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Steam-Blanched Highbush Blueberry (*Vaccinium corymbosum* L.) Juice: Phenolic Profile and Antioxidant Capacity in Relation to Cultivar Selection

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High-quality standards in blueberry juice can be obtained only taking into account fruit compositional variability and its preservation along the processing chain. In this work, five highbush blueberry cultivars from the same environmental growing conditions were individually processed into juice after an initial blanching step and the influence was studied of the cultivar on juice phenolic content, distribution and relative antioxidant activity, measured as scavenging capacity on the artificial free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). A chromatographic protocol was developed to separate all main phenolic compounds in berries. A total of 15 glycosylated anthocyanins, catechin, galactoside, glucoside, and rhamnoside quercetin 3-derivatives, and main benzoic and cinnamic acids were identified. The total content and relative distribution in anthocyanins, chlorogenic acid, and quercetin of each juice were dependent upon cultivar, and the total content was highly correlated (rxy = 0.97) to the antioxidant capacity. A selective protective effect of berry blanching in juice processing can be observed on more labile anthocyanin compounds.

KEYWORDS: ***Vaccinium corymbosum* L.; blueberry juice; blanching; cultivar; antioxidant capacity; anthocyanin; phenolics; HPLC; DPPH•**

INTRODUCTION

Blueberry processing into juice is an important tool not only to increase berries commercial life but also to enrich the diet with healthy compounds. Scientific evidence, supported by epidemiologic studies, has emerged about the influence of diet on chronic degenerative diseases. Through diet, health-promoting phytochemicals are taken in and, among them, phenolic compounds, especially flavonoids, play a chief role because of their antioxidant, anti-inflammatory, and cell-regulatory properties (*1*). Pigmented fruits are naturally rich in phenolic compounds and blueberry (*Vaccinium* sp.) in a special way. Blueberries are characterized by a high content in phenolic acids and anthocyanins, and they express one of the highest in vitro antioxidant activities among berries and fruit in general (*2, 3*). A lot of scientific investigations have confirmed the healthy properties of blueberries in the prevention of carcinogenesis, heart diseases, age-related dysfunctions, and infections (*2, 4, 5*). New scenarios are presented, and new questions are being opened on bioavailability of phenolic compounds and indirect and synergic effects elicited in vivo by these phytochemicals, which are not exclusively mediated by antioxidant pathways (*6*).

Blueberry juice abundance in bioactive phenolic compounds is related to two factors: the first connected with the synthesis of secondary metabolites by the plant, and the second connected with the processing juice technology. Phenolic compounds are secondary metabolites of plants involved in defense mechanisms, pigmentation, and allelopathic phenomena (*7*). Inheritance of phenolics in *Vaccinium* species and cultivar has been suggested in previous studies, while the reciprocal influence of environmental growing conditions and genotype on these compounds needs further investigation (*8–11*).

The impact of juice processing on fresh berry phytochemicals has been studied, with results showing a detrimental effect of technology explained through oxidative degradation because of tissue disorganization after milling and through mechanical loss of compounds, especially anthocyanin pigments associated with the press-cake residue (*12, 13*). In a previous study on blueberry juice processing (*14*), it was proven that a steam-blanching step of berries before milling was effective in improving stability and recovery of phenolic bioactive compounds. Blanching acts by inactivating oxidative enzymes of processed fruits (*15*) and physically improving the permeability of pigmented pericarp cells.

It is in our interest to improve knowledge on the nutraceuticals characterizing raw fruit and preserve these compounds right through the processing chain up to the end product. New quality parameters should be introduced, related to the abundance in

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biologically active compounds, besides traditional parameters related to the physical−chemical aspects of the product.

To this aim, in this work, five highbush blueberry cultivars from the same environmental growing conditions were individually processed into juice after an initial blanching step and the influence was studied of the cultivar on juice phenolic content, distribution, and relative antioxidant activity. To this purpose, a chromatographic protocol was specifically developed to separate all main phenolic compounds.

MATERIALS AND METHODS

Chemicals. Methanol, acetone, and formic acid were from Merck (KGaA, Darmstadt, Germany). Acetonitrile was from Fluka (Buchs, Switzerland). All solvents were HPLC-grade. Depectinizing enzyme Cytolase M102 was from Genencor International (Palo Alto, CA). Commercial standards of gallic acid, catechin, chlorogenic acid, cyanidin 3-glucoside, caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, quercetin 3-galactoside, quercetin 3-glucoside, *o*-coumaric acid, and quercetin 3-rhamnoside were from Sigma-Aldrich (St. Louis, MO). Stabilized artificial free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was from Fluka (Buchs, Switzerland).

Plant Material. Highbush blueberries (*Vaccinium corymbosum* L.) of the five cultivars "Bluegold", "Bluechip", "Bluecrop", "Coville", and "Berkeley", grown at Centro Ricerche CRESO (Boves, Cuneo, Italy) were used. All samples, cultivated in the same environmental growing conditions, were harvested at commercial maturity and individually quickly frozen on stage ($T = -50$ °C; air speed = 4.5 m/s)

Berry Extraction. Two extraction media containing water and formic acid (90:10, v/v) or acetone, water, and formic acid (60:30:10, v/v/v), respectively, were tested. The acetone extraction for blueberry total phenolics was chosen on the basis of literature data, reporting a superior extraction rate of this solvent compared to others (*16*).

The phenolic profile was studied of two blueberry cultivars, "Berkeley" and "Coville", taken as a reference. Blueberries (15 g) were added with the extracting solution (25 mL) and homogenized using an Ultraturrax (20 300 rpm) blender (IKA Labortechnik, Staufen, Germany). Homogenate was left at room temperature for 15 min and centrifuged at 6000 rpm for 5 min (PK 130 Centrifuge, ALC International, Milan, Italy). The pellet following centrifugation was washed with 25 mL of the formic acid or formic acid/acetone solution and centrifuged; the resulting supernatant was combined with the initial extract. Organic solvent was removed in a Jouan RC 10.10 centrifugal evaporator (Jouan, Winchester, VA) and replaced by water. Extractions were made in duplicate.

Juice Preparation. An aliquot (1 kg) of fruit for each blueberry cultivar was steam-blanched (85 °C) for 3 min and tap-water-cooled in a pilot steam-blanching tunnel (Ghizzoni Dante and Figlio, Felino, Parma, Italia), before being processed into juice, as previously reported by Rossi and co-workers (*14*). Blanched berries were milled with a commercial hand blender Philips HR 1361 (Eindhoven, Nederland), added with 0.70 g/kg of depectinizing enzyme, and after a rest of 1 h at room temperature, pressed in a filter bag press (Bertuzzi, Brugherio, Italy); a pasteurization step (90 °C, 1 min) was provided at the end of the process.

Analyses. *HPLC Analysis of Phenolics.* Phenolic profiles of samples were studied by a RP-HPLC system equipped with a multiwavelength detector. The method previously reported by Rossi and co-workers (*14*) for anthocyanins (450–560 nm) was modified to obtain also the separation of other nonanthocyanin phenolic compounds (250–350 nm). The chromatographic system consisted of a PU 1580 pump (Jasco Co., Tokyo, Japan), a 250 × 4.6 mm i.d., 5 μm, Inertsil ODS-3 column, thermostatted at 40 °C, and a Jasco MD 2010 Plus photodiode array detector. For system control and data handling, a Jasco HSS-1500 HPLC system control program and software package were used. The mobile phases consisted of acetonitrile (A) and water/formic acid (90:10, v/v) (B). Elution was performed at a flow rate of 0.5 mL/min by the following linear gradient steps: start condition 8% A in B, then 11% A in 25 min, 23% A in 22 min, 45% A in 16 min, 75% A in 3 min, 75% A for 8 min, and 8% A in 5 min. A 12 min post run for conditioning (8% A) was provided between injections. Before injection (20 μL), the samples were diluted 1:10 (v/v) in mobile-phase B and filtered through a 0.45 μm HA membrane filter (Millipore Corp., Bedford, MA).

Identification and Quantification of Phenolics. Blueberry anthocyanin monoglucosides were identified by coelution after spiking the samples with an extract of *Vitis vinifera* L. cv. "Sangiovese". Anthocyanin profile of this grape cultivar, already described in the literature (*17*), is in fact naturally characterized by the exclusive presence of glucosylated forms. Anthocyanin monoarabinosides and monogalactosides were identified on the basis of their UV/vis spectra and by comparing to bibliographic data (*14*). All of the anthocyanin monoglycosides were quantified as cyanidin 3-glucoside by measuring detector response to the commercial standard. Benzoic acids (270–280 nm), cinnamic acids (305–330 nm), catechin (270–280 nm), and flavonol glycosides (350–380 nm) were identified by comparing UV/vis spectra and retention times to those of commercial standards. Their concentration was determined by measuring detector response to standard compounds.

Spectrophotometric Analysis of Anthocyanins. Total monomeric anthocyanins were estimated by a pH-differential method (*18*), and total anthocyanins and polymeric color were measured by a hydrogen peroxide bleaching method (*19*). A UV4 UV/vis spectrometer (Unicam, Cambridge, U.K.) was used for spectroscopic measurements. Monomeric anthocyanin content was calculated as cyanidin 3-glucoside, using an extinction coefficient of 26 900 L cm^{-1} mol^{-1} and a molar mass of 449.2 g/mol. Results were expressed as milligrams of equivalent cyanidin 3-glucoside per 100 mL of juice.

DPPH Assay. Relative antioxidant capacity of juice samples from the five blueberry cultivars was measured (517 nm) as scavenging activity on the stabilized artificial free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), adapting the Yen and Duh method (*20*). A DPPH• methanolic solution (2 mL; 50 mg/L) was placed in a 10 mm cuvette of a spectrophotometer (Unicam UV/vis Spectrometer UV4) and mixed with 0.5 mL of diluted sample; the quenching kinetic was monitored over 15 min. The proper sample dilution was assessed in a preliminary study, where the DPPH• methanolic solution was added with different juice dilutions (1, 2, 4, 10, 20%, v/v) and DPPH• residual absorbance after 10 min reaction was plotted. The 4% (v/v) juice dilution was chosen to ensure a percentage of free radical remaining not quenched at the steady state of the reaction. Data were corrected by subtracting two blanks: DPPH• absorbance without tested juice sample (B1) and added juice absorbance without DPPH• (B2). This last control was necessary because of the interference of blueberry juice anthocyanin absorbance (515 nm) at the assay wavelength.

Statistical Analysis. Analysis of variance and Tukey HSD multiple range test were used to determine statistically significant differences ($p \leq 0.05$). Correlation studies were performed using a linear regression analysis.

RESULTS AND DISCUSSION

Chromatographic Separation. Through the chromatographic protocol developed, the main compounds characterizing raw berries and some minor constituents were separated.

The ability of the organic solvent to increase phenolic extraction from berries is evidenced in **Figure 1**. No extraction improvement is observed for chlorogenic acid, localized in the berry pulp vacuoles and easily soluble, while an increase is observed for anthocyanins (32%) and flavonols (22%), compounds localized in the epidermal tissue and associated with cell-wall materials. These results provide evidence of a different availability of blueberry phenolic compounds, related to their histochemical localization, confirming the partition into an easily available water-soluble fraction and a fraction more tightly bound to cell structures, extractable through organic solvents (*21*). On this basis, we focused our analysis on the phenolic profile of blueberry acetone extracts.

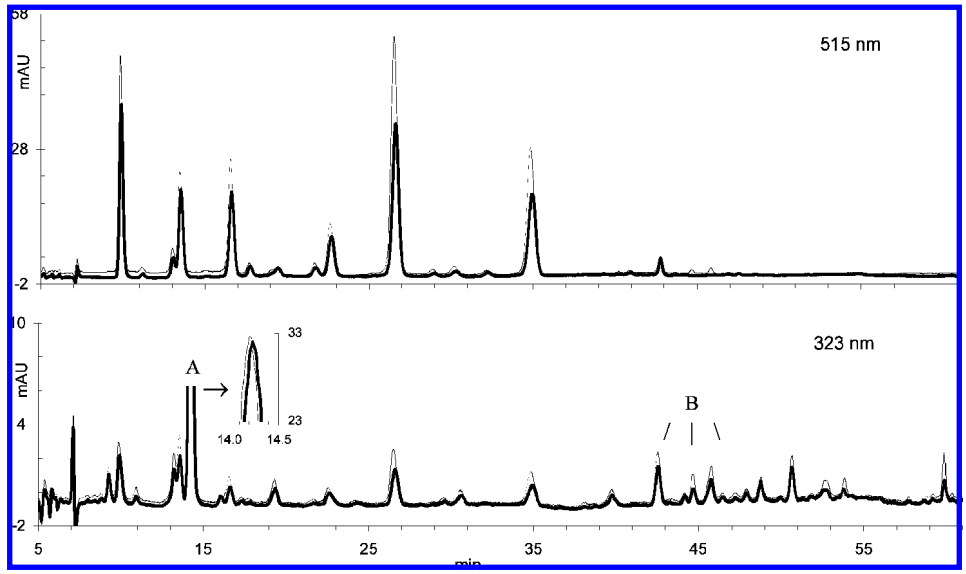

Figure 1. HPLC separation of phenolic compounds from blueberry* extracts. Anthocyanin detection, 515 nm; chlorogenic acid (A) and flavonols (B) detection, 323 nm. Extraction media = water plus formic acid (bold solid line); acetone plus formic acid (thin solid line). (*) cv. "Berkeley".

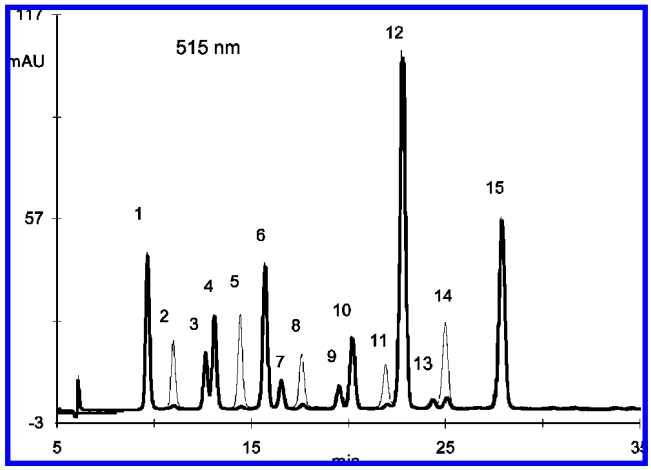

Figure 2. Chromatographic detection of glycosylated anthocyanins. Peaks attribution: 1, delphinidin 3-galactoside; 2, delphinidin 3-glucoside; 3, cyanidin 3-galactoside; 4, delphinidin 3-arabinoside; 5, cyanidin 3-glucoside; 6, petunidin 3-galactoside; 7, cyanidin 3-arabinoside; 8, petunidin 3-glucoside; 9, peonidin 3-galactoside; 10, petunidin 3-arabinoside; 11, peonidin 3-glucoside; 12, malvidin 3-galactoside; 13, peonidin 3-arabinoside; 14, malvidin 3-glucoside; and 15, malvidin 3-arabinoside. (Bold solid line) Blueberry extract. (Thin solid line) Blueberry extract plus *Vitis vinifera* L. cv "Sangiovese" extract.

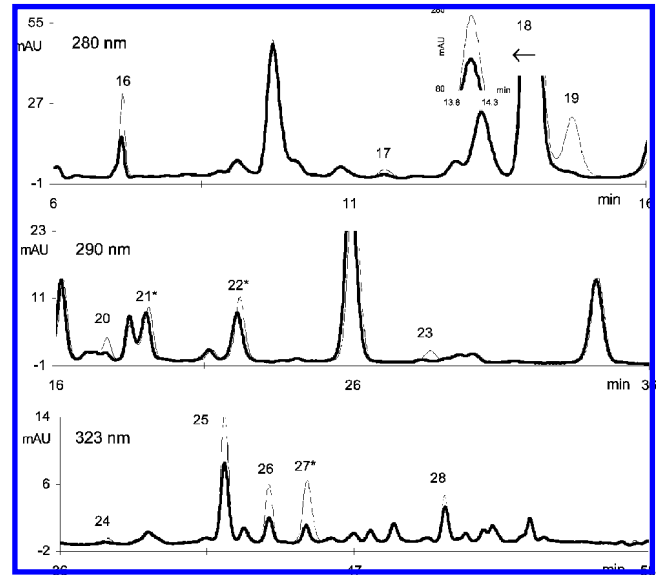

Figure 3. HPLC detection of phenolic compounds from blueberry extract alone (bold solid line) and plus the addition of 13 authentic standards (thin solid line). Peaks assignement: 16, gallic acid; 17, catechin; 18, chlorogenic acid; 19, cyanidin 3-glucoside; 20, caffeic acid; 21*, vanillic acid; 22*, syringic acid; 23, *p*-coumaric acid; 24, ferulic acid; 25, quercetin 3-galactoside; 26, quercetin 3-glucoside; 27*, *o*-coumaric acid; and 28, quercetin 3-rhamnoside. (*) No corresponding spectra sample/standard.

In the visible region (520 ± 10 nm), 15 peaks were detected, corresponding to the glycosylated forms of the five anthocyanidins characterizing highbush blueberry fruits: delphinidin, cyanidin, petunidin, peonidin, and malvidin, in their elution order. Using an extract of *Vitis vinifera* L. cv. "Sangiovese" as the natural standard (*18*), glucosidic derivatives were identified (**Figure 2**). Through the analysis of spectra, slight differences were observed in the maximum of absorbance of glycosidic derivatives of cyanidin and peonidin (515 nm), delphinidin and petunidin (523 nm), and malvidin (527 nm); similar observations were also found in the literature (*22*). These data, together with data on polarity and elution order of glucosidic functions (galactoside < glucoside < arabinoside), contributed in the identification of arabinosidic and galactosidic derivatives, leading to an anthocyanin profile consistent with bibliographic data (*23–26*).

In **Figure 3**, chromatographic separation of phenolic compounds in the UV region (280–323 nm) is shown and peaks assignment and increase because of standards addition are evidenced. Gallic acid, catechin, chlorogenic acid, caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, galactoside, glucoside, and rhamnoside quercetin 3-derivatives, and *o*-coumaric acid were identified. Sample peak assignment failed for vanillic, syringic, and *o*-coumaric acids because there was no correspondence between the absorbance spectra of the sample and standards, as a result of the interference of not identified compounds.

Juices Phenolic Analysis. The innovative berry-blanching technology was applied on blueberries from the five cultivars "Bluegold", "Berkeley", "Coville", "Bluechip", and "Bluecrop",

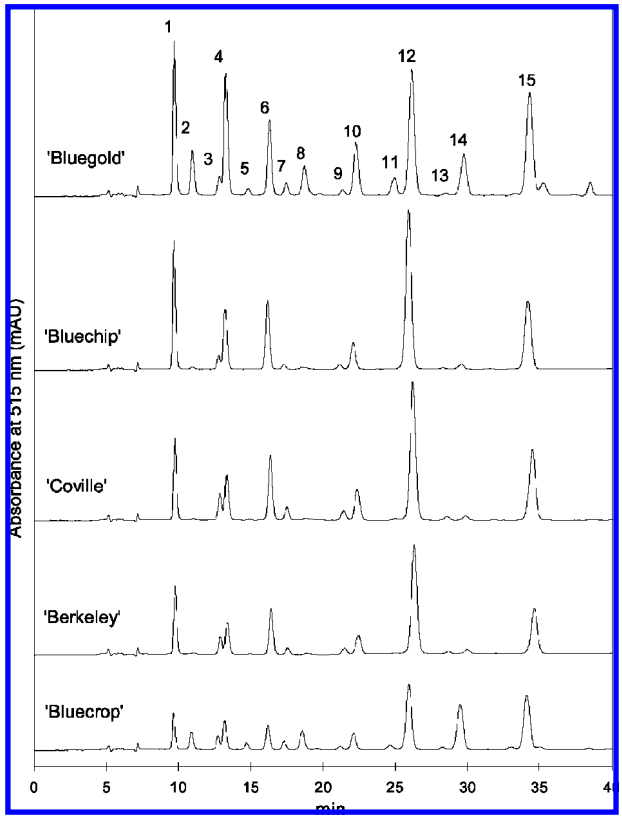

Figure 4. Anthocyanin chromatographic profiles of juices from blueberry cultivars. Peak attribution: 1, delphinidin 3-galactoside; 2, delphinidin 3-glucoside; 3, cyanidin 3-galactoside; 4, delphinidin 3-arabinoside; 5, cyanidin 3-glucoside; 6, petunidin 3-galactoside; 7, cyanidin 3-arabinoside; 8, petunidin 3-glucoside; 9, peonidin 3-galactoside; 10, petunidin 3-arabinoside; 11, peonidin 3-glucoside; 12, malvidin 3-galactoside; 13, peonidin 3-arabinoside; 14, malvidin 3-glucoside; and 15, malvidin 3-arabinoside.

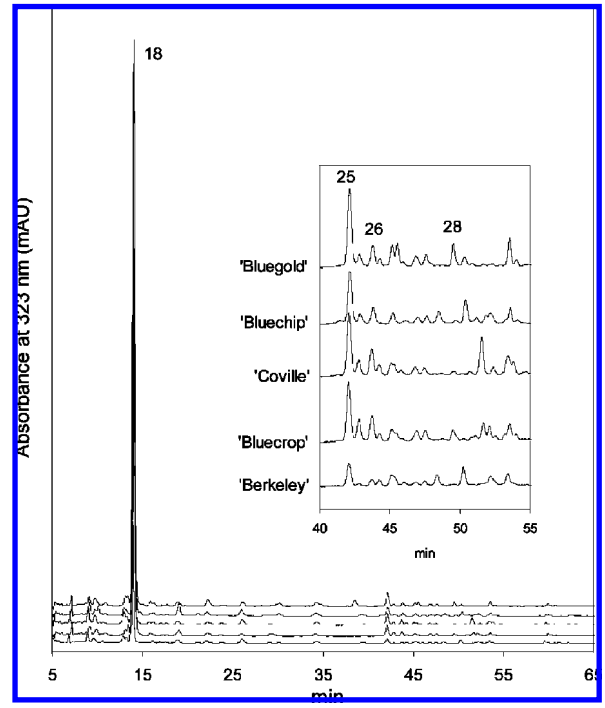

Figure 5. Chromatographic separation of phenolic compounds in juices from blueberry cultivars. Peak attribution: 18, chlorogenic acid; 25, quercetin 3-galactoside; 26, quercetin 3-glucoside; and 28, quercetin 3-rhamnoside.

and five juice samples were obtained with a juice yield ranging from 68 to 72%. Through the chromatographic protocol developed on extracts, we focused on the most representative phenolic compounds in the juices: anthocyanin, chlorogenic acid, and quercetins (**Figures 4** and **5**). What is noticeable is that each juice deriving from one cultivar was characterized not only by its own total content in phenolics and anthocyanin but also by its own relative percentage distribution in these last compounds. Significant variability among samples was observed for chlorogenic acid content, followed by anthocyanin and quercetin content (**Table 1**). A large variation was also observed in the percentage distribution of anthocyanin glycosidic functions (**Table 2**), with glucoside derivatives being more abundant in juice samples from cv. "Bluegold" and "Bluecrop", while galactoside derivatives being more abundant in juice samples from cultivars "Coville", "Berkeley", and "Bluechip". Similar results, found also by Cho and co-workers on berries (*11*), were explained through the particular enzymatic background (glycosyl transferase) that each cultivar has, which could be influenced by common ancestors. As can be seen by **Table 2**, a lower variability among juices was observed in the percentage distribution of the five blueberry anthocyanins.

Spectrophotometric techniques confirmed a large variation in total anthocyanin content (92.5–213.4 mg/100 mL juice) and total monomeric anthocyanin content (46.8–115.8 mg/100 mL juice) among cultivars (**Figure 6**), deriving percent polymeric color ranging from 45.7 to 49.5%, in agreement with data reported in the literature for blueberry juices (*13, 27*). The same trend was observed among samples for total, monomeric, and glycosylated anthocyanins, showing a good agreement between spectrophotometric and chromatographic data. Spectrophotometric data on monomeric anthocyanin content, also including acylated forms, are always higher than chromatographic data on glycosylated anthocyanin content; partial interference of polymeric compounds at the spectrophotometric assay wavelength was also suggested (*13*).

Table 1. Chlorogenic Acid, Quercetin, and Glycosylated Anthocyanins Content (mg/100 mL) in Juices from Blueberry Cultivars

	cultivar				
compound[a]	"Bluegold"	"Coville"	"Bluechip"	"Bluecrop"	"Berkeley"
chlorogenic acid	85.0 a	69.3 b	42.7 d	57.3 c	21.0 e
quercetin	2.2 ab	1.5 b	2.7 ab	1.4 b	2.0 ab
glycosylated anthocyanin	61.2 a	40.8 b	43.9 b	30.3 c	30.8 c
total	148.4 a	111.6 b	89.3 c	89.0 c	53.8 d

[a] Means ($n = 4$) within a row followed by different letters are significantly different ($p \leq 0.05$).

Table 2. Percentage Distribution of Anthocyanin Glycosides and Glycosylated Anthocyanin Compounds in Juices from Blueberry Cultivars[a]

	cultivar				
compound	"Bluegold"	"Coville"	"Bluechip"	"Bluecrop"	"Berkeley"
galactoside	42.5 ± 0.5	58.8 ± 1.1	61.9 ± 2.0	37.2 ± 0.9	61.3 ± 2.1
glucoside	19.0 ± 1.2	5.8 ± 0.3	5.7 ± 0.3	28.6 ± 1.3	7.1 ± 0.5
arabinoside	38.6 ± 0.8	35.4 ± 1.0	32.4 ± 1.9	34.2 ± 1.0	31.6 ± 2.5
delphinidin	28.6 ± 0.8	18.0 ± 0.8	23.6 ± 0.7	16.6 ± 2.5	19.2 ± 1.0
cyanidin	4.7 ± 0.5	7.6 ± 1.3	4.2 ± 0.5	8.1 ± 1.0	7.3 ± 0.5
petunidin	19.7 ± 0.7	19.2 ± 1.2	17.9 ± 1.0	16.4 ± 0.7	18.5 ± 0.9
peonidin	4.8 ± 0.3	4.6 ± 0.5	3.1 ± 0.2	5.6 ± 0.5	4.5 ± 0.3
malvidin	42.4 ± 1.0	50.6 ± 1.4	51.2 ± 0.8	53.5 ± 1.3	50.5 ± 1.4

[a] Each value is expressed as mean ± standard deviation (SD) ($n = 4$).

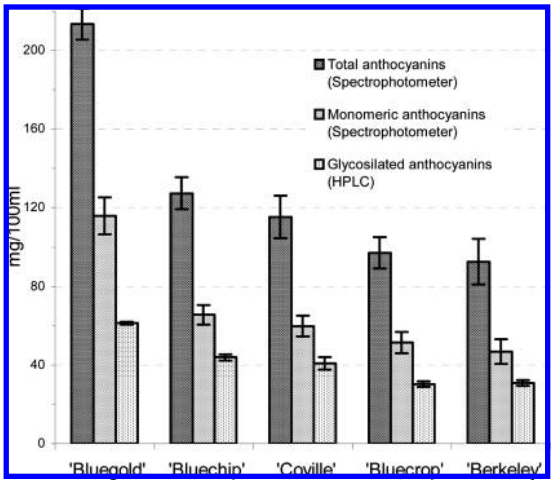

Figure 6. Anthocyanin content of juices from blueberry cultivars. Each histogram represent a mean ± standard error ($n = 4$).

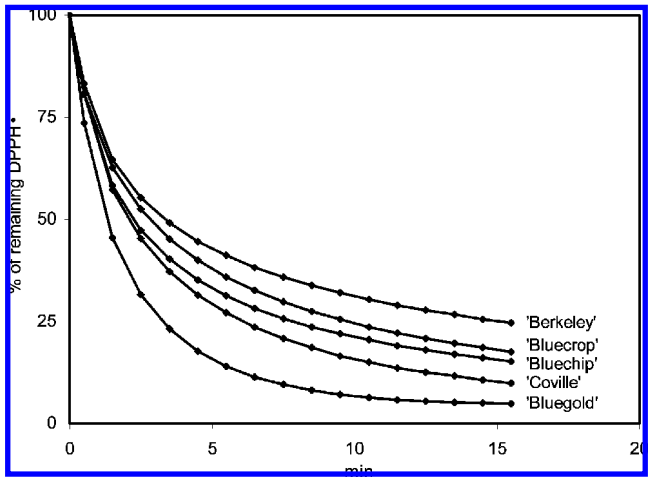

Figure 7. Quenching kinetic of juices from blueberry cultivars on free-radical DPPH•.

Juices DPPH Assay. Relative antioxidant capacities of juices from single berry cultivars were tested on the artificial free-radical DPPH•, monitoring its quenching kinetic over 15 min. The DPPH• assay is a simple procedure, suitable for a basic screening to assess the relative ranking in antioxidant capacity among different samples (*28*). In **Figure 7**, results are reported, expressed as the remaining percentage of DPPH• not quenched along the reaction time for each cultivar. A gradual increase in the quenching activity was observed among samples, from "Berkeley" to "Bluegold" juice. The antioxidant capacity of juices, expressed as a percentage of quenched free radical at 15 min, well-correlated among all cultivars with juice phenolic content, expressed as the sum of total glycosylated anthocyanins, chlorogenic acid, and quercetins (rxy = 0.97). A lower correlation was observed between juice antioxidant capacity and the content in glycosylated anthocyanins alone (rxy = 0.75), total monomeric anthocyanins by the pH differential method (rxy = 0.68), or total anthocyanins by the bleaching method (rxy = 0.68). These results on juices are in agreement with data previously reported by Prior et al. (*9*) on fresh berries, showing the importance of a pool of phenolic compounds besides anthocyanin pigments, concurring to the whole antioxidant capacity of a fruit juice and differently expressed in each blueberry cultivar. When different assays are applied, the nature and activity of single antioxidants should be better elucidated.

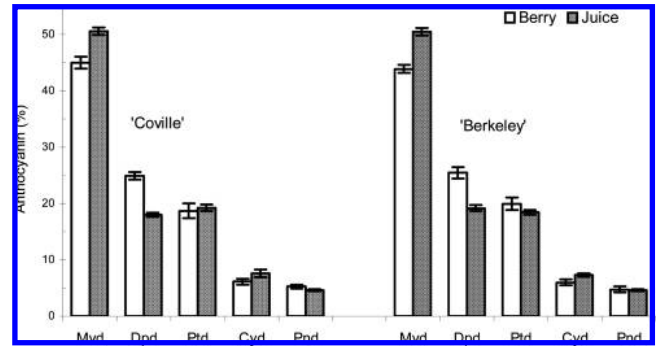

Figure 8. Antocyanin percentage distribution in berries and deriving blanching-processed juice from blueberry cv. "Coville" and cv. "Berkeley". Mvd, malvidin; Dpd, delphinidin; Ptd, petunidin; Cyd, cyanidin; and Pnd, peonidin. Each histogram represent a mean ± standard error ($n = 4$).

Juice Processing and Anthocyanin Profile Changes. Processing technology directly acts on the recovery of bioactive compounds from berries into juice. On the two blueberry cultivars "Berkeley" and "Coville", individual anthocyanin changes and total anthocyanin (HPLC) recovery were studied. When we compared anthocyanin percentage distribution in blueberry fruit and blanching-processed juices, we observed that each anthocyanin pigment differently responds to the technological treatment applied (**Figure 8**). Delphinidin glycosides were in fact the most unstable anthocyanins, with their proportion changing with processing from 24.9 to 18.0% (cv. "Coville") and from 25.4 to 19.2% (cv. "Berkeley"). Delphinidin derivatives decreased their content into juice in favor of malvidin derivatives (cv. "Coville" from 45.0 to 50.6%; cv. "Berkeley" from 43.9 to 50.5%) and cyanidin derivatives (cv. "Coville" from 6.1 to 7.6%; cv. "Berkeley" from 6.0 to 7.3%). These percentage fluctuations follow the same trend as already reported in the literature for blueberry juices traditionally processed (*12, 13*), but they are more contained, especially with regard to delphinidin. This may be due to the protective effect of blanching, mediated by enzymatic (PPO) inactivation, on more labile phenolic compounds. Furthermore, from our results, the total anthocyanin (HPLC) recovery ("Berkeley" 38.6%; "Coville" 46.3%) was higher than recovery data found in the literature for traditional blueberry juice (*12, 13*). The above-mentioned findings all together suggest that the anthocyanin profiles of blueberry juices and raw blueberry are more similar if a berry-blanching step is introduced in processing.

In conclusion, our data indicate that blueberry cultivar selection could be a useful source of variability in bioactive compounds, to be exploited to obtain a juice product that fits better with new healthy-quality parameters. Furthermore, from our findings, blanching could be considered a valid step along the processing chain to improve pigment stability and extraction rate, therefore contributing to the achievement of a juice technology more respectful of the fruit original abundance and distribution in bioactive compounds. Further investigation on blueberries from the same cultivar but different harvest locations and times could help to elucidate the influence of environmental growing conditions on the final juice composition. When a proper processing technology is joined with suitable raw fruit material, new quality standards could be reached.

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