

Quantification and Purification of Mulberry Anthocyanins with Macroporous Resins

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Total anthocyanins in different cultivars of mulberry were measured and a process for the industrial preparation of mulberry anthocyanins as a natural food colorant was studied. In 31 cultivars of mulberry, the total anthocyanins, calculated as cyanidin 3-glucoside, ranged from 147.68 to 2725.46 mg/L juice. Extracting and purifying with macroporous resins was found to be an efficient potential method for the industrial production of mulberry anthocyanins as a food colorant. Of six resins tested, X-5 demonstrated the best adsorbent capability for mulberry anthocyanins (91 mg/mL resin). The adsorption capacity of resins increased with the surface area and the pore radius. Residual mulberry fruit juice after extraction of pigment retained most of its nutrients, except for anthocyanins, and may provide a substrate for further processing.

INTRODUCTION

Edible pigments are important food additives which can increase the acceptability of a food product. The safety of synthetic pigments has been questioned, leading to a reduction in the number of permitted colorants [1]. Due to this limitation and to the worldwide tendency towards the consumption of natural products, the interest in natural colorants has increased significantly [2]. Anthocyanins provide attractive colors such as orange, red, and blue. They are water-soluble, which facilitates their incorporation into aqueous food systems. These qualities make anthocyanins attractive natural food colorants. Besides their color attributes, anthocyanins have been reported to be beneficial to health as potent antioxidants and to improve visual acuity [3]. They have also been observed to possess antineoplastic, radiation-protective, vasotonic, vasoprotective, anti-inflammatory, chemo- and hepato-protective activities [3]. The literature provides an abundance of data on most of the plant species considered as potential sources of anthocyanin food colors. Bridle and Timberlake [4] suggested several sources including some of the principal commercially available anthocyanin colorants, that is, grape (*Vitis* sp.), elderberries (*Sambucus nigra*), red cabbage (*Brassica oleracea*), roselle (*Hibiscus sabdariffa*), and other sources including blood orange (*Citrus sinensis*), black chokeberry (*Aronia melanocarpa*), and sweet potato (*Ipomoea batatas*).

In many countries, especially in China, mulberry (*Morus* spp., Moraceae) is used for its foliage to feed the silkworm (*Bombyx mori* L.). As with many other forage

crops, mulberry breeding has focused on enhancing foliage production through heterosis breeding [5]. Mulberry fruit is rich in anthocyanins and can be considered as a potential source for production of a natural red food colorant. The major anthocyanins identified in the fruit extract are cyanidin 3-glucoside and cyanidin 3-rutinoside [6]. Due to its small size, relatively low output, and short storage life, this fruit received relatively little attention. However, application of new technologies in breeding and processing may offer a commercially profitable production of anthocyanins from mulberry fruit.

Adsorbent resins are durable nonpolar or slightly hydrophilic polymers having high adsorption capacity with possible recovery of the adsorbed molecules, relative low cost, and easy regeneration. They are currently used for adsorption of flavonoids and other components extracted from many plants. Nonpolar styrene-divinylbenzene (SDVB) resins have been used to recover hesperidin from citrus peel or the wastewater flowing from centrifuges of essential oil separation (yellow water) [7, 8] and anthocyanins from pulp wash of pigmented oranges [9]. They have also been used to remove naringin and limonin from citrus juices and to recover cold pressed grapefruit oil from wastewater [10]. Resins are effective adsorbent material for anthocyanins from different sources and have been widely used in research and in the production of anthocyanins [4, 9, 11, 12, 13].

The objective of this study was to evaluate the relative anthocyanin contents in different cultivars of mulberry, and to investigate a potential industrial process for the extraction of mulberry anthocyanins for use as a natural

TABLE 1. Physical and chemical properties of resins.

Resin	Pore radius (Å)	Surface area (m ² /g)	Particle diameter (mm)	Polarity
D3520	85 ~ 90	480 ~ 520	0.3 ~ 1.25	Nonpolar
D4020	100 ~ 105	540 ~ 580	0.3 ~ 1.25	Nonpolar
X-5	290 ~ 300	500 ~ 600	0.3 ~ 1.25	Nonpolar
NKA-9	155 ~ 165	250 ~ 290	0.3 ~ 1.25	Polar
AB-8	130 ~ 140	480 ~ 520	0.3 ~ 1.25	Low polar
D101A	90–100	500–550	0.3 ~ 1.25	Nonpolar

food colorant. Six resins, having different chemical and physical properties, were investigated for adsorption of mulberry anthocyanins.

MATERIALS AND METHODS

Plant material and sample treatment

Samples for the evaluation of anthocyanin content in various cultivars

Mulberry fruits were obtained from South China Mulberry Resource Garden in Dafeng Agricultural Experimental Base of the Guangdong Academy of Agricultural Sciences (Guangdong, China). For each cultivar, 250–300 g fully mature mulberry fruits were harvested and stored at 4°C until further treatment. All treatments were performed on the day of harvesting. After weighing, mulberry fruits were mixed in an electric blender, filtered with a nylon filter cloth, and centrifuged at 5000g for 15 minutes. The supernatant was collected and assayed for anthocyanin content, soluble solid substances, and total acid content.

Samples for evaluation of adsorbent capacities of resins

Mulberry fruits for juice production and anthocyanin purification were obtained from two mulberry bases: Doumen Base and Huadou Base, situated in Doumen District, Zhuhai and Huadou District, Guangzhou, China, respectively. Fruits were collected at the fully mature stage. In order to maintain freshness, mulberry juice factories were constructed in the vicinity of the mulberry bases. Mulberry fruits were rinsed with 0.9% saline water and then by tap water, before being squeezed. The resulting juice was centrifuged, pasteurized, and stored in sterilized bags at 4°C (for immediate further processing) or at –18°C (for later processing).

Resins: their pretreatment and activation

The resins tested were D3520, D4020, X-5, NKA-9, D101A, and AB-8 (Chemical Industrial Company affiliated to Nankai University, Tianjin, China). All resins were cross-linked polystyrene copolymers. Their physical and chemical properties are shown in Table 1.

The resins were pretreated and activated according to the manufacturer's recommendation. Firstly, they were rinsed with distilled water and filtered with nylon filter cloth to retain those with a particle diameter larger than

0.3 mm. They were then soaked overnight in 2 bed volumes (BV) of 95% ethanol. After soaking, the resins were introduced into a glass column and rinsed with a further 2 BV of 95% ethanol. Subsequently they were rinsed with 2 BV of distilled water to dispel the ethanol, 1 BV of 4% (w/v) sodium hydroxide, 2 BV of distilled water, 1 BV 4% (v/v) hydrochloric acid, and finally by distilled water until the pH of the eluent became neutral.

Purification of mulberry anthocyanins

Raw mulberry juice was centrifuged at 5000g for 15 minutes to produce a bright (nonturbid) supernatant. Different volumes of the supernatant were passed through resin columns depending on the adsorbent capability of each resin. Anthocyanins and other phenolics were adsorbed onto the column; sugar, acids, and other water-soluble compounds were eluted with more than 2 BV of distilled water until the wash water was clear. The adsorbed material was then eluted with acidified ethanol (0.5% (v/v) of hydrochloric acid) until there was no color in the eluent. The eluent was concentrated on a rotary evaporator under reduced pressure at 60°C and the resulting concentrate was lyophilized to form a pigment powder.

In order to select the best resin for capturing mulberry anthocyanins, the anthocyanins in the eluates were tested spectrophotometrically as a function of time. The resins were considered to be saturated when, during column loading, the anthocyanin contents of the juice and the eluent were equal.

To determine the optimum ethanol concentration for elution of the adsorbed anthocyanins, 5 g aliquots of resin saturated with anthocyanins were added to a range of 250 mL flasks with different concentrations of acidified ethanol, and incubated on a shaker for 1 hour at 25°C, 125 rpm. The contents of anthocyanins in these eluates were measured spectrophotometrically.

Total anthocyanin content

The total anthocyanin content was determined using the pH differential method [14]. An F755B UV spectrophotometer (Shanghai, China) and 1 cm path length disposable cuvettes were used for spectral measurements at 420, 538, and 700 nm. Pigment content was calculated as cyanidin 3-glucoside (cyd 3-glc), using an extinction coefficient of 29 600 L cm⁻¹ mg⁻¹ and molecular weight of 448.8.

Determination of pH, titratable acidity, and soluble solid contents

The pH measurements were made using a digital pH meter (pB-20, Sartorius, Germany) calibrated with pH 4 and 7 buffers. Titratable acidity was measured by the titrimetric method. Titratable acidity of mulberry was expressed as % citric acid. Soluble solid contents were expressed as refractive index and were measured with a 2 WAJ Abbe refractometer (Shanghai, China).

HPLC analysis of mulberry anthocyanins

The HPLC analysis of anthocyanins was performed as described by Konczak-Islam et al [15]. The pigment solutions were filtered through a 0.45 μm syringe-driven filter unit (Millipore Corporation Bedford, Mass). The HPLC system consisted of two LC-10AD pumps, SPD-M10A diode array detector, CTO-10AS column oven, DGV-12A degasser, SIL-10AD autoinjector, and SCL-10A system controller (Shimadzu, Japan) equipped with Luna (3 μm C18(2), 4.6 mm \times 100 mm, Phenomenex, Calif) column at 35°C. The following solvents in water with a flow rate of 1 mL/min were used: A (1.5% phosphoric acid) and B (1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile). The elution profile was a linear gradient elution with 25%–85% solvent B in solvent A for 100 minutes. The chromatograms were monitored at 530 nm and recorded, and the relative concentrations of individual pigments were calculated from the peak areas.

RESULTS AND DISCUSSION

Total anthocyanin content in different cultivars of mulberry

Thirty-one cultivars of mulberry were analyzed with respect to their fruit weight, soluble solid substances ($^{\circ}\text{Brix}$), total acids, and total anthocyanins. These characteristics varied between cultivars despite all samples having been collected at a mature stage and from the same place (Table 2). The total anthocyanin content of the evaluated cultivars varied between 147.68 (cultivar Yuiyou-26) and 2725.46 mg/L juice (cultivar 7403). The anthocyanin content was also found to depend on climate and production area. It was observed that anthocyanin content of the same mulberry cultivar was much higher on a sunny day than on a rainy day (data not shown). As was observed with respect to the anthocyanin content, other mulberry fruit parameters such as average fruit weight, soluble solid substances, and total acids varied across the different cultivars studied.

Adsorbent capability of different resins to mulberry pigments

The single strength juice was used as the starting material for the production of purified mulberry pigment. The columns were loaded with juices to obtain a complete

saturation, rinsed with deionized water, and subsequently eluted with acidified ethanol solution. The adsorption capacities of the resins were evaluated based on the spectrophotometrical measurement of anthocyanins levels in the eluates (Figure 1). The 6 resins, ranked according to their adsorption capacities (highest to lowest), were as follows: X-5>AB-8>D4020>D101A>D3520>NKA-9. The ranking appears to reflect the different physicochemical properties of these resins.

Among the nonpolar resins tested, the mulberry anthocyanin adsorbing capabilities primarily depended on the pore radius and, to a lesser extent, on the surface area. Resin X-5, with the largest pore radius (290–300 Å) and surface area (500–600 m^2/g), had the best adsorption capability. Its total adsorption capacity (91 mg/mL of resin) was more than double that of any other resin. The total adsorption capacity of the X-5 resin evaluated under the conditions of this experiment was more than 100 times that reported by Di Mauro et al [9]. Similarly the nonpolar resin D3520, with the smallest pore radius (85–90 Å), showed the second lowest adsorption capacity and it is suggested that too small pore size did not allow penetration of anthocyanins into the reticulate of resin [9]. NKA-9, the only polar resin tested and with the smallest surface area (250–290 m^2/g), had the lowest mulberry anthocyanin adsorption capacity.

These findings are consistent with the results reported by Di Mauro et al [9] although there are some differences in the exact ranges of pore radius and surface area studied.

Eluent efficacy of different concentrations of acidified ethanol

In devising a potential industrial production process for mulberry anthocyanins as an edible colorant, it is necessary to take into consideration not only the resin, but also the effluent solvent and its concentration. As shown in Figure 2, the acidified ethanol could effectively elute anthocyanins from the resin at concentration higher than 30% (v/v). The solutions with low concentrations of ethanol have a higher boiling point and are therefore more difficult to concentrate than those with high concentrations. In order to design an accelerated anthocyanin recovery process, a higher concentration of ethanol is recommended.

Elution of mulberry pigment with acidified ethanol

Acidified 80% (v/v) ethanol was found to be able to elute most of the anthocyanins adsorbed by the resin. Using this eluent, the overall recovery rate was higher than 99%. Of a total of 600 mL eluent, the first 150 mL contained only 1.5% of the total anthocyanins (due to washing water still present in resins); the middle 250 mL contained 96%, while the last 200 mL contained 2.5% (Figure 3). Although high concentrations of ethanol can effectively elute the pigment adsorbed by the resins, the product cannot be completely dissolved in the water, suggesting a presence of some impurities. In order to

TABLE 2. Average fruit weight, content of soluble solid substances, total acids, and total anthocyanins in fruit of various mulberry cultivars.

Cultivar	Average fruit weight (g)	Soluble solid contents ($^{\circ}$ Brix)	Titrateable acids (g/L)	Total anthocyanins (mg/L)
7403	3.49	10.00	7.30	2725.46
7848	4.09	7.75	4.51	1033.79
Bei-1-13	5.34	3.25	1.87	1496.99
Bei-2-5	7.20	8.75	5.81	792.13
Bei-2-8	8.64	11.75	5.40	1419.79
Bei-2-17	N/A	6.90	2.97	399.42
Bei-3-5	5.02	11.50	2.66	909.60
Da-10	4.83	11.50	4.70	1533.91
Guiyou-10-19	7.68	10.50	3.96	1594.26
Guiyou-15	6.22	8.25	4.84	976.73
Guiyou-46	4.53	6.00	3.46	322.22
Guiyou-70	6.46	6.50	4.57	496.76
Guiyou-75	N/A	7.80	3.71	651.16
Guo-1	4.27	10.00	1.73	594.10
Guo-2	4.98	9.75	1.79	1758.79
Heipisang	7.18	5.75	7.11	1218.40
Miao-66	N/A	9.80	4.33	771.99
Nanjian-6	3.76	10.75	1.07	1379.51
Shangshan-6	4.00	10.25	3.03	1496.99
Tang-10	3.91	10.5	5.13	1382.87
Xuan-27	7.89	9.00	7.36	882.75
Yueyou-18	7.82	9.50	3.25	1319.10
Yueyou-25	4.91	11.50	2.59	1033.79
Yueyou-26	5.72	8.50	3.94	147.68
Yueyou-32	4.77	10.75	3.40	1507.06
Yueyou-34	6.98	7.00	5.44	1084.14
Yueyou-36	N/A	9.70	2.60	1322.45
Yueyou-46	5.86	7.50	2.63	808.91
Yueyou-51	6.26	9.75	2.70	1362.73
Yueyou-87	7.38	9.00	2.21	758.56
Zhan-1432	5.86	7.25	3.52	1060.65

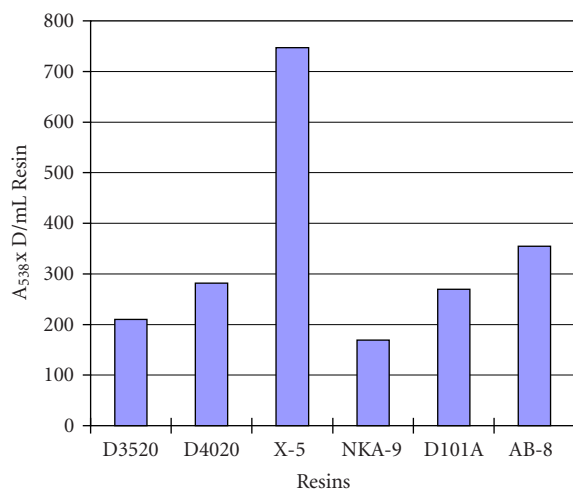


FIGURE 1. The mulberry pigment adsorbent capabilities of different resins (D = dilution factor).

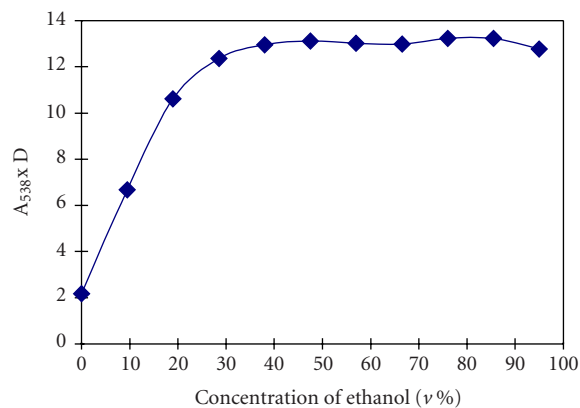


FIGURE 2. The elution efficacy of different concentrations of acidified ethanol (D = dilution factor).

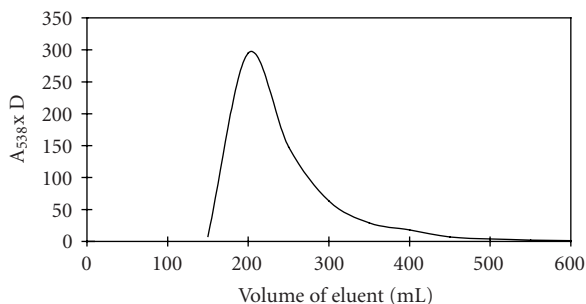


FIGURE 3. The elution of anthocyanins with acidified 80% (v/v) ethanol applied to mulberry pigment adsorbed onto resin ($D =$ dilution factor).

obtain a water-soluble pigment, different concentrations of acidified ethanol (10~80%) (v/v) were evaluated and the resulting eluent was concentrated, dried, and solubility in water was tested. The results showed that pigment eluted with less than 30% (v/v) acidified ethanol was completely water-soluble; however, it accounted for about 80% of the total weight of pigments.

Comparison of mulberry pigment before and after purification with resin

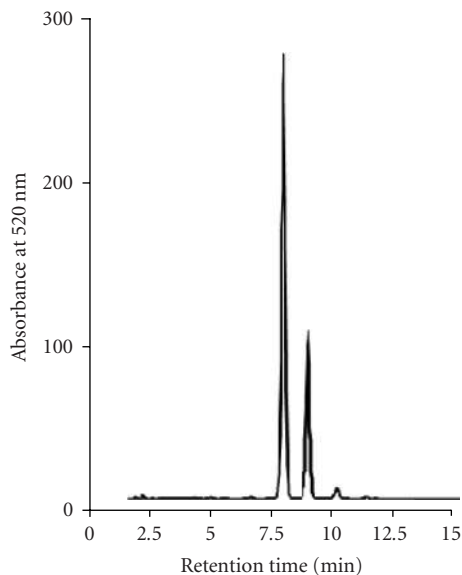
Crude mulberry juice is rich in anthocyanins and, after concentration, can be directly used as a natural pigment. However, the concentration of anthocyanins in such juice is low, with a color value usually less than 4.0. That is due to the presence of nonpigment components such as mono-, di-, and polysaccharides, minerals, proteins, or organic acids in large proportions. Instead of the concentrated juice, pigment powder can be produced by spray drying, but the resulting product is very hygroscopic, becomes sticky and hard to dissolve in water. These products have a relatively short shelf life (data not presented).

Contrary to the crude mulberry juice, the eluent of pigment purified with resins can be easily concentrated to obtain a high color value, usually more than 100, and lyophilize easily. During the purification with resins, most of the impurities are removed. Removal of the impurities can also decrease the enzymatic and the nonenzymatic reaction causing the browning of pigment and thus increase the stability of potential food colorant.

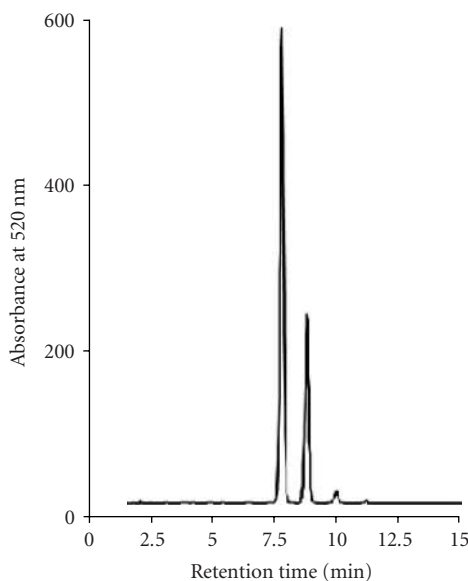
When tested by HPLC under the same conditions (520 nm), the HPLC profiles of the purified and crude pigment extracts were identical, consisting of four peaks (Figure 4). This suggests that pigment purification using the resins did not change the composition of the anthocyanin mixture.

Comparison of raw mulberry juice before and after extracting mulberry pigment with resin

Besides the anthocyanin pigments the crude mulberry juice contains also other components. If in an industrial setting the crude juice was used only for the production



(a)



(b)

FIGURE 4. HPLC chromatogram of mulberry anthocyanins (a) before purification (crude pigment); and (b) after purification (purified pigment).

of pigment, environmental waste would be generated. We have evaluated the total sugar, total acids, total anthocyanins, some vitamins, and the pH of the mulberry juice before and after purification with X-5 resin under unsaturated adsorption conditions (Table 3).

The results indicated that almost all anthocyanins were adsorbed by the resins, while total sugar, total acids, and vitamins (vitamin B1) remained in the raw juice

TABLE 3. Components in raw mulberry juice before and after purification with X-5 resin.

Components	Before	After	Retained proportion (%)
pH	4.70	4.65	—
Total acids (g/L)	7.10	5.86	82.5
Total soluble substance (%)	11.80	11.00	93.2
VB1(mg/L)	27.92	27.45	98.3
Total anthocyanins (mg/L)	384.07	11.52	3.0

under unsaturated adsorption. Therefore, it can be considered after removal of the anthocyanins the residual juice to be fermented in order to produce products such as juice, wine, and sauce, enhancing the overall value of the mulberry fruit.

Our results indicate that the utilization of mulberry anthocyanins as a natural food colorant is possible and it may enhance the overall profitability of mulberry plant from being only the source of foliage for silkworm to a promising pigment source for food applications.

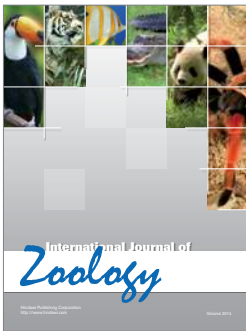
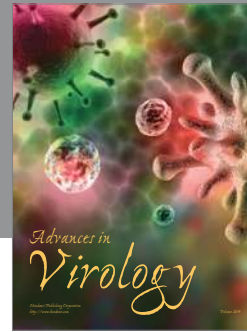
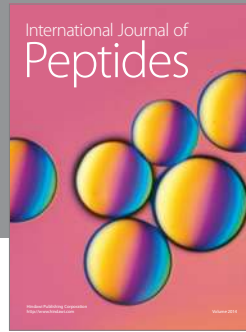
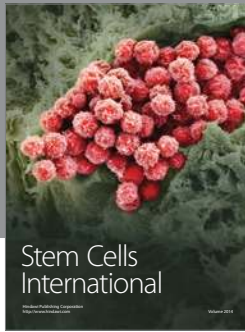
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