SUPPLEMENTARY MATERIAL

Variation of anthocyanins and other major phenolic compounds throughout the ripening of four Portuguese blueberries (*Vaccinium corymbosum* L) cultivars

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Abstract

Blueberries are widely recognized as one of the richest sources of bioactive compounds, among which are anthocyanins. Though the berries ripeness has been reported as affecting the phytochemical composition of fruits. Therefore, the present work aimed to evaluate the variation of anthocyanins, and other major phenolics, throughout five ripening stages in four blueberry cultivars. The results showed that the antioxidant capacity and anthocyanin content increased during ripening, reaching the highest values when the blueberries are collected from bunches comprised of 75% ripe blueberries. Antagonistically, the amount of phenolic acid decreases while the quercetin-3-glucoside levels remain stable. Furthermore, Goldtraube blueberries appear to possess, systematically, higher amounts of phenolic compounds than the other cultivars studied. Thus, when seeking the highest yield of anthocyanins, the preferred harvest should occur in bunches that contain ca 75% of ripe blueberries and, considering the cultivars assayed, the Goldtraube cultivar appears the richest in phenolic compounds.

Keywords: *Vaccinium corymbosum*; anthocyanins; ripening; blueberry

Experimental Section

Samples

In a production area that possessed all four test cultivars (latitude: 40°47'24.66" N; longitude: 8°23'17.21" W; elevation above the sea level: 549 m), twenty four blueberry bushes were randomly selected (6 per cultivar with the agronomical characteristics

listed in table 1S). Bushes from the different cultivars were arranged in a 2X2 rectangular formation comprised of evenly spaced rows of plants.

Samples were collected from Goldtraube, Duke, Bluecrop and Ozarkblue blueberry bushes at different ripening stages: S1 – green fruits with a reddish crown (unripe fruit); S2 – light pink blueberries with no observable green area (unripe fruit); S3 blueberries that came from a bunch containing only 25% of ripe blueberries (ripe fruit); S4 – blueberries collected from bunches that had 75% or more of their blueberries ripe (ripe blueberries); S5 – blueberries that fell from the bush (overripe blueberries).

At each ripening stage, blueberries from the 6 different bushes (20 g collected from each bush) were collected from random areas of each plant and immediately transported to the laboratory for analysis.

Extracts

The blueberry pulp was homogenized (10% (w/v), 24 000 rpm, 1 min) with acidified methanol (0.01% HCl) using an Ultra-turrax T18 (IKA, Staufen, Germany) and left to extract at room temperature in a light tight container. After 12 h, the mix was centrifuged (6026g, 4 °C, for 15 min) and filtered through a 4-7µm filter (Prat Dumas, Couze St. Front, France). Each extraction was performed in triplicate.

Total anthocyanins content (ACY), Total antioxidant capacity (TAC) and Total Phenolic Content (TPC)

The total monomeric anthocyanins were assessed using the differential pH method as described by (Jakobek et al. (2007)) and the results, expressed in cyanidin-3-O-glucoside (C3Glu) equivalents, were calculated as described by the same authors (Jakobek, et al., 2007).

The ABTS radical cation (ABTS•+) assay, to assess the TAC of extracts, and the Folin - Ciocalteu phenol's reagent, to evaluate the TPC, were used as described by Gião et al. (2007).

Compound identification and quantification

The different phenolic compounds were analysed using reverse – phase high performance liquid chromatography coupled with a diode-array detection system (Waters Series 600, Mildford, Massachusetts) equipped with a reverse phase Symmetry C18 column (250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size, kept at 30 °C) and a guard column containing the same stationary phase. Chromatographic separation was carried out using a linear gradient of 2 solvents (A- 5% methanol, 2.5% formic

acid, 92.5% ultra-pure water; B- 25% methanol, 25% formic acid, 50% ultra-pure water) varying as illustrated in table 2S. Injection volume was 40 µL. Detection was achieved using a diode array detector measuring at wavelengths ranging from 200 to 600 nm in 2 nm intervals. The different peaks were analysed by comparison of retention times and spectra with that of pure phenolic and anthocyanin glycoside standards all acquired from Extrasynthese (Genay Cedex, France) except for delphinidin-3-arabinoside which was acquired from ChemFaces (Hubei, China). Quantification was performed using calibration curves (Table 4S) of the presumed compounds considering a detection limit of 2.5 mg/ 100 g of fresh blueberries. Three independent analysis were performed for each of the triplicate extracts.

Statistical Analysis

The normality of the distribution was assessed using the Shapiro-Wilk normality test. As the results proved to follow a normal distribution, a comparison of the results was performed using One-Way ANOVA coupled with Scheffe's Post-Hoc test (the differences were considered significant when $p < 0.05$). Additionally, the correlation between different variables was assessed using Pearson's correlation (the differences were considered significant when $p < 0.05$).

References

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Tables

Table S1 – Growth conditions and age of the different cultivars.

	Duke	Bluecrop	Goldtraube	Ozarkblue
Plant's age	13	17	12	13
Average soil pH	4.6	5.1	5.1	4.6
Average organic matter content	11%	12%	12%	11%
Soil type	Humic Dystrudept	Humic Dystrudept	Humic Dystrudept	Humic Dystrudept
Average daily light during ripening	ca.9.2 h	ca. 9.2 h	ca. 9.2 h	ca. 9.2 h
Sample harvest period	4 th – 8 th of June	11 th – 16 th of June	18 th – 23 rd of June	25 th – 30 th of June
Average high temperature (°C)	28 °C	28 °C	28 °C	28 °C
Average low temperature (°C)	12 °C	12 °C	12 °C	12 °C

Table S2 – Flow conditions used for HPLC analysis

Time (min)	% Solvent A	% Solvent B	Flow (mL/min)
0 to 60	100 - 40	0 - 60	0.65
60 to 65	40 – 90	60 – 10	0.50
65 to 70	90 - 100	10 - 0	0.50

Table S3 – Correlation between the total antioxidant capacity, phenolic and anthocyanin content

		ACY – TAC	ACY – TPC	TAC – TPC
Duke	Pearson's R	0.980	0.898	0.955
	p-value	< 0.01	< 0.01	< 0.01
Bluecrop	Pearson's R	0.051	- 0.464	- 0.058
	p-value	0.857	0.082	0.837
Goldtraube	Pearson's R	0.932	0.896	0.972
	p-value	< 0.01	< 0.01	<0.01
Ozarkblue	Pearson's R	0.900	0.756	0.884
	p-value	< 0.01	0.001	< 0.01

Table S4 – Standards and chromatographic information regarding the calibration curves

Standard	Retention Time	Maximum absorbance (λ)	Calibration curves		
			Slope	y-Intercept	R ²
Cyanidin-3-arabinoside	42.43	516.2	185958,0	2078,7	0,989
Cyanidin-3-galactoside	37.73	516.2	184409,3	104057,5	0,993
Cyanidin-3-glucoside	40.04	515.6	163200,7	89177,5	0,995
Delphinidin-3-arabinoside	33.41	523.5	124339,2	216344,0	0,996
Delphinidin-3-galactoside	35.17	522.9	166883,9	151461,9	0,979
Delphinidin-3-glucoside	29.67	523.5	190244,0	137988,3	0,979
Malvidin-3-galactoside	38.01	528.1	159352,2	65858,3	0,977
Malvidin-3-glucoside	39.53	527.2	166282,4	13416,4	0,981
Petunidin-3-galactoside	44.28	516.2	141320,5	-609036,5	0,998
Petunidin-3-glucoside	43.90	525.6	198294,5	69479,6	0,991
Peonidin-3-arabinoside	44.77	517.4	129169,8	-9872,4	0,987
Peonidin-3-galactoside	44.93	516.2	141320,49	-609036,5	0,998
Gallic acid	32.85	322.5	5299,2	-9570,7	0,999
Chlorogenic acid	33.5	326.7	218281,8	85772,5	0,999
<i>p</i> -Coumaric acid	45.29	309.8	464264,8	-868221,6	0,994
Quercetin-3-Glucoside	51.40	256.2	100826,0	-559537,5	1,000

Table S5 – Anthocyanin content, in mg per 100 g of fresh fruit, for each stage and cultivar according to the differential pH (TAC) and HPLC analysis

	Ripening Stage	Anthocyanin content	
		TAC	HPLC
Duke	S1	nd	nd
	S2	6.28 ± 2.87	nd
	S3	93.99 ± 0.81	524.26 ± 16.98
	S4	124.68 ± 5.01	684.37 ± 56.60
	S5	77.65 ± 4.84	617.00 ± 29.37
Bluecrop	S1	6.195 ± 3.47	nd
	S2	10.64 ± 2.63	nd
	S3	42.51 ± 0.01	188.76 ± 41.40
	S4	56.83 ± 2.06	194.57 ± 13.69
	S5	70.27 ± 2.14	245.76 ± 19.66
Goldtraube	S1	1.35 ± 0.59	nd
	S2	6.87 ± 1.46	nd
	S3	61.56 ± 1.67	427.17 ± 11.04
	S4	158.58 ± 3.06	572.76 ± 35.14
	S5	159.97 ± 1.11	564.16 ± 16.06
Ozarkblue	S1	nd	nd
	S2	2.93 ± 1.72	nd
	S3	46.60 ± 2.02	259.6 ± 18.54
	S4	95.63 ± 3.34	523.48 ± 23.13
	S5	49.18 ± 0.53	185.77 ± 8.23

nd, not detected.