Supplementary Information

Uncovering the biosynthetic pathways of key flavor and color compounds in pomegranate using pathway-based metabolomics

Jin-Pyo An [a], Dongjoo Kim [a], Xuebo Song [a], John M. Chater [b], Claire C. Heinitz [c], Yu Wang [a,*]

[a]Food Science and Human Nutrition Department, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA

[b]Horticultural Science Department, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA

[c]United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository - Tree Fruit & Nut Crops & Grapes, One Shields Ave, University of California, Davis, Davis, CA 95616, USA

***Corresponding author**: Yu Wang, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, Florida 33850, United States; orcid.org/0000-0002-2003-270X; Email: yu.wang@ufl.edu

Table of Contents

Table S1. Optimal MS/MS parameters for anthocyanin quantification

Compound	Retention time (min)	Polarity	Q1 (m/z)	Q3 (m/z)	CE (V)
Compound 1	4.91	positive	627	303	27
Compound 2	6.25	positive	465	303	31
Compound 3	6.40	positive	611	303	26
Compound 4	5.56	positive	611	287	28
Compound 5	6.85	positive	449	287	27
Compound 6	7.02	positive	595	287	31
Compound 7	6.45	positive	625	301	32
Compound 8	7.45	positive	433	271	22
Genistein d4 (Internal standard)	16.15	positive	275	154	28

Table S2. UHPLC-MS parameters for targeted metabolomics.

Column	Parameter	Value	Parameter	Value
RP (C-30)	Column temperature	40°C	Vaporizer temperature	275 °C
	Spray voltage (pos)	3.0 kV	Sweep gas flow rate	1
	Spray voltage (neg)	2.5 kV	Sheath gas flow rate	35
	Capillary temperature	275°C	AGC target	2e5
	Aux gas flow rate	10	Isolation window	1.7 m/z
HILIC-Z	Column temperature	40°C	Vaporizer temperature	290 °C
	Spray voltage (pos)	3.0 kV	Sweep gas flow rate	1
	Spray voltage (neg)	2.5 kV	Sheath gas flow rate	40
	Capillary temperature	290°C	AGC target	2e5
	Aux gas flow rate	10	Isolation window	1.7 m/z

Table S3. UHPLC-MS parameters for fatty acid analysis

Column	Parameter	Value	Parameter	Value
RP (C-18)	Column temperature	40°C	Vaporizer temperature	290 °C
	Spray voltage (pos)	3.0 kV	Sweep gas flow rate	1
	Spray voltage (neg)	2.5 kV	Sheath gas flow rate	40
	Capillary temperature	290°C	AGC target	2e5
	Aux gas flow rate	10	Isolation window	1.7 m/z
	Resolution	70,000		

Table S4. UHPLC-MS parameters for punicalagin analysis

Method	Parameter	Value	Parameter	Value
MS	Column temperature	25°C	Vaporizer temperature	300 °C
	Spray voltage (neg)	2.5 kV	Sweep gas flow rate	1
	Spray voltage (neg)	2.5 kV	CID Gas (mTorr)	2
	Capillary temperature	325°C	Sheath gas flow rate	40
	Aux gas flow rate	12		
SRM	Precursor ion	1083	RF lens (V)	248
	Product ion (quan)	601	Collision Energy (quan)	47
	Product ion (qual)	781	Collision Energy (qual)	35
	Q1 resolution	0.7	Q3 resolution	0.7

Table S5. Optimal MS/MS parameters for punicalagin α and β

Compound	Retention time (min)	Polarity	Q1 (m/z)	Q3 (m/z)	CE (V)
Punicalagin α	10.39	negative	1083.03	601.00	47
Punicalagin β	10.61	negative	1083.03	781.07	35
Genistein d_4	8.3	positive	275.1	219.1	25

no	Volatiles	CAS	Chemical formula	Exact mass
1	Hexanal	66-25-1	$C_6H_{12}O$	100.0888
2	β-pinene	127-91-3	$C_{10}H_{16}$	136.1252
3	Ethyl-2-butenoate	10544-63-5	$C_6H_{10}O_2$	114.0680
4	Limonene	138-86-3	$C_{10}H_{16}$	136.1252
5	3-Methyl-1-butanol	123-51-3	$C_5H_{12}O$	88.0888
6	2-Hexenal	505-57-7	$C_6H_{10}O$	98.0731
7	2-Pentylfuran	3777-69-3	$C_9H_{14}O$	138.1044
8	γ-Terpinene	99-85-4	$C_{10}H_{16}$	136.1252
9	o-Cymene	527-84-4	$C_{10}H_{14}$	134.1095
10	Sulcatone	110-93-0	$C_8H_{14}O$	126.1044
11	1-Hexanol	111-27-3	$C_6H_{14}O$	102.1044
12	(Z)-3-Hexen-1-ol	928-96-1	$C_6H_{12}O$	100.0888
13	Nonanal	124-19-6	$C_9H_{18}O$	142.1357
14	(E) 2-Octenal	2548-87-0	$C_8H_{14}O$	126.1044
15	2-Ethyl-1-hexanol	104-76-7	$C_8H_{18}O$	130.1357
16	(E)-2-Nonenal	18829-56-6	$C_9H_{16}O$	140.1201
17	Linalool	78-70-6	$C_{10}H_{18}O$	154.1357
18	1-Octanol	111-87-5	$C_8H_{18}O$	130.1357
19	cis-Bergamotene	18252-46-5	$C_{15}H_{24}$	204.1878
20	β-Caryophyllene	87-44-5	$C_{15}H_{24}$	204.1878

21	Terpinen-4-ol	562-74-3	$C_{10}H_{18}O$	154.1357
22	Methyl benzoate	93-58-3	$C_8H_8O_2$	136.0524
23	p-Tolualdehyde	104-87-0	C_8H_8O	120.0575
24	1-Nonanol	143-08-8	$C_9H_{20}O$	144.1514
25	L-α-Terpineol	10482-56-1	$C_{10}H_{18}O$	154.1357
26	Methyl salicylate	119-36-8	$C_8H_8O_3$	152.0473
27	2,4-Dimethylbenzaldehyde	15764-16-6	$C_9H_{10}O$	134.0731

Table S6. Chemical information of volatile compounds

Table S7. Linearity, LOD, and LOQ for anthocyanin quantification

Compound	Calibration Range (µg /mL)	Slope	Intercept	r^2	*LOD*[a]	*LOQ*[b]
Compound 1	0.1-1000	16.6014	-8.9571	0.9998	0.014	0.043
Compound 2	0.1-1000	60.3187	-18.5947	0.9998	0.017	0.052
Compound 3	0.1-1000	41.7565	-19.2963	0.9999	0.027	0.082
Compound 4	0.1-1000	30.8500	0.0767	1.0000	0.015	0.045
Compound 5	0.1-1000	90.9162	15.5747	0.9999	0.017	0.051
Compound 6	0.1-1000	58.7399	3.2889	0.9999	0.031	0.095
Compound 7	0.1-1000	0.2174	-0.0687	0.9995	0.011	0.035
Compound 8	0.1-1000	47.4895	4.7021	0.9999	0.010	0.033

Table S8. Precision and Accuracy for anthocyanin quantification

Anthocyanin	Conc. (μg/mL)	Intraday precision RSD (%)[a]	Interday precision RSD (%)[b]	Accuracy (%)
1	0.5	4.09	6.76	97.6
	10	4.02	5.68	95.1
	200	3.55	6.13	104.7
2	0.5	3.05	7.82	94.2
	10	5.13	6.68	103.6
	200	3.65	7.01	105.8
3	0.5	3.56	6.14	95.8
	10	4.18	7.34	97.3
	200	3.20	5.11	103.5
4	0.5	4.77	4.74	96.4
	10	2.65	5.68	104.5
	200	2.12	5.97	105.1
5	0.5	3.68	6.30	97.2
	10	3.35	5.67	102.4
	200	2.97	7.35	101.9
6	0.5	5.67	8.01	94.6
	10	5.99	7.34	93.1
	200	4.51	6.51	96.0
7	0.5	3.51	7.45	93.6
	10	4.71	6.25	105.4
	200	2.98	5.64	94.6
8	0.5	2.64	4.58	95.0
	10	4.74	5.01	106.8
	200	5.53	6.47	105.2

The precision was demonstrated as relative standard deviation (RSD) and accuracy was evaluated by comparing measured concentrations to nominal values. [a]Intra-day precision was evaluated by analyzing the samples on the same day. [b]Inter-day precision was assessed by analyzing three samples over three consecutive days. Three replicate analyses were performed.

Table S9. Relative abundance and their ratio of punicalagin α and β

no.	Punicalagin	Relative abundance			
		Azadi	Eversweet	Poinciana	Wonderful
1	Punicalagin α	7.28 ± 1.09	6.98 ± 0.89	8.02 ± 1.24	3.47 ± 0.50
2	Punicalagin β	7.95 ± 1.22	7.27 ± 1.09	8.53 ± 1.34	3.83 ± 0.61
3	Ratio (punicalagin α/β)	0.92	0.96	0.94	0.90

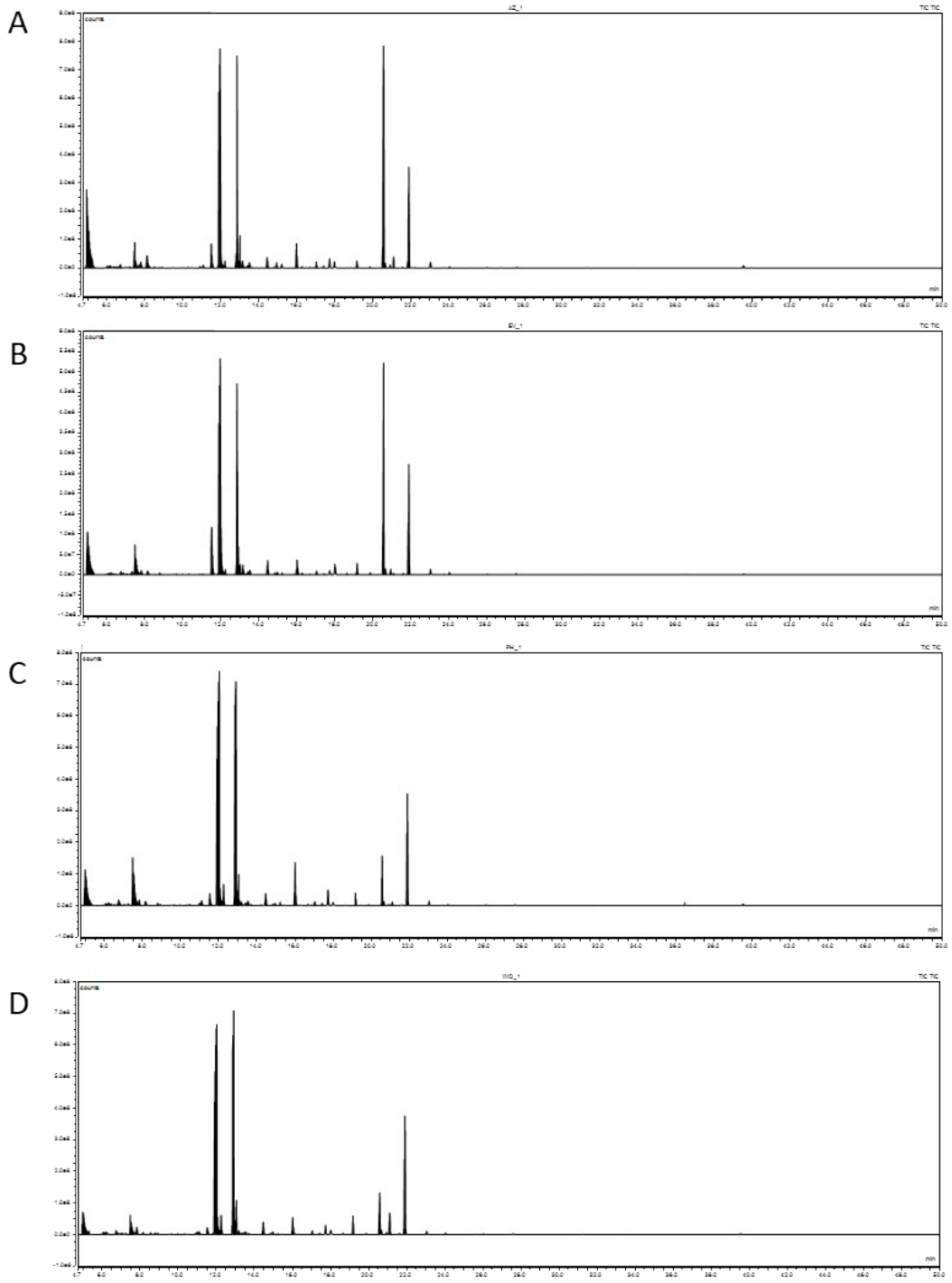


Fig. S1. Gas Chromatograms of four pomegranate cultivars (A: Azadi, B: Eversweet, C:Phoenicia, D: Wonderful).

A

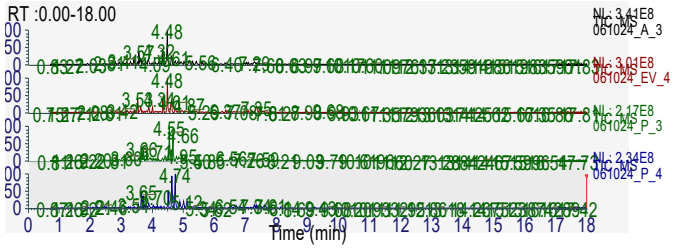
B

C

D

Fig. S2. LC-MS chromatograms (C-30 column) using a t-SIM mode (A: Azadi, B: Eversweet, C:Phoenicia, D: Wonderful).

A



B

C

D

Fig. S3. LC-MS chromatograms (HILIC column) using a t-SIM mode (A: Azadi, B: Eversweet, C:Phoenicia, D: Wonderful).

Punicalagin α

Punicalagin β

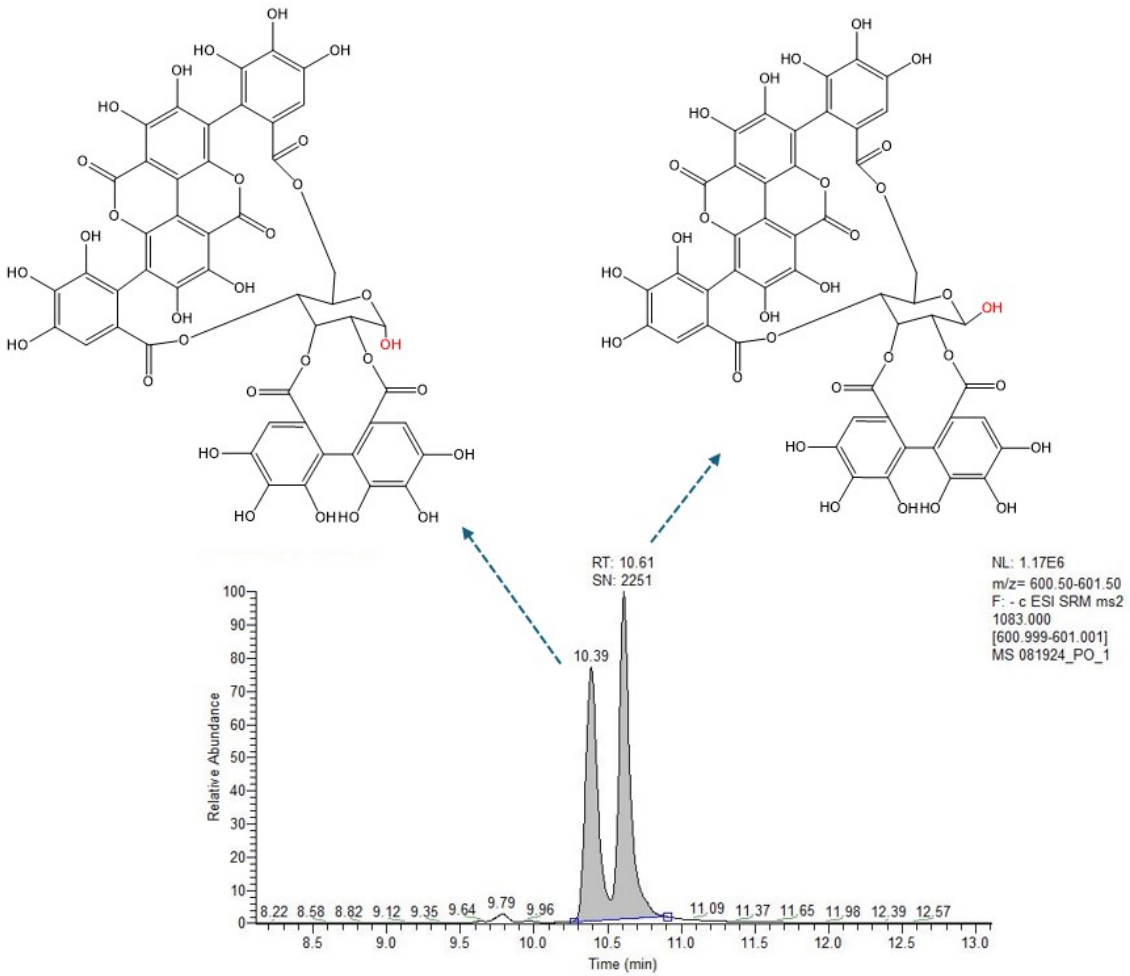


Figure S4. Chromatogram and chemical structure of punicalagin α and β

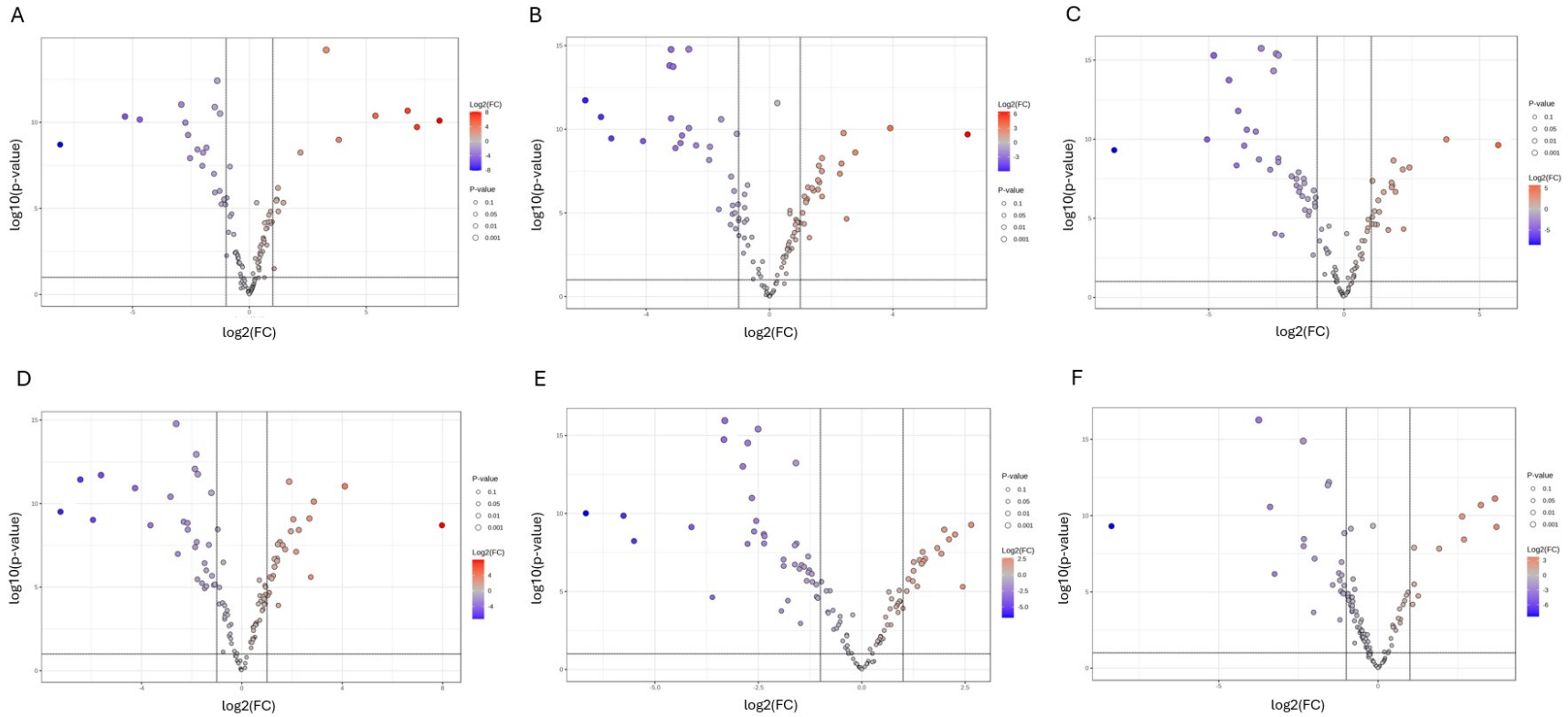


Fig. S5. Volcano plot comparing each cultivar. (A) 'Azadi' vs 'Eversweet'; (B) 'Azadi' vs 'Phoenicia'; (C) 'Azadi' vs 'Wonderful'; (D) 'Eversweet' vs 'Phoenicia'; (E) 'Phoenicia' vs 'Wonderful'; (F) 'Phoenicia' vs 'Wonderful'. The color represented its Log2(FC) value, while the point size represented the P-value.

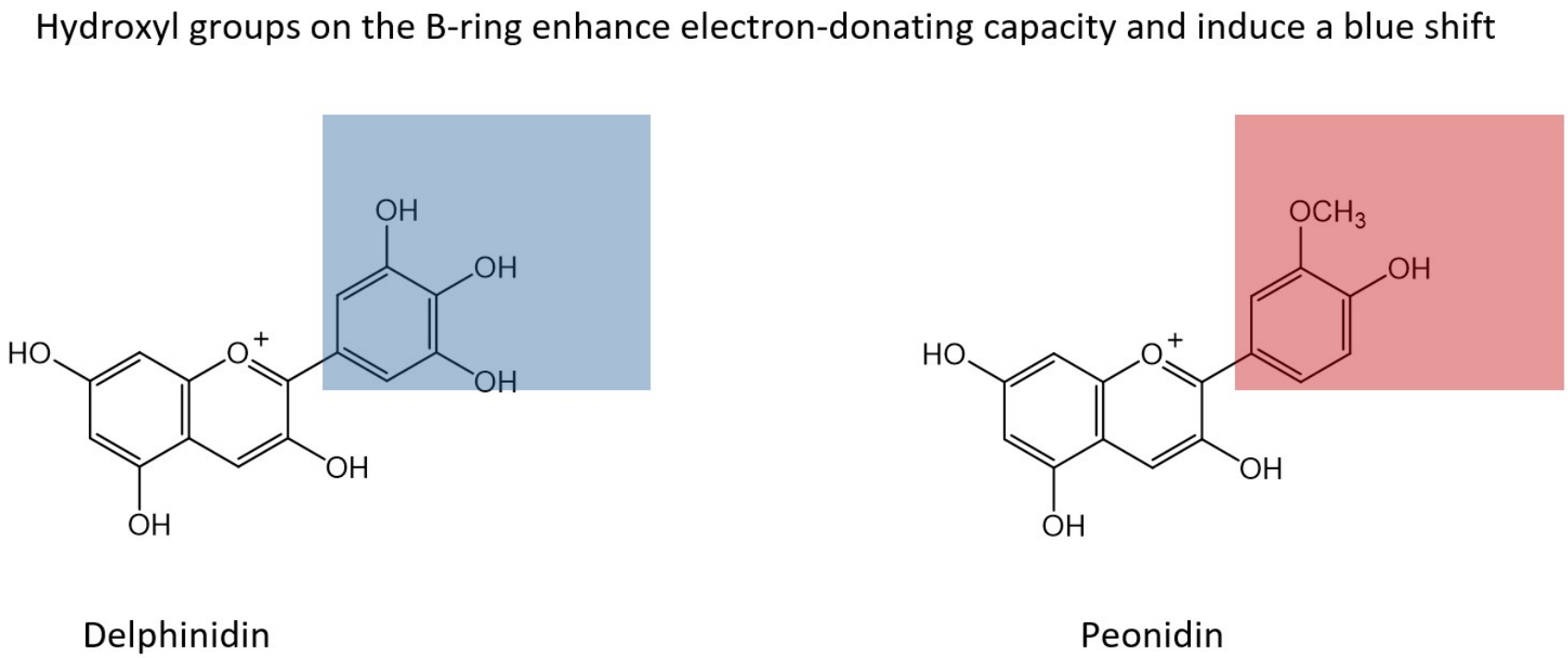


Fig. S6. Effect of B-ring functional groups on anthocyanin color.