials.

The flavonoid standards, including the anthocyanin cyanidin-3-galactoside, and flavonols quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside and quercetin aglycone were purchased from Indofine Chemical Company (Somerville, NJ). Other cranberry flavonol standards were isolated using a semi-prep HPLC system described by Singh et al. [20]. Citric acid, malic acid, quinic acid and benzoic acid standards were obtained from Sigma (St. Louis, MO). Individual PAC standards were isolated using Sephadex® LH-20 and BAKERBOUND® Diol gravity column chromatography [20].

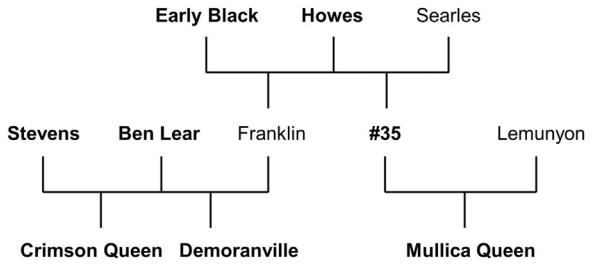

Fig. 1. Pedigree chart of cranberry cultivars. Cultivars in **bold font** were analyzed.

2.3. Extraction of cranberry flavonoids and organic acids

For flavonoid quantification, about 10 g of fruits were weighed and crushed in 40 ml 80% aqueous acetone with 0.1% acetic acid in laboratory blender for 1 min, followed by 30 min sonication and filtration with filter paper. Liquid extracts were dried in rotary evaporator under high vacuum and 35 °C water bath and re-dissolved in methanol to a final volume of 6 ml. Samples were filtered through 0.45 μm Spin-X® centrifuge filter tube before analysis. For flavonoid characterization and isolation, acetone extracts were further partitioned with n-hexane and ethyl acetate and pre-purified in Sephadex® LH-20 column as previously described [20].

For organic acid identification and quantification, about 3 g of fruit were weighed and crushed in 30 ml distilled water in a blender for 1 min and heated in 90 °C water bath for 10 min. After filtration through filter paper, aqueous extracts were collected for HPLC analysis.

2.4. HPLC apparatus and conditions

Cranberry flavonols were analyzed in Dionex UltiMate® 3000 LC system. A Gemini® 150 × 4.6 mm C18 110 Å, 5 μm LC column was used and flavonols were detected at 366 nm. Cranberry PACs were analyzed in Waters Alliance® LC system. A Develosil® 250 × 4.6 mm 100Diol-5, 5 μm LC column was used and PACs were detected in both PDA detector at 280 nm and fluorescence detector with excitation/emission wavelengths at 280/308 nm. Organic acids were analyzed in a Dionex® HPLC system with AS50 Autosampler, AS50 Thermal Compartment, PDA-100 Detector and GP40 Gradient Pump. A Waters Atlantis® 250 × 4.6 mm dC18, 5 μm LC column was used and organic acids were detected at 210–230 nm in PDA detector. All solvent systems and elution gradients are summarized in Table 1.

2.5. MS spectrometry

An Applied Biosystems API 3000™ triple-quad LC–MS/MS mass spectrometer coupled with the Dionex UltiMate® 3000 LC system was used in LC/MS-MS analysis. MS data was obtained under atmospheric pressure chemical ionization (APCI) in negative ion detection mode, with following parameters: Curtain gas: 12 psi, Nebulizer gas: 7 psi, Nebulizer current: −2.0 mA, Entrance potential: −10 V, Focusing potential: −300 V, Declustering potential: −60 V, Collision energy: −50 V, Collision cell exit potential: −5.0 V, Source temperature: 500 °C. The same LC methods were used for flavonol and PAC separation.

2.6. Characterization and quantification of flavonols, proanthocyanidins and organic acid

Characterization of specific compounds was carried out by comparing their HPLC peak retention time, UV–vis absorbance spectra, fluorescence spectra and/or MS data with those of standards. Due to the detection limit of APCI-MS, only PAC oligomers with degree-of-polymerization less than 8 can be identified by mass spectra. PAC polymers greater than heptamers were identified by comparing their HPLC peak retention time patterns with previously published data [21]. For compound quantification, HPLC standard curves were first prepared using purchased or isolated standards. Flavonols, PACs (oligomers and polymers) and organic acid were then quantified by calculating peak area corresponding to relevant standard curves.

2.7. Quantification of total monomeric anthocyanins

Anthocyanins were quantified using a pH differential spectrophotometric method [22] with slight modification. Cranberry flavonoid