

# Changes of Total Anthocyanins and Proanthocyanidins in the Developing Blackberry Fruits

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**Abstract:** Anthocyanin and proanthocyanidin pigments of blackberry fruits at five developing stages were isolated and determined by using UV-visible detection and the high-throughput colorimetric method. There were both rich anthocyanin and proanthocyanidin compounds in the ripen blackberry fruits, ranked at 1.426 mg.g<sup>-1</sup> and 4.41 mg.g<sup>-1</sup> respectively. Distinctive quantitative patterns were observed between the anthocyanins and proanthocyanins. The former kept at a relative flat level in the early stage, not only the green and green-to-red stage, but also the red stage. It was enriched dramatically in the last red-to-black stage and peaked in the final full ripen black fruits. On the contrary, proanthocyanidin content had a gradually decreasing pattern in concentration. As for a single berry fruit, total proanthocyanidin reached the maximum level at the red-to-black stage but dramatically decreased during the last ripening process.

**Keywords:** Blackberry; Anthocyanin; Proanthocyanidin; Developing Stage.

## Introduction

Flavonoid is among one of the three main classes of pigments for coloration in plants<sup>1</sup>. It comprises more than 5000 different kinds of plant secondary metabolites<sup>2</sup>. Anthocyanins, which demonstrate a wide spectrum of colors from pale yellow to deep blue, and the colorless polymerized compounds which are classified as proanthocyanidins (also known as condensed tannins), are the two common flavonoid

metabolites. They are both end-products of the well-known flavonoid pathway<sup>3</sup>. The health-promoting properties of both have been implicated in a wide range. Anthocyanins have been reported to be involved in enhancing immune function<sup>4</sup>, protecting against age-related neurological disorders<sup>5</sup> and exhibiting anti-cancer properties<sup>6</sup>. Proanthocyanidin concentrations are believed to be an essential factor affecting astringency and bitterness tastes of persimmon<sup>7</sup>, apple<sup>8</sup>, grape<sup>8,9</sup> and other fruits as well as

their products like grape wine<sup>10,11</sup>. Their pharmacological effects have also been demonstrated, e.g. antiviral, antimicrobial, anti-HIV, radical scavenging, anti-oxidative, anti-complementary and anti-tumor promoting properties, as well as cardiogenic and anti-arteriosclerotic activities<sup>12</sup>.

Studies of model plants such as *Arabidopsis thaliana* or strawberry flavonoid pathway gave us an outline of material flow of biosynthesis of anthocyanins and proanthocyanidins<sup>13-15</sup>. Proanthocyanidins were believed to be diverged from the anthocyanin path and the two shared the same substrates, leucoanthocyanidins<sup>16,17</sup>. Moreover, these two compounds might be under the same regulation mechanism, for example, controlled by the same transcription factors like *myb10* family<sup>18-20</sup>.

Blackberry is an important economic and medicinal purpose fruit in both Europe and North America<sup>21</sup>. Due to its high anthocyanin and phenolic contents, the composition of anthocyanins and proanthocyanidins, and their antioxidant activities in fresh fruits as well as their produced products were widely studied<sup>22-28</sup>. Veazie et al.<sup>23</sup> depicted in detail the ripening physiology such as soluble solids content, titratable acidity, anthocyanin content as well as ethylene production in his report on an erect thornless blackberry 'Navaho'. Due to the lack of molecular correlation support for anthocyanins and proanthocyanidins by that time, the dynamic changes in the fruit developing stages of these two substances had not yet been taken into considerations. In the current study, anthocyanins of the 5 typical stages representing the respective developing maturity were determined by conventional pH differential method. And the newly established high throughput DMACA assay was taken to indicate the proanthocyanidin alternations in blackberries. The changing patterns of the two and the potential mechanisms underlying were also discussed here.

### Plant materials

The tetraploid blackberry cultivar 'Arapaho' (ARK631 × ARK883)<sup>29, 30</sup> was used in this study. All fruits were harvested from plants at the orchard of Sichuan

Agricultural University (Sichuan, China) (29°59'10.56"N, 102°58'51.54"E). They were introduced from AFS Academy of Forestry Sciences (China) in 2004. Five typical different maturity stages based on visual appearance of the fruit was investigated referred to the previous report<sup>23</sup>: green, green to red (part green-part red), red, red to black (part red-part black) and black (full black). Fruits were collected from late June to mid-August, 2011. Sample plots were in a completely randomized design with 3 blackberry plants per blot. Berries of each stage were typically about 20 g. Samples were transported to the lab in a cooled container, counted and weighted, followed by immediately frozen before stored in a refrigerator (-80 ± 5°C).

### Reagents and standards

4-dimethylaminocinnamaldehyde (DMACA) and the standard procyanidin B2 (HPLC purity ≥99.5%) was purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemical reagents and solvents were either purchased from Sangon Biotech Co. (Shanghai, China) or from Bioneec-tech Co. (Chengdu, China). They were high-performance liquid chromatography (HPLC) grade or analytical grade quality. Ultrapure Milli-Q water with electrical conductivity of 18.2 MΩ cm<sup>2</sup> obtained through a Millipore Direct-Q™ 5 filter system (Millipore Corp., Bedford, MA) was used throughout this study.

### Anthocyanin extraction

Extractions of anthocyanin from blackberry fruits at different stage followed procedures described by Kao<sup>27</sup> with little adaptation: Five grams of frozen fruit were ground into fine powder in liquid nitrogen to prevent oxidation and divided into two equivalents. One was used for anthocyanin extraction and the other for proanthocyanidin analysis. The powder was transferred and mixed gently with 25 ml of extraction solvent (400 ml of acetone/400 ml of methanol/200 ml of water/10 ml of acetic acid) in a 50 ml polypropylene screw-top centrifuge tube (Sangon, Shanghai, China). Purged

with nitrogen and tightly capped. Secondary, the mixture was incubated in a water bath at 60°C for 1h followed by 3 min sonication (Scientz-II D, Ningbo, China).

After the samples were cooled to room temperature, they were centrifuged at  $13000 \times g$  for 15 min at 4°C (Eppendorf 5804R, Germany) to remove any cell debris. The upper aqueous phase was transferred into a new tube and ready for analyzing instantly.

### Extraction of proanthocyanidin

The other equivalent frozen powders were transferred into a new 50 ml tube and extracted with acetone 75 ml combined with 24.5 ml ultrapure water and 0.5 ml acetic acid as described by Prior *et al.*<sup>31</sup>: Purged with nitrogen and capped tightly, followed by shaking for 1 h at room temperature on a platform shaker (HQL150C, Zhongke, Wuhan, China). Subsequently, all samples were subjected a 3 min sonication (Scientz-II D, Ningbo, China) and centrifuged at  $13000 \times g$  at 20° C for 10 min. (Eppendorf 5804R, Germany). The supernatant was collected for analysis.

### Quantification for total anthocyanin

Total anthocyanin analysis was carried out by using a pH differential method described by Wrolstad *et al.*<sup>32</sup>. A Unico-2102c UV spectrophotometer (Unico, Shanghai, China) and 1 cm pathlength disposable cells were used for spectral measurements. Dilute the samples in series so that the optional values remain in the linear range of the apparatus. Prepare two dilutions of the crude blackberry extract for each developing stage, one with potassium chloride buffer (0.025M, pH 1.0) and the other with sodium acetate buffer (0.4M, pH 4.5) by using a predefined dilution factor. Let all samples equilibrate at room temperature for 15 min. Absorbencies were read at 510 and 700 nm, against a blank cell filled with distilled water. The monomeric anthocyanin pigment concentration in the extract was expressed as cyanidin-3-glucoside, which was proved to be the most abundant anthocyanin component in blackberry<sup>26</sup>. Total anthocyanin content was calculated by using the following formula:

Anthocyanin content ( $\text{mg} \cdot \text{L}^{-1}$ )

$$= \frac{A \times MW \times DF \times 1000}{\epsilon \times l}$$

Where,

$$A = (A_{510\text{nm}} - A_{700\text{nm}})_{\text{pH}1.0} - (A_{510\text{nm}} - A_{700\text{nm}})_{\text{pH}4.5}$$

cyanidin-3-glucoside molar weight (MW) = 449.2, molar absorptivity ( $\epsilon$ ) = 26900 and cell pathlength (l) = 1 cm.

### Measurement of proanthocyanidin

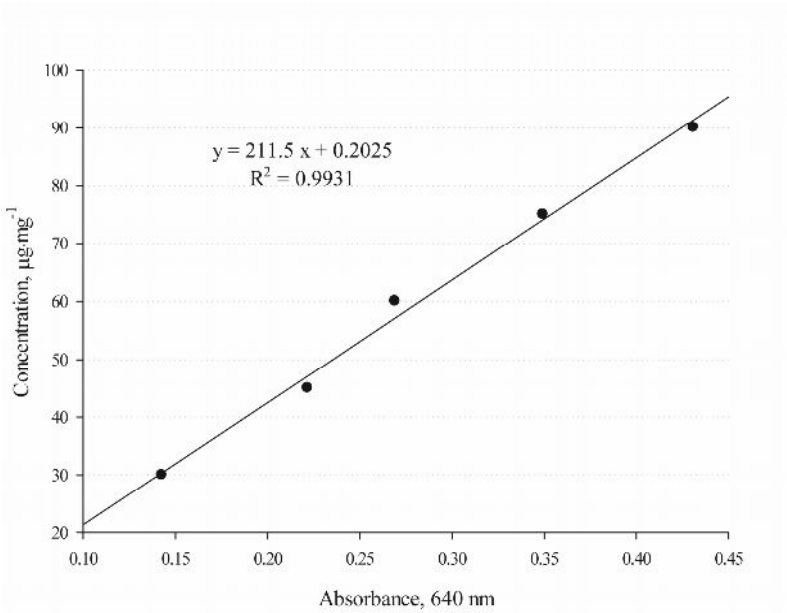
Quantification of proanthocyanidins was performed on a 96-well plate using the validated standard DMACA assay<sup>31</sup>: 70  $\mu\text{l}$  of 80% ethanol (blank), 70  $\mu\text{l}$  samples with a predefined dilution factor or 70  $\mu\text{l}$  serial diluted standard procyanidin B2 was piped into marked wells. Add 210  $\mu\text{l}$  of  $1 \text{ g} \cdot \text{L}^{-1}$  DMACA into each well within 2 min. The plate was read at 640 nm every minute for 30 min by a microplate reader (Bio-Rad model 680 plus 640nm optical filter, Hercules, CA, USA). The peak absorbance which usually occurred after 4 min but before 20 min was obtained. Corrected absorbencies were calculated by subtracting average blank absorbance. A standard curve was generated from the standards. And proanthocyanidin concentrations were then calculated by using the regression equation ( $Y = a + bX$ ) between procyanidin B2 concentration (Y) ( $\mu\text{g}$ ) and the corrected maximum absorbance (X).

### Total anthocyanin and proanthocyanidin per berry

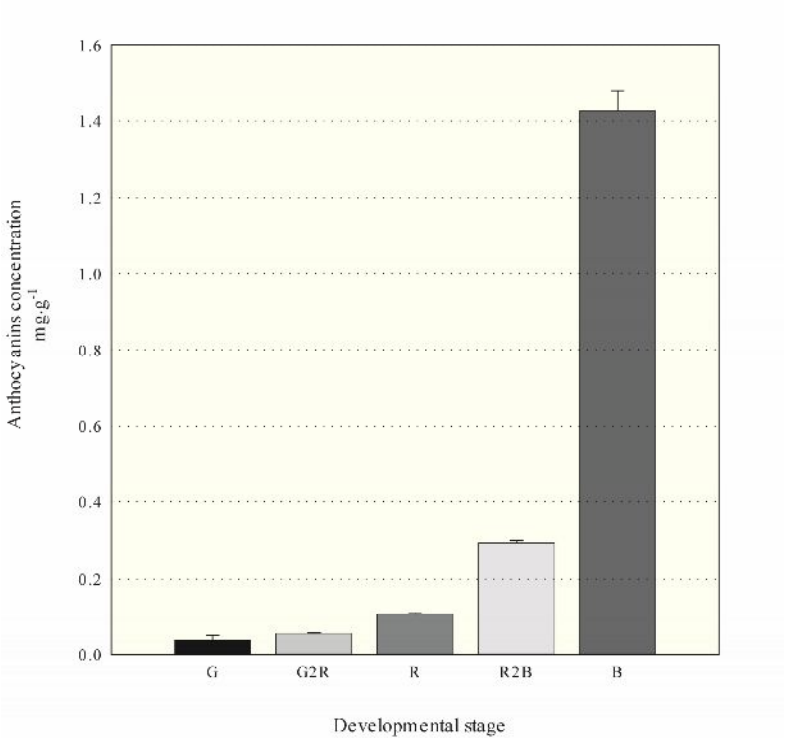
The mean berry weight was computed according to the gross fruits weight for each stage and their total counts. The derived results were used to indicate the individual anthocyanin and proanthocyanidin changes by multiplying the corresponding concentrations.

### Statistics

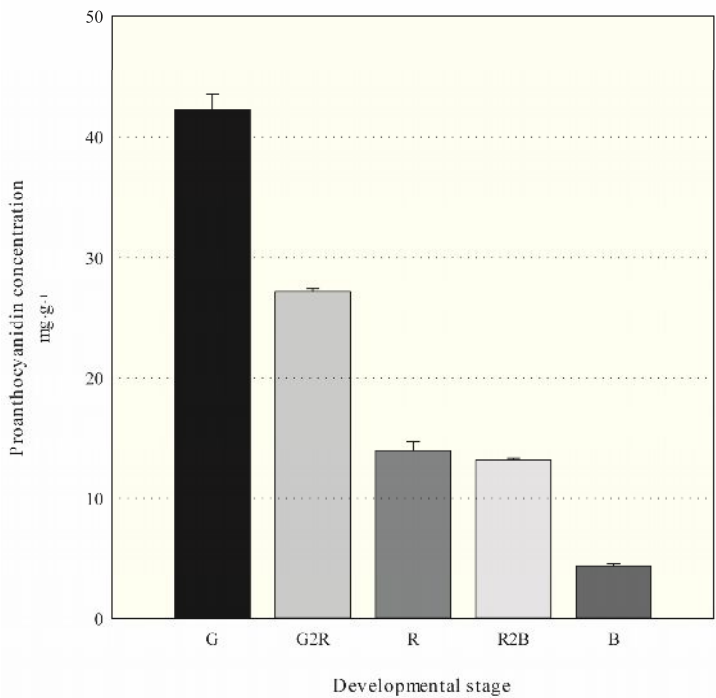
All chemical tests were triplicated and the data collected was analyzed with the SPSS 17.0 for windows software package. Results were expressed as means  $\pm$  standard deviations.



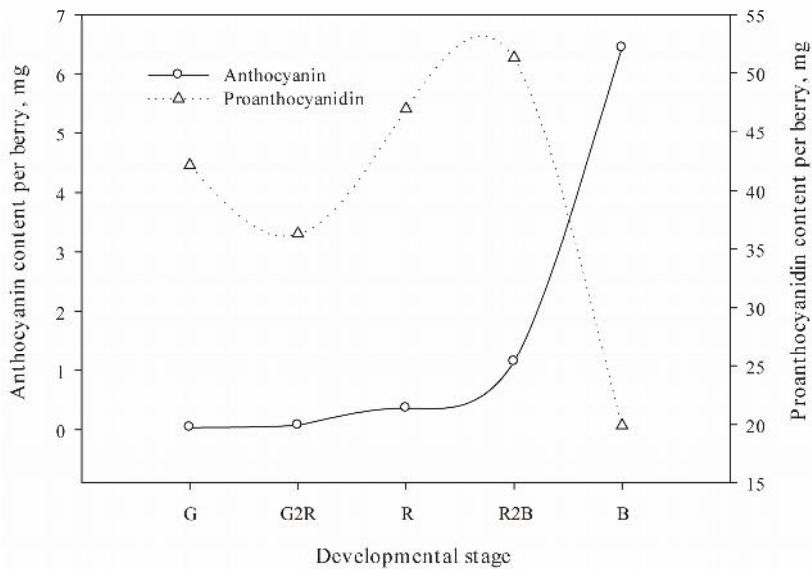
**Fig. 1** Calibration plot for proanthocyanidin determination



**Fig. 2** Anthocyanin concentration in the 5 berry developing stages; G: green; G2R: green to red; R: red; R2B: red to black; B: black



**Fig. 3** Pronthocyanin concentration in the 5 berry developing stages; G: green; G2R: green to red; R: red; R2B: red to black; B: black



**Fig. 4** Change patterns of anthocyanin and proanthocyanidins in a single berry fruit; G: green; G2R: green to red; R: red; R2B: red to black; B: black

## Results and Discussion

Total anthocyanin pigments were not detectable until the fruit reached the red stage. Values increased from 0.106 to 1.426 mg·g<sup>-1</sup> fresh weight (FW) from red to ripen fruit (black) stage. This could be much higher than the total anthocyanin reported for blackberry (0.4 mg·g<sup>-1</sup> FW for 'Arapaho')<sup>27</sup>. Many factors may count for such results, such as sampling differences, sample preparation, measurement variation<sup>25</sup>. Compared with high-performance liquid chromatography<sup>33</sup>, or electrospray mass spectrometry and tandem mass spectrometry<sup>26</sup>, the conventional method based on reversible structural transformation within different pH buffer was much more economic and easier manipulated. It was competent for comparing studies within the same species in terms of total anthocyanin, with reliable indications<sup>34</sup>.

In this study, the newly established high-throughput plate reader based DMACA method was used to determine proanthocyanidins. In 2010, Prior et al.<sup>31</sup> validated a standard proanthocyanidin quantification method and proved to be reproducible, highly specific and accurate in a certain manner. Later, this was tested in selected chocolate and confectionery products by Payne et al.<sup>35</sup> and found to be effective too. With the recommendations that the procyanidin B2 was the preferred standard while using this method<sup>31</sup>, a standard calibration curve was established (Fig.1). Then proanthocyanidins in samples were calculated accordingly. Blackberry proanthocyanidins were previously demonstrated in their ripening stage or products ( $27 \pm 17.5$  mg·100g<sup>-1</sup> FW or 5.33 mg·100ml<sup>-1</sup> juice)<sup>36,37</sup> in the forms of gallocatechin(GC) or catechine(C) or in other. The results of our study indicated that it was much underestimated than one could expect. In the final ripening stage, proanthocyanidins (expressed as procyanidin B2 equivalent) ranked 4.41 mg·g<sup>-1</sup> FW. The highest concentration was found in the early unripen stage, reached 42 mg·g<sup>-1</sup> FW.

The distinct patterns were obtained thus from the data in the five stages. The most characterizing pattern for anthocyanin along with maturity lied in the last stage. Total anthocyanins dramatically accumulated while the

berry turned black (Fig. 2). This was concordant with that was observed in the previous study on blackberry as well as in the reports of other fruit species<sup>38-40</sup>. In contrast, proanthocyanidins demonstrated a gradually decreasing trend (Fig. 3). The highest concentration was found in the early unripen fruit. This was the first report in the fruit of blackberries. This pattern was almost like that was disclosed in strawberries and persimmons<sup>7, 41</sup>. However, the proanthocyanidins changing styles were totally different in the developing grape berries, which exhibited an increasing pattern.

In a single fruit, the anthocyanin growth of 'Arapaho' was monophasic, but was biphasic for proanthocyanidins (Fig 4). By the stage of green to red, gross proanthocyanidins in an individual berry dropped to approximately 2/3 of that in the green stage. Afterwards, proanthocyanidins slightly increased to the maximum level when berries got shiny red and ready to turn black, at a time when anthocyanins remained at a slight increasing level. When it came to the last phase of the fruit ripening, the total Proanthocyanidin content decreased sharply. On the other hand, the anthocyanins dramatically accumulated. The competition of the shared substrates for synthesizing the two compounds may explain such a phenomenon. In the studies of strawberry, genes involving the synthesis of proanthocyanidins had a relative increasing transcription abundance in the turning stage but possessed a significant descending trend in the full ripening stage<sup>39</sup>. However, no molecular evidence had been found so far to explain what had happened in the blackberry fruits, in which a more severe accumulation or decreasing pattern was obtained.

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