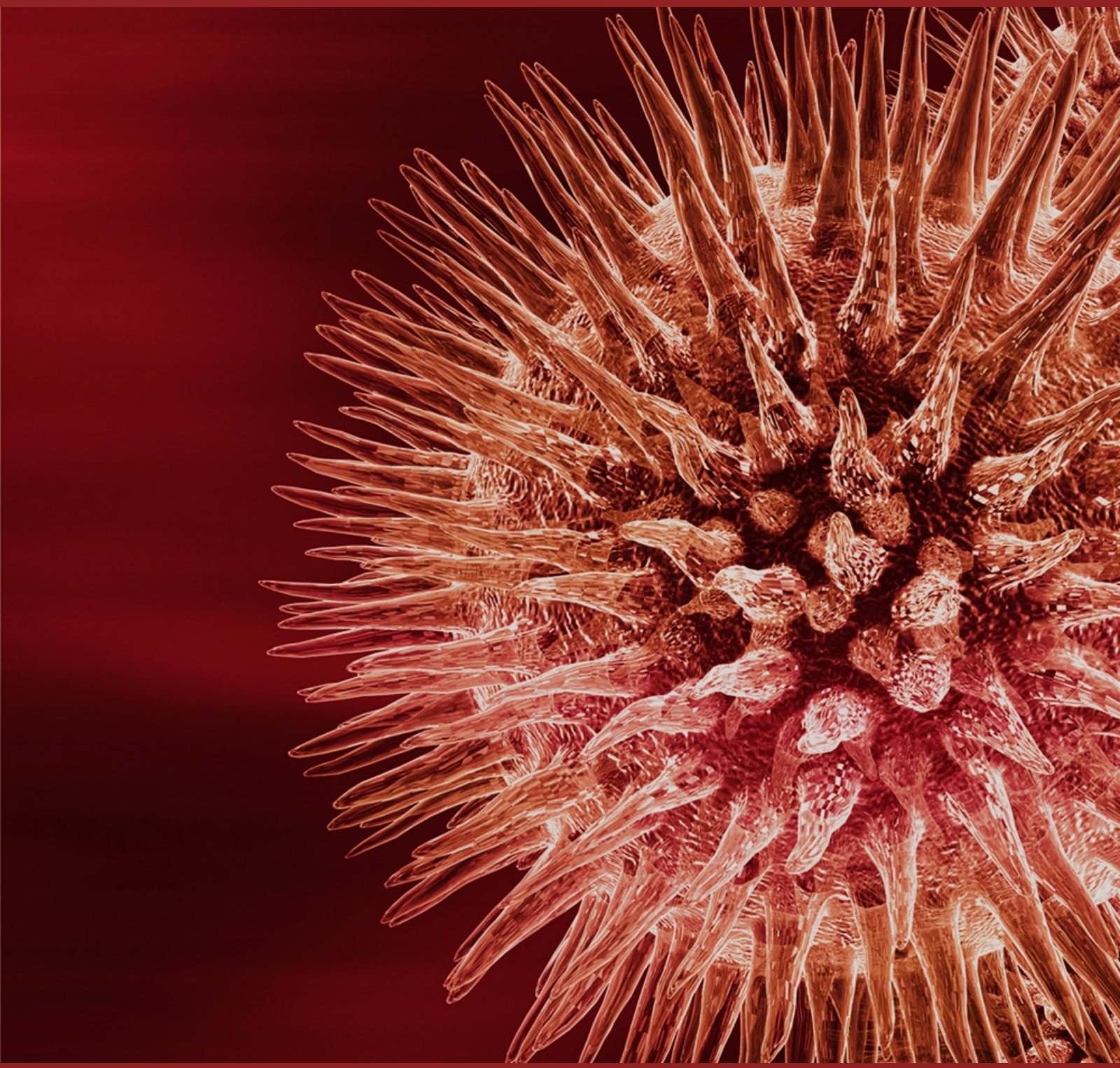
Journal of Biomedicine and Biotechnology

Anthocyanins—More Than Nature's Colour

Guest Editors: Izabela Konczak and Wei Zhang



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EDITORIAL

Anthocyanins—More Than Nature's Colours

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Research over the past decade has produced incontrovertible evidence for a vast array of health benefits arising from the consumption of fruits and vegetables. In an endeavor to identify the active health-promoting ingredients, many researchers have focused on the properties of the flavonoids, a large class of phenolic compounds that is abundant in such foods. Most prominent among the flavonoids are the anthocyanins—universal plant colorants responsible for the red, purple, and blue hues evident in many fruits, vegetables, cereal grains, and flowers. Represented by over 600 molecular structures as identified to date, anthocyanins are of particular interest to the food colorant industry due to their ability to impart vibrant colours to the product. Now it seems highly likely that they also enhance the health-promoting qualities of foods.

Anthocyanins were incorporated into the human diet many centuries ago. They were components of the traditional herbal medicines used by North American Indians, the Europeans, and the Chinese, and were habitually derived from dried leaves, fruits (berries), storage roots, or seeds. Anthocyanin-rich mixtures and extracts (though not purified compounds) have been used historically to treat conditions as diverse as hypertension, pyrexia, liver disorders, dysentery and diarrhoea, urinary problems including kidney stones and urinary tract infections, and the common cold. They have even been purported to yield improvements to vision and blood circulation.

Recent studies using purified anthocyanins or anthocyanin-rich extracts on in vitro experimental systems have confirmed the potential potency of these pigments. Demonstrable benefits include protection against liver injuries; significant reduction of blood pressure; improvement of eyesight; strong anti-inflammatory and antimicrobial activities; inhibition of mutations caused by mutagens from cooked food; and suppression of proliferation of human cancer cells. Along with other phenolic compounds, they are potent scavengers of free radicals, although they can also behave as pro-oxidants. Because of their diverse physiological activities, the consumption of anthocyanins may play a significant role in preventing lifestyle-related diseases such as cancer, diabetes, and cardiovascular and neurological diseases.

Many questions remain. We do not know, for example, whether these apparent health benefits stem from anthocyanins alone, or from their synergistic interactions with other phenolic compounds. Are the health-promoting qualities of anthocyanin-phenolic mixtures preserved across the various food systems? What is the fate of anthocyanin molecules after consumption? Reports on bioavailability of anthocyanins indicate that less than 1% of consumed anthocyanins is detectable in human plasma and urine. Are the health-protective qualities observed in in vitro studies also displayed in vivo? If so, what might be the mechanism of the biological activity of anthocyanins?

The Third International Workshop on Anthocyanins organized by the Cooperative Research Centre for Bioproducts and Food Science Australia in Sydney, Australia, January 27–29, 2004, provided a forum for discussing the nutritional, physiological, and therapeutical functions of anthocyanins, and the opportunities for development of novel anthocyanin-based functional foods in compliance with regulatory requirements. Through scientific presentations and dialogue among researchers, industry managers, and invited consumers, one aim of the workshop was to popularize the application of anthocyanins as natural food colorants with nutraceutical qualities. Biotechnological progress in meeting the requirements of the food colorant industry and consumers, such as in the genetic engineering for production of selected anthocyanins with enhanced stability and/or health-beneficial properties, was described. Plant cell cultures were suggested as an excellent research tool to explore the "anthocyanin enigma" wherein interactions between anthocyanins and other phenolic compounds or metals can facilitate or even enhance the physiological activities of anthocyanin-rich extracts. Indeed, insightful comparisons were drawn between the effects of anthocyanins on animal cells and their native functions in plant cells. Display and degustation of anthocyanin-based food products was provided by Wild

(Germany), Nutrinova Australia Ltd, Kingfood Australia Ltd, Tarac Technologies Ltd (Australia), and The Natural Confectionery Co (Australia), and served as an encouraging example to both researchers and industry managers through their search for novel anthocyanin-based food products promoting good health.

The program of IWA2004 (International Workshop on Anthocyanins) offered 6 plenary lectures and 19 oral presentations in sessions covering anthocyanins in plant cells—function, biosynthesis, and regulation; application of plant cell cultures and bioprocessing for accumulation of anthocyanins with enhanced commercial/health properties; health beneficial effects of anthocyanins; development of anthocyanin-based functional foods; and anthocyanins and the mystery of red wine color. The presentations were accompanied by 26 posters. We would like to thank 170 authors from 13 countries (Australia, China, Finland, France, India, Japan, Germany, Nepal, New Zealand, Portugal, Taiwan, UK, and USA) for their contributions to the program of IWA2004.

This *special issue* of the *Journal of Biomedicine and Biotechnology* combines selected works presented at IWA2004. It reflects the diversity in presentations and discussion, and aims to disseminate information gathered during the workshop. We thank 63 authors of the submitted papers for their contribution. The preparation of this special issue would not have been be possible without the generous support of 40 experts in various areas of anthocyanin research coming from 19 countries, who extensively evaluated the manuscripts submitted and through their constructive questions and suggestions significantly contributed towards the present form of this issue of the *Journal of Biomedicine and Biotechnology.*

Izabela Konczak
Wei Zhang

Izabela Konczak is a Research Scientist with the Food Ingredients and Functionality team of Food Science Australia, Sydney. Her recent projects focus on application of biotechnology for production of selected secondary metabolites with potential application in food and pharmaceutical industries as well as discovery of novel bioactive phytochemicals, studies on their physiological activities, bioavailability, and possible application as nutraceuticals. With 4 years of professional experience in Europe (Poland), followed by over 10 years of postdoctoral research in Japan, New Zealand, Australia, and USA, she has developed interdisciplinary skills across biotechnology, including cell line development, accelerated accumulation of specific secondary metabolites in plant cell cultures, biotransformation, bioprocessing, and genetic modification. She is the author of commercially applied plant cell cultures based technology for rapid multiplication of Sandersonia hybrids (New Zealand) and the Coinventor of technology for production of anthocyanins in sweet potato cell cultures (patents in Japan and Australia). After joining Food Science Australia and the Cooperative Research Center for Bioproducts in the year 2000, she initiated and coorganized the International Workshop on Anthocyanins series aiming at popularizing the knowledge on health-preventive properties of anthocyanin-rich extracts of fruits and vegetables and their enhanced application as natural food colorants and ingredients of functional food products.

Wei Zhang was born on 11 April 1969. He has been a Biochemical Engineer since 1989 and received his PhD degree in biochemical engineering from Dalian Institute of Chemical Physics, Chinese Academy of Sciences, in 1994. He has a strong theoretical and experimental expertise in the areas of integrated bioprocessing, membrane bioreactor, plant cell culture, immobilized cell system, modelling and supercomputer simulation of bioprocesses as well as marine bioproducts engineering. His postdoctoral research career has brought him to work in a number of top universities and institutions including the University of Tokyo (Japan), the University of Cambridge (UK), and Cornell University (USA). As a result, he has published a total of over 100 publications in refereed journals and conferences (in more than 80% as first author). His international experience includes working in China, Korea, Japan, Australia, UK, and USA that brings enormous opportunities for international R&D collaborations. He has been involved in organizing committees of several international conferences as member or chair. He has been giving seminars and presentations in various national and international conferences and research laboratories. He is currently a Senior Lecturer in bioprocessing and Head of Plant Cell Culture Laboratory at the CRC for Bioproducts, Flinders University.

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RESEARCH ARTICLE

LC/PDA/ESI-MS Profiling and Radical Scavenging Activity of Anthocyanins in Various Berries

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Anthocyanin extracts of two blueberries, *Vaccinium myrtillus* (bilberry) and *Vaccinium ashei* (rabbiteye blueberry), and of three other berries, *Ribes nigrum* (black currant), *Aronia melanocarpa* (chokeberry), and *Sambucus nigra* (elderberry), were analyzed by high-performance liquid chromatography coupled with photodiode array detection and electrospray ionization - mass spectrometry (LC/PDA/ESI-MS). Both bilberry and rabbiteye blueberry contained 15 identical anthocyanins with different distribution patterns. Black currant, chokeberry, and elderberry contained 6, 4, and 4 kinds of anthocyanins, respectively. The radical scavenging activities of these berry extracts were analyzed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH). All these extracts showed potent antiradical activities.

INTRODUCTION

Anthocyanins (Figure 1) are representative plant pigments widely distributed in colored fruits and flowers. They also exhibit antioxidant activities and therefore may contribute to the prevention of heart disease, cancer, and inflammatory disease [1, 2, 3, 4, 5, 6]. Berries have been known to contain anthocyanin pigments abundantly and thus have been used globally as a medicine or a source of health food/dietary supplement. The amounts and distribution of anthocyanins in the berries differ depending on their plant species, cultivation conditions, and producing districts. Consequently, the antioxidant activity may be different among various berry extracts, in particular, the berry anthocyanin extracts in the commercial market. Although a number of researches for anthocyanin profiling and antioxidant activity of various berries have been reported [7, 8, 9, 10], comprehensive comparison of various berry anthocyanin extracts circulating in the Japanese market is still worth investigation.

In the present study, the extracts of two blueberries, *Vaccinium myrtillus* (bilberry) and *Vaccinium ashei* (rabbiteye blueberry), and three other berries, *Ribes nigrum* (black currant), *Aronia melanocarpa* (chokeberry), and *Sambucus nigra* (elderberry), were analyzed by high-performance liquid chromatography (HPLC) coupled with photodiode array detection and electrospray ionization - mass spectrometry (LC/PDA/ESI-MS). Bilberry is one of a lowbush wild blueberry found in Northern Europe natively. Its extract has been recognized as a medicine in Italy in the field of ophthalmology [11, 12]. Rabbiteye blueberry is a cultivated species for eating raised in relatively warm regions like Japan. Oral intake of a mixture of black currant anthocyanins, prepared from black currant fruit, has been claimed to be beneficial to visual functions [13]. Elderberry extract was reported to possess some anti-inflammatory activity [14] and antivirus activity against influenza viruses [15]. LC/PDA/ESI-MS, a powerful tool for the analysis of anthocyanins [7, 16, 17], allowed the simultaneous determination of all anthocyanins in plant extracts. We used LC/PDA/ESI-MS for comprehensive profiling of anthocyanins in berry extracts. The radical scavenging activity was also examined in those berry extracts.

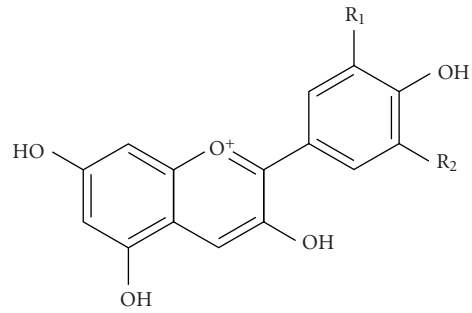

FIGURE 1. Structural formula of anthocyanidins. Cyanidin, R_1 = OH, R_2 = H; delphinidin, R_1 = R_2 = OH; peonidin, R_1 = OCH_3, R_2 = H; petunidin, R_1 = OCH_3, R_2 = OH; malvidin, R_1 = R_2 = OCH_3.

MATERIALS AND METHODS

Reagents

Chemicals and HPLC solvents were purchased from Sigma-Aldrich, Wako Pure Chemical Industries, Ltd, or Kanto Kagaku, and were of at least analytical grade. Anthocyanin standards (delphinidin chloride, cyanidin chloride, cyanidin 3-glucoside chloride, peonidin chloride, peonidin 3-glucoside chloride, malvidin chloride, malvidin 3-glucoside chloride, and malvidin 3-galactoside chloride) were purchased from Extrasynthese.

Preparation of extracts

Commercially frozen fruits (300 g) of bilberry, rabbiteye blueberry, and black currant were purchased. The fruits were homogenized in 450 mL of 90% ethanol (0.1% H_2SO_4) and stirred overnight at room temperature. After centrifugation at 3 000 rpm for 5 minutes, the supernatants were filtered and applied to a column of nonionic polymeric absorbent (Amberlite XAD-7, Rohm and Haas, Philadelphia, Pa) followed by washing with water. Anthocyanin fraction was then collected by elution with aqueous ethanol (0.05% citric acid). Commercial juice concentrates of chokeberry and elderberry were also purchased and applied to the purification steps as above. Purified fractions obtained from the five berries containing anthocyanins were concentrated and freeze-dried to powder.

Total anthocyanidin contents in berry powders

The powders of each berry extract containing anthocyanins dissolved in methanol (2% HCl) were hydrolyzed at 80°C for 30 minutes to obtain corresponding anthocyanidins. Peak values of absorbance around 500–600 nm of each anthocyanidin solution were detected using UV-1700 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) to determine the anthocyanidin amounts as delphinidin equivalents.

LC/PDA/ESI-MS analysis of anthocyanins in berry extracts

Powders of each extract were dissolved in H_2O to the concentration of 2 mg/mL followed by filtration with 0.45 μm nylon membrane and applied to LC/PDA/ESI-MS analysis [17]. The analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, Calif) using a Capcell Pak C18 UG120 5 mm column (4.6 mm × 150 mm, Shiseido, Tokyo, Japan) followed by a Finnigan LCQ^{DECA} mass spectrometer with electron spray ionization source (Thermo Electron, San Jose, Calif). HPLC conditions were as follows: solvent A, 0.1%TFA/H_2O; solvent B, 50%CH_3CN/0.1%TFA/50%H_2O; linear gradient, initial percentage of B (15%) to 60 minutes (30%); column temperature, 40°C; flow rate, 0.5 mL/min. Ultraviolet-visible absorption spectra of anthocyanins were detected by a photodiode array detector (PDA) in the range of 250–600 nm. MS parameters were as follows: ionization mode, positive; sheath gas, nitrogen; capillary temperature, 320°C; capillary voltage, 5.0 kV; full scan acquisition, from 50 to 1000 *m/z* at 2 scan/s. Tandem MS analysis was carried out with helium as the collision gas.

Measurement of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The method reported by Blois [18] was generally followed. Each berry powder was dissolved and diluted in ethanol at concentrations of 2.0, 1.0, 0.5, 0.2, and 0.1 mg/mL. Fifty μL of the diluted extracts were added to 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) ethanol solution (100 μM) and left to stand for 5 minutes at room temperature followed by measurement of the absorbance of the resulting solutions at 517 nm using the spectrophotometer described earlier. The DPPH radical scavenging activity obtained by each berry powder was compared with that of Trolox, an analog of vitamin E.

RESULTS AND DISCUSSION

Anthocyanin contents of five berry extracts were analyzed using LC/PDA/ESI-MS. Figure 2 shows a typical chromatogram of the anthocyanins on PDA with selective wavelength (500–550 nm absorption). Extracts of bilberry and rabbiteye blueberry showed 13 identical peaks, although their intensities were different (Figures 2a and 2b). Six, four, and two peaks were obtained from the extracts of black currant, chokeberry, and elderberry, (Figures 2c, 2d, and 2e).

The peaks were also analyzed and identified continuously by mass spectrometry. Anthocyanins of bilberry extract were confirmed to contain five anthocyanin aglycons, that is, delphinidin, cyanidin, petunidin, peonidin and malvidin (Figure 1). Mass chromatograms of each aglycon (Figure 3) indicated that bilberry and rabbiteye blueberry possessed 15 anthocyanins; nevertheless, the extracts showed only 13 peaks on the PDA chromatogram. This is due to the coelution of two different metabolites. Namely, peak numbers 16 and 19 on PDA chromatogram (Figures 2a and 2b) each consisted of two independent anthocyanins, pceonidin 3-glucoside (16a) and malvidin 3-galactoside (16b) on peak 16, and peonidin 3-arabinoside (19a) and malvidin 3-glucoside (19b) on peak 19. A similar result was observed in the case of elderberry extracts. On PDA chromatogram, only two peaks could be detected (Figure 2e, peak numbers 1 and 7). However, mass chromatogram revealed that those peaks each consisted of two independent anthocyanins, cyanidin 3,5-diglucoside (1a) and cyanidin 3-sambubioside-5-glucoside (1b) on peak 1, and cyanidin 3-sambubioside (7) and cyanidin 3-glucoside (8) on peak 7 (Figure 4). One additional large peak was also detected on mass chromatogram of $m/z = 611$ at 58 minutes (Figure 4), although the peak might reflect some flavonoid other than anthocyanin since the compound had no absorbance

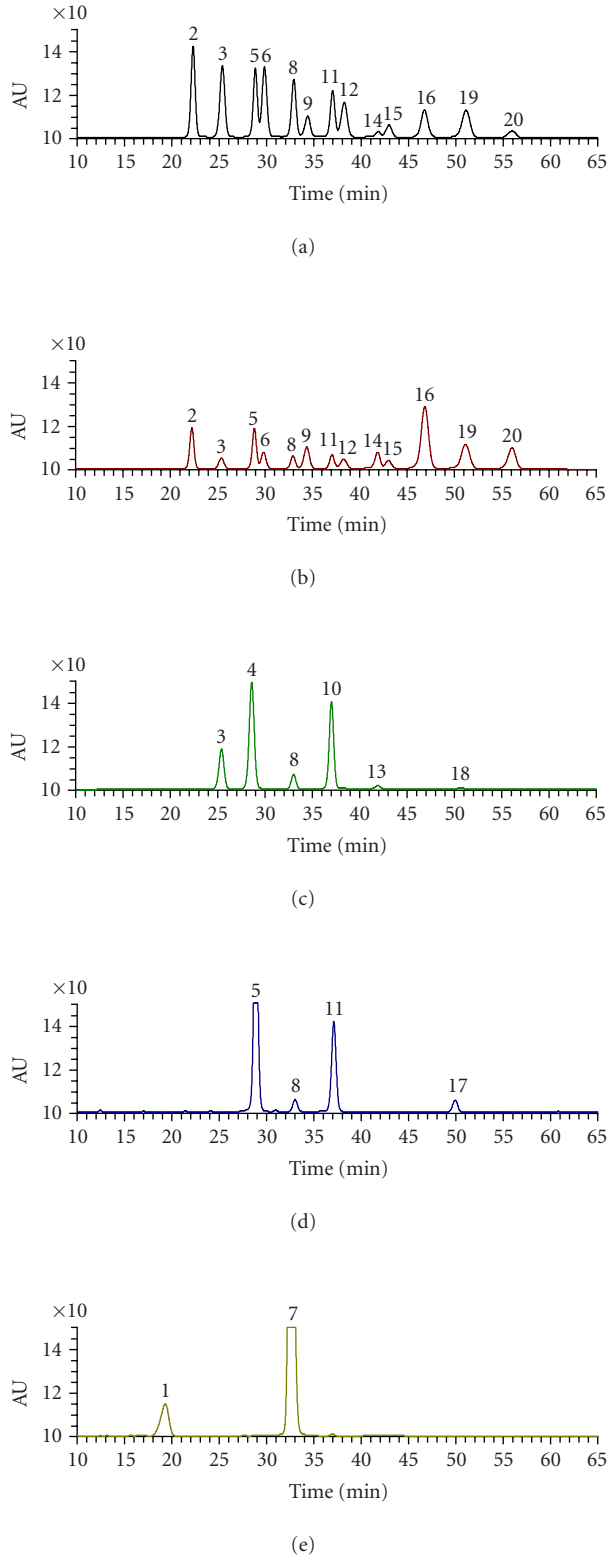

Figure 2. HPLC chromatogram of berry extracts on PDA. The chromatograms were obtained on PDA of selective wavelength (500 to 550 nm). Numbers on the chromatograms indicate detected peak numbers corresponding to the numbers in Table 1. (a) Bilberry; (b) Rabbiteye; (c) Black currant; (d) Chokeberry; (e) Elderberry.

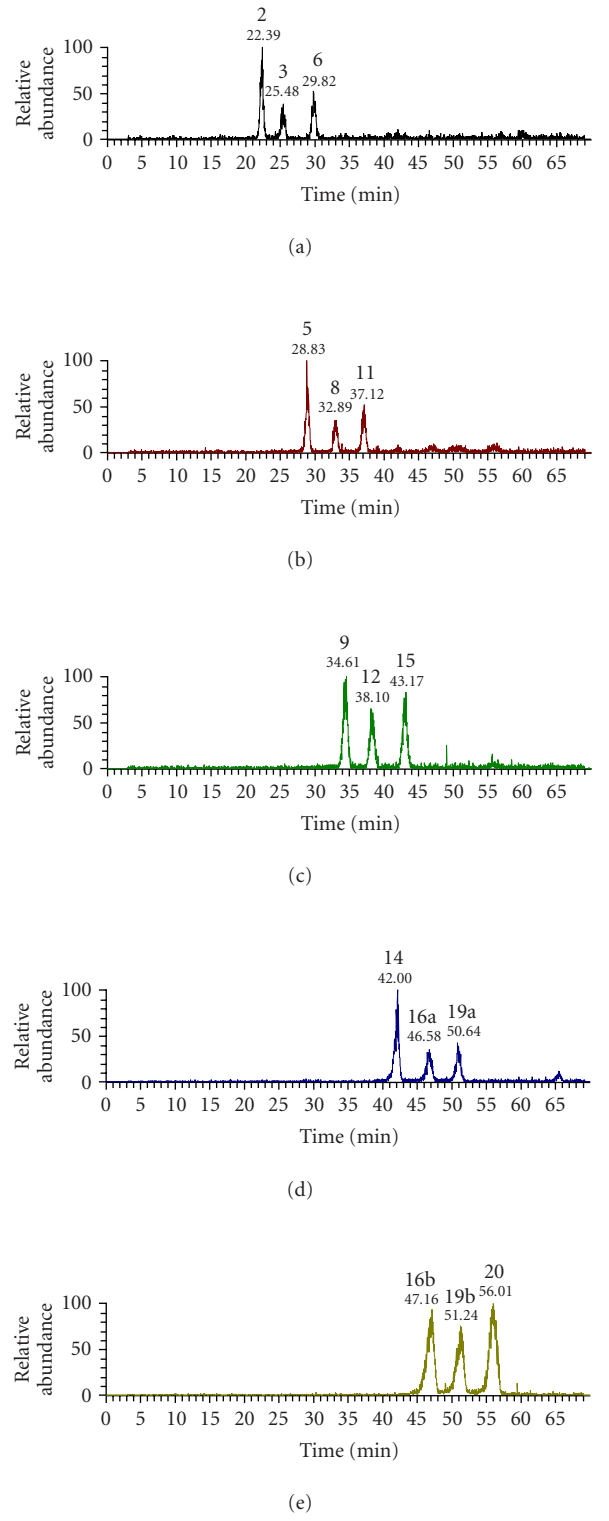

Figure 3. Mass chromatogram of bilberry extract selected by m/z of each aglycon. Mass chromatogram revealed that bilberry extract contains 15 anthocyanins; nevertheless, the extract showed only 13 peaks on PDA. The peaks of 16 and 19 in Figure 2 overlap with 16a and 16b, and 19a and 19b, respectively. (a) Delphinidin (m/z = 303); (b) cyanidin (m/z = 287); (c) petunidin (m/z = 317); (d) peonidin (m/z = 301); (e) malvidin (m/z = 331).

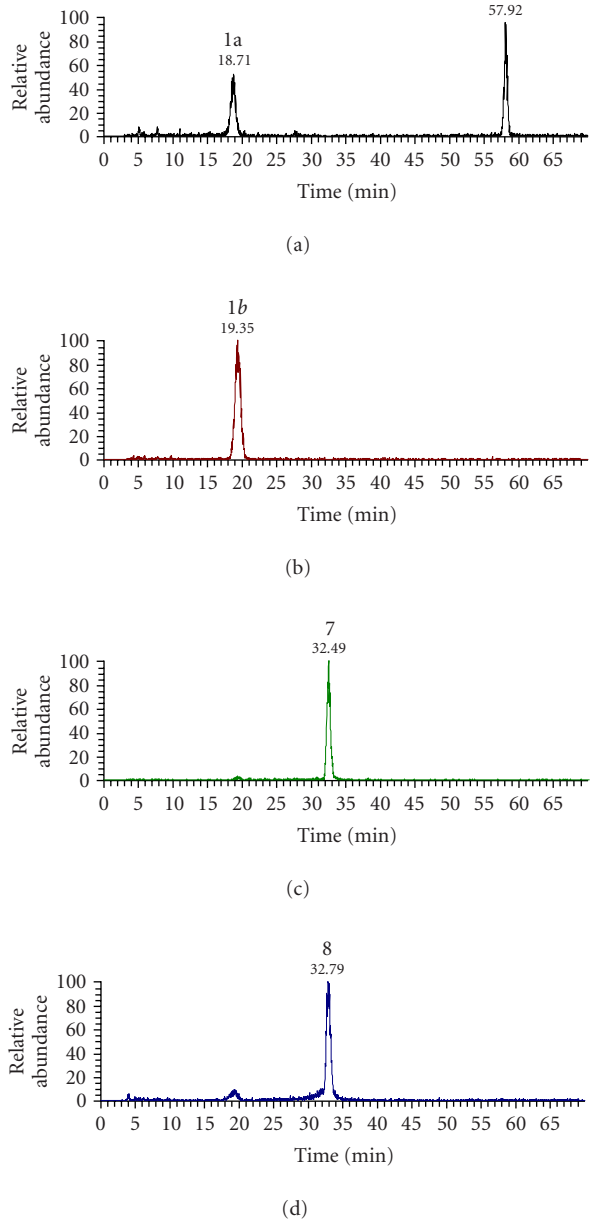

FIGURE 4. Mass chromatogram of elderberry extract selected by m/z of each anthocyanin. Mass chromatogram revealed that elderberry extract contains 4 anthocyanins; nevertheless, the extract showed only 2 peaks on PDA. The peaks of 1 and 7 in Figure 2 overlap with 1a and 1b, and 7 and 8, respectively. (a) Cyanidin 3,5-diglucoside (m/z = 611); (b) cyanidin 3-sambubioside-5-glucoside (m/z = 743); (c) cyanidin 3-sambubioside (m/z = 581); (d) cyanidin 3-glucoside (m/z = 449).

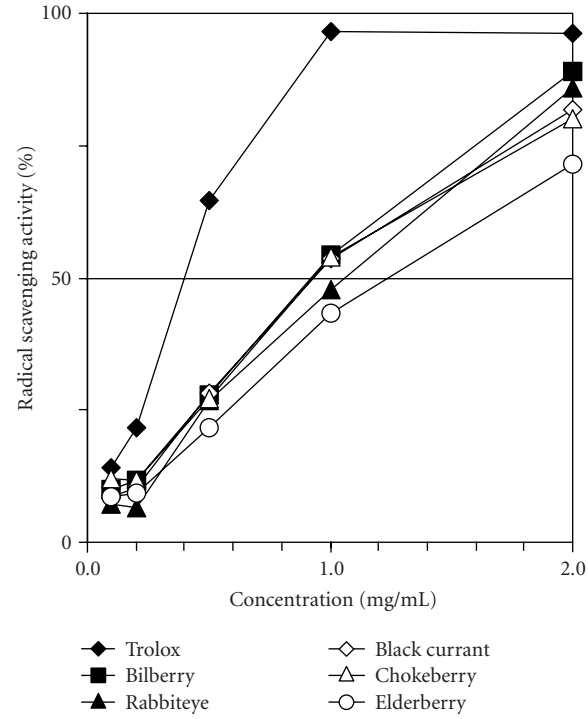

FIGURE 5. Radical scavenging activities of berry extracts. The berry extracts were incubated with DPPH for 5 minutes, and the absorbance at 517 nm due to DPPH radical was determined. Trolox was used as a positive control.

at 500–550 nm on PDA (Figure 2). These results implied that in LC-MS analysis, complete separation by HPLC is not necessarily required since mass chromatography can separate compounds accurately.

Total anthocyanidin contents of berry powders prepared in the present study calculated as delphinidin equivalents per 100 mg of powder after hydrolysis were determined as follows: 28.8 mg (bilberry), 24.8 (rabbiteye), 16.0 (black currant), 19.1 (chokeberry), and 30.4 (elderberry).

Table 1 summarizes the data of LC/PDA/ESI-MS and anthocyanin composition in five berry extracts. Anthocyanins found in bilberry and rabbiteye blueberry consisted of delphinidin, cyanidin, petunidin, peonidin, and malvidin attached with galactose, glucose, or arabinose at the C-3 position [7, 11]. The amount of delphinidin and cyanidin glycosides detected from the extract of bilberry was more than that of rabbiteye blueberry. Delphinidin and cyanidin 3-rutinosides were the main pigments found in black currant [7, 19]. A small amount of petunidin and peonidin 3-rutinosides were detected as well, although they were tentative and remained to be identified. Chokeberry contained only cyanidin as an aglycon, which attached mainly with galactose and arabinose [8, 20]. Plenty of cyanidin 3-sambubioside and cyanidin 3-glucoside were found in elderberry extract [21, 22]. In addition, some of their 5-glucosides were also detected, suggesting that only elderberry possesses a cyanidin 5-glucosyltransferase activity among those berries [23, 24].

Figure 5 shows radical scavenging activity of the berry extracts using DPPH. All five berry extracts exhibited potent radical scavenging activities, though weaker than that of Trolox. They showed relatively similar activities;

Table 1. Profiling of anthocyanins in various berries by HPLC/PDA/ESI-MS. Peak intensity (shown as +) was determined from each peak height on PDA chromatogram. Retention time (Rt) on PDA chromatogram. Plus and minus marks stand for the following: (barely detected) +/− < + < ++ < + + + < + + ++ < + + + + + (abundant).

Peak no	Compound name	Rt (min)	Molecular (m/z)	Fragment (m/z)	Bilberry	Rabbiteye	Black currant	Chokeberry	Elderberry
1a	cyanidin 3,5-diglucoside	19.27	611	449, 287					++
1b	cyanidin 3-sambubioside-5-glucoside		743	449, 287					
2	delphinidin 3-galactoside	22.27	465	303	++++	++			
3	delphinidin 3-glucoside	25.35	465	303	+++	+	++		
4	delphinidin 3-rutinoside	28.51	611	303			++++		
5	cyanidin 3-galactoside	28.83	449	287	+++	++		+++++	
6	delphinidin 3-arabinoside	29.79	435	303	+++	+			
7	cyanidin 3-sambubioside	32.51	581	449, 287					+++++
8	cyanidin 3-glucoside	32.89	449	287	+++	+	+	+	
9	petunidin 3-galactoside	34.33	479	317	++	++			
10	cyanidin 3-rutinoside	36.89	595	287			++++		
11	cyanidin 3-arabinoside	36.99	419	287	+++	+		++++	
12	petunidin 3-glucoside	38.21	479	317	++	+			
13	petunidin 3-rutinoside	41.75	625	317			+/−		
14	peonidin 3-galactoside	41.78	463	301	+	+			
15	petunidin 3-arabinoside	42.93	449	317	+	+			
16a	peonidin 3-glucoside	46.66	463	301	++	+++			
16b	malvidin 3-galactoside		493	331					
17	cyanidin 3-xyloside	49.72	419	287				+	
18	peonidin 3-rutinoside	50.47	609	301			+/−		
19a	peonidin 3-arabinoside	51.07	433	301	++	++			
19b	malvidin 3-glucoside		493	331					
20	malvidin 3-arabinoside	55.91	463	331	+	++			

however, the activity of bilberry was the highest and that of elderberry was slightly low. Extracts of black currant and chokeberry showed nearly identical levels of radical scavenging activity to bilberry extract; nevertheless, total amounts of anthocyanidins in black currant or chokeberry were nearly half those of bilberry, implying that antioxidant activity is not necessarily parallel with the amount of anthocyanidin aglycons. As is generally well known, berries contain a large amount of phenolic compounds that act as antioxidants besides anthocyanins [8, 10, 25, 26]. Since not only anthocyanins but also such phenolic compounds were likely to be extracted from each berry into the powders we prepared in the present study, the radical scavenging activities of berry powders were contributed by both anthocyanins and other phenolics.

There are already a number of reports on the antioxidant activity of berry extracts by several methods such as oxygen radical absorbance capacity (ORAC) [8, 25] or DPPH radical scavenging capacity [7], indicating that bilberry and black currant possess almost equal antiradical activities. Chokeberry was also shown to possess strong antioxidant activity [8]. Moreover, delphinidin 3-glucoside (found abundantly in bilberry and black currant), delphinidin 3-rutinoside (found only in black currant), and cyanidin 3-glucoside (found abundantly in bilberry and elderberry) were reported to have relatively strong antiradical activity among various anthocyani(di)ns [27]. From these reports and our results, extracts of bilberry, black currant, and chokeberry can be regarded as nice candidates for materials of health-beneficial functional foods from the view of the radical scavenging activity.

Since different berries contain unique patterns of anthocyanins, these berries are good resources of the novel genes involved in anthocyanin production. The genes and enzymes responsible for modification and storage are largely unknown [28]. Molecular study on production of berry anthocyanin would identify new genes necessary for the unique pattern of each berry anthocyanin.

ACKNOWLEDGMENT

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RESEARCH ARTICLE

The Change of Total Anthocyanins in Blueberries and Their Antioxidant Effect After Drying and Freezing

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This study examined the effects of freezing, storage, and cabinet drying on the anthocyanin content and antioxidant activity of blueberries (*Vaccinium corymbosum* L). Fresh samples were stored for two weeks at 5°C while frozen samples were kept for up to three months at −20°C. There were two drying treatments, one including osmotic pretreatment followed by cabinet drying and the other involving only cabinet drying. Total anthocyanins found in fresh blueberries were 7.2 ± 0.5 mg/g dry matter, expressed as cyanidin 3-rutinoside equivalents. In comparison with fresh samples, total anthocyanins in untreated and pretreated dried blueberries were significantly reduced to 4.3 ± 0.1 mg/g solid content, 41% loss, and 3.7 ± 0.2 mg/g solid content, 49% loss, respectively. Osmotic treatment followed by a thermal treatment had a greater effect on anthocyanin loss than the thermal treatment alone. In contrast, the frozen samples did not show any significant decrease in anthocyanin level during three months of storage. Measurement of the antioxidant activity of anthocyanin extracts from blueberries showed there was no significant difference between fresh, dried, and frozen blueberries.

INTRODUCTION

Anthocyanins, natural pigments which are responsible for the blue, purple, violet, and red colours of fruit, are one of the major flavonoid classes [1]. The major sources of anthocyanins in edible plants are families Vitaceae (grape) and Rosaceae (cherry, plum, raspberry, strawberry, blackberry, apple, peach, etc.). Other plant families which contain anthocyanin pigments are Solanaceae (tamarillo and eggplant), Saxifragaceae (red and black currants), Cruciferae (red cabbage), and Ericaceae (blueberry and cranberry) [2]. Blueberries contain the following anthocyanins: malvidin 3-galactoside, delphinidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-arabinoside, malvidin 3-arabino-side, cyanidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-arabinoside, delphinidin 3-glucoside, malvidin 3-glucoside, peonidin 3-glucoside, peonidin 3-galactoside, peonidin 3-arabinoside, and peonidin 3-glucoside [3].

Flavonoids such as flavones, isoflavones, flavonones, anthocyanins, and catechins have strong antioxidant capacity [4]. These compounds can be found in cereal grains, tubers, tea, coffee, fruits, and vegetables. The berries, such as blueberries, are significant sources of anthocyanins. Methanol extracts from *Vaccinium angustifolium* L and cultivar Fundy possess higher antioxidant activity than sweet cherry, potato (purple), wheat germ, and ginseng root [5].

The consumption of wild blueberries, a food source with high in vitro antioxidant properties, is associated with a diet-induced increase in ex vivo serum antioxidant status [6]. Anthocyanins in grape juice reduced in vitro oxidation of human low-density lipoprotein (LDL) [7]. Potential bioavailability, in humans, of several anthocyanins from red wine was tested. Within 12 hours after 300 mL of wine consumption, 1.5%–5.1% of the ingested anthocyanins were found in urine. Two compounds among the wine anthocyanins were unchanged while the others, which were considered by the authors, seemed to have undergone molecular modifications [8]. Frozen, liquid-extracted, and freeze-dried powders, made from wild blueberries, were used in a study of bioactive properties, which included antioxidant activity, cardioprotective capacity, and cancer chemoprevention activity. Many fractions of the extracts showed antioxidant activities, especially those rich in anthocyanins and proanthocyanidins [9]. Anthocyanins have been found to significantly suppress the growth of cultured tumour cells and have been shown to have greater inhibitory effect than other flavonoids [10, 11].

Blueberries are commercialised in different ways, mainly as fresh or frozen products. Freezing and drying are two possible methods to preserve blueberries but the severity of both processes might destroy anthocyanins or their antioxidant effects. Blueberries are known for their bioactive properties such as antioxidant activity, cardiovascular protection, antidiabetic properties, vision

improvement properties, and inhibition of carcinogenesis and mutagenesis [12]. Thus, the aim of this study was to determine and to compare total anthocyanins and their antioxidant effects in frozen or dried blueberries and to compare them with the values found in fresh berries.

MATERIALS AND METHODS

Samples

Fresh blueberries (*Vaccinium corymbosum* L) were supplied by Blueberry Farms of Australia P/L, Corindi Beach, New South Wales, Australia.

Treatments

Fresh blueberries were kept at 5°C for up to two weeks before extraction (FR2). Several batches of blueberries were frozen and kept at −20°C up to 3 months. The samples were taken and examined at 1-month (FZ1M) and 3-month (FZ3M) storage. There were 2 replicates for each sampling point.

Two batches of blueberries weighing 1 kg each were dried. The first batch, PT, had been treated with 60% w/w sugar and 1% w/w NaCl solution for 4 hours and slowly dried in a cabinet dryer at 90°C for 90 minutes, followed by 70°C for 120 minutes, and finally 50°C for 120 minutes. The second batch, UN, was dried directly without any pretreatment using the same temperature profile.

Dry matter was determined by drying 5–10 g blueberry sample in a vacuum oven at 70°C, 85 kPa for 72 hours. The dried blueberries were weighed again and the dried matter that remained was determined. Total anthocyanins and antioxidant effect from dried samples (UN and PT) were compared with those of frozen and fresh samples.

Anthocyanin extraction

Samples weighing 20 g of fresh, frozen, and proportionally reduced amounts (based on moisture loss during drying) of dried blueberries were blended in a food processor for 1 minute with 150 mL of a mixture of methanol, acetic acid, and distilled water (M:A:W) at a ratio of 25:1:24. Frozen blueberries were thawed in a refrigerator (at about 5°C) overnight prior to the extraction. Half of the well-blended solution was centrifuged at 21 900 g (12 000 rpm) for 20 minutes at 20°C. The remaining residue from centrifugation after the supernatant was removed was mixed thoroughly with 75 mL M:A:W, centrifuged, and the supernatant was separated. Each sample was extracted 3 times. The clear liquid from the 3 extractions was evaporated under vacuum at 35°C. The residue from vacuum evaporation was redissolved with 5 mL of 3% (w/v) formic acid in water. This aqueous solution was adsorbed on a C18 Sep-Pak cartridge. The cartridge was washed with 5 mL of 3% (w/v) formic acid in water and eluted with 3.5 mL of 3% (w/v) formic acid in methanol. The anthocyanins eluted from the cartridge were evaporated under vacuum at 35°C until dryness [13].

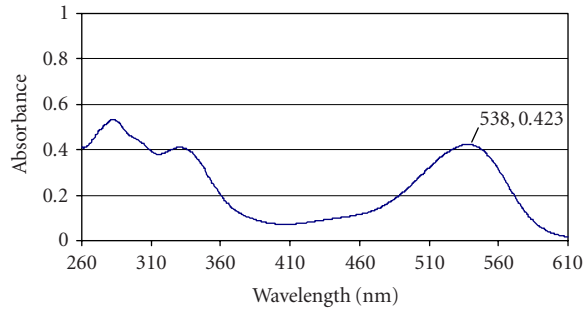

Figure 1. Scan spectrum of blueberry extracts in MeOH:HCl.

Determination of total anthocyanins

The residue was diluted to the volume of 25 mL by mixing with the mixture of methanol and 0.1 M HCl at a ratio of 85:15 (MeOH:HCl). The anthocyanin solution was diluted to the appropriate concentration for measurement of absorbance in the Cary 100 scanning UV-Vis spectrophotometer using 1 cm path length quartz cells at 538 nm. Total anthocyanins were expressed as cyanidin 3-rutinoside equivalents [14]. The molar absorptivity of cyanidin 3-rutinoside was equal to 31085 at 530 nm in MeOH:HCl. This molar absorptivity has been determined experimentally.

Antioxidant effects

The antioxidant activity of the anthocyanin extracts was measured using a free radical method of Brand-Williams et al [15]. The free radical used in this study was 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The UV-1601 UV-Vis spectrophotometer was used to determine the concentration of DPPH. 3.9 mL of 6×10^{-5} mol/L DPPH in methanol (spectrophotometric grade) were put into the disposable cuvette with 0.1 mL of anthocyanin extract. The decrease of absorbance was measured at 0 minute, 1 minute, and every 5 minutes at 515 nm for 2 hours or until the absorbance became steady. The remaining DPPH concentration (C_{DPPH}) was calculated using the following equation [15]:

$$C_{DPPH} = \left(Abs_{515} + 2.58 \times 10^{-3}\right) \times 12509^{-1}. \quad (1)$$

Statistical analysis

The data were analysed by analysis of variance (ANOVA) method and Duncan, multiple-range test at 5% level of significance using SPSS.

RESULTS AND DISCUSSION

Total anthocyanins

A spectrum of the blueberry extract is presented in Figure 1. In the anthocyanin extracts, the peak in the visible region was recorded at 538 nm while the peaks in the UV range were at 280 and 320 nm. The presence of these

Table 1. Anthocyanin content in evaluated samples.

Blueberry samples	Total anthocyanins mg/g dry matter
Fresh blueberries (FR0)	$7.2 \pm 0.5^{a**}$
Fresh blueberries 2-week storage at 5°C (FR2)	5.7 ± 0.5^{ab}
Untreated dried (UN)	4.3 ± 0.1^{bc}
Pretreated dried (PT)	3.7 ± 0.2^{c}
Stored frozen for 1 month (FZ1M)	8.1 ± 0.1^{a}
Stored frozen for 3 months (FZ3M)	7.9 ± 1.3^{a}

*Total anthocyanin as cyanidin 3-rutinoside equivalent.

**Mean ± standard deviation of duplicate samples. The means that have the same superscript are not significantly different ($P < .05$).

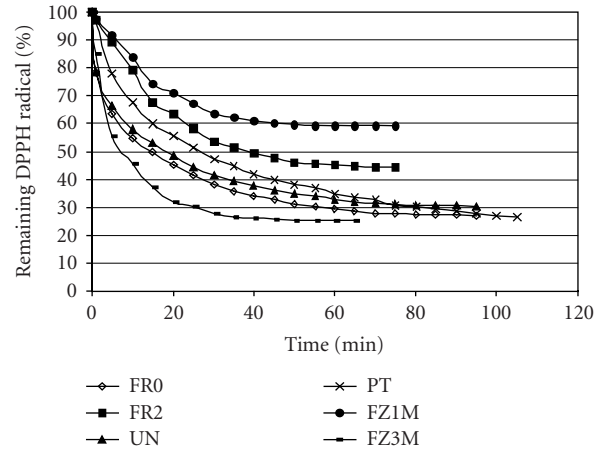

Figure 2. Kinetic behaviour of reducing DPPH radical of anthocyanins found in blueberry extract after the following treatments: FR0 : fresh blueberries; FR2: fresh blueberries kept at 5°C for two weeks; UN : untreated blueberries dried in a cabinet dryer; PT : osmotically pretreated blueberries dried in a cabinet dryer; FZ1M : frozen blueberries kept at −20°C for 1 month; FZ3M : frozen blueberries kept at −20°C for 3 months.

peaks reflects the fact that blueberries contain a mix of anthocyanins and other phenolic compounds.

One sample of fresh blueberries, (FR0), was extracted immediately after having been received from the grower while another sample, (FR2), was kept at 5°C for 2 weeks before extraction. Total anthocyanins in FR2 were slightly lower than those in FR0; namely, 5.7 and 7.2 mg/g dry matter, respectively (Table 1). The blueberries that had been stored for two weeks at 5°C were softer. In a study by Sapers and Phillips [16], the leakage of anthocyanins was proportional to the percentage of soft berries in the whole sample. In comparison with other fruits such as plums, the concentration of anthocyanins found in 2-week refrigerated blueberries was higher (5.7 ± 0.5 mg/g dry matter) than that found in fresh plums, which was in the range of 2.6–5.2 mg/g dry matter [14].

The total anthocyanin content in both dried samples UN and PT was less than that in fresh berries (Table 1). The percentage of loss of anthocyanins in UN was 41% while it increased to 49% in PT. Slightly higher reduction in anthocyanin content was observed in PT than in UN. However, the difference was not statistically significant. This difference was caused by the 4-hour pretreating step that leached out some anthocyanins. Anthocyanin leakage might happen due to dewaxing, which was caused by stirring and soaking during the osmotic pretreatment. This observation is comparable to the study by Sapers and Phillips [16] who found that dewaxing weakened the berry cuticle and allowed the skin to rupture. This permitted some leakage from the exposed edges or undersurface of the torn skin to the osmotic solution that caused anthocyanin loss before the drying step. Also, the thermal processing destroyed some anthocyanins. A study about the evolution of anthocyanins in raspberries during jam making, in which heat was used, showed that 17%–40% of anthocyanins were lost [17].

As for the appearance, the PT samples presented a more shiny aspect than the UN samples. Furthermore, the moisture content after drying of PT (33.6% wet basis) was lower than that of UN (36.9% wet basis).

Anthocyanin contents of frozen samples were found stable over 3 months of storage (Table 1). The fruits, which were stored frozen for 1 month (FZ1M) and 3 months (FZ3M), showed no significant difference from FR0.

Antioxidant effect

The results of the kinetic behaviour of blueberry extracts are shown in Figure 2. After adding the blueberry extract to the DPPH solution, the absorbance was increased due to the colour of the extracts. The slope of the equations may be a useful parameter to define the antioxidant capacity. The steeper the slope, the lower the amount of antioxidant that is necessary to decrease by 50% the initial DPPH concentration [18]. The steepest slope was that of FZ3M (Table 2). This means a lower amount of the extract was necessary to decrease the initial DPPH concentration. FZ1M showed the lowest antioxidant activity (though not the lowest anthocyanin content), while there was no significant difference in antioxidant effect between FZ3M and FR0.

Antioxidant activity can also be assessed by the oxygen radical absorbance capacity (ORAC). The ORAC method estimates the antioxidant capacity of a sample by taking the oxidation reaction to completion whereas DPPH estimates the stable free radical and thus is more appropriate to characterise the antioxidant activity in a food sample. In a study of the commercial frozen lowbush blueberries, which contained lower levels (60%–80%) of "blue" than the other samples, it was found that the antioxidant activity (ORAC) was comparable to that of the fresh fruits [19]. This result supported an earlier study on variation in ORAC based on variety, maturity, and source, done by Prior et al [20].

Table 2. Average slope values of blueberry extracts.

Blueberry extracts	Slope* (% DPPH/min)
FR0	-0.0110^{ab}
FR2	-0.01035^{ab}
UN	-0.0103^{ab}
PT	-0.0116^{ab}
FZ1M	-0.0076^{a}
FZ3M	-0.0145^{b}

*Slopes that have the same superscript are not significantly different ($P < .05$).

As for the dried products, UN and PT, samples showed no significant difference in antioxidant activity from the fresh berries even though the anthocyanin contents shown in Table 1 were lower than those in the fresh samples. According to similar studies [20, 21], the correlation coefficient between ORAC and the total phenolics was higher than the correlation coefficient between ORAC and total anthocyanins. In a study of total phenolics in blueberries [22], chlorogenic acid, a major colourless phenolic of blueberries, was found at the level of 60–100 mg/100 g of fresh berries and significantly contributed to ORAC [23]. The anthocyanins breakdown products from drying process might act as antioxidants without being affected by the thermal process.

CONCLUSIONS

The amount of total anthocyanins in the frozen samples, expressed as cyanidin 3-rutinoside equivalents, was not significantly different from that in the fresh samples. In contrast, the concentration of anthocyanins in dried blueberries (UN and PT) was significantly reduced in comparison with that in fresh blueberries while antioxidant activity of the extracts did not differ from that of the fresh fruit. Fruit drying resulted in reduction of the total anthocyanin level by 41%. When drying was preceded with osmotic dehydration, 49% of anthocyanins were lost. There was no significant difference in antioxidant activity between the anthocyanin extracts of the frozen or dried samples and the fresh fruit. Antioxidant activity in blueberries is an appealing characteristic to consumers. Any processing method that maintains the level of compounds known for their health benefits will be of interest to the food industries.

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RESEARCH ARTICLE

Sour Cherry (*Prunus cerasus* L) Anthocyanins as Ingredients for Functional Foods

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In the recent years many studies on anthocyanins have revealed their strong antioxidant activity and their possible use as chemotherapeutics. The finding that sour cherries (*Prunus cerasus* L) (also called tart cherries) contain high levels of anthocyanins that possess strong antioxidant and anti-inflammatory properties has attracted much attention to this species. Here we report the preliminary results of the induction of anthocyanin biosynthesis in sour cherry callus cell cultures. The evaluation and characterization of the in vitro produced pigments are compared to those of the anthocyanins found in vivo in fruits of several sour cherry cultivars. Interestingly, the anthocyanin profiles found in whole fruit extracts were similar in all tested genotypes but were different with respect to the callus extract. The evaluation of antioxidant activity, performed by ORAC and TEAC assays, revealed a relatively high antioxidant capacity for the fruit extracts (from 1145 to 2592 μmol TE/100 g FW) and a lower one for the callus extract (688 μmol TE/100 g FW).

INTRODUCTION

Anthocyanins, one of the major groups of pigments belonging to the secondary metabolite group of flavonoids, are often responsible for the orange, red, and blue colors in fruits, vegetables, flowers, and other storage tissues in plants. Thus, they have become important as food additives. However, interest in anthocyanins has recently intensified because of their possible health benefits. One of the best known properties of flavonoids in general is their strong antioxidant activity in metabolic reactions due to their ability to scavenge oxygen radicals and other reactive species. This feature makes flavonoids a potential tool for use in studies on oxidative stress, the ageing process, and cancer [1], especially since it has been reported that anthocyanins inhibit the growth of cancer cells [2] and act as chemotherapeutics for numerous diseases [3]. The finding that sour cherries (*Prunus cerasus* L) contain significant levels of anthocyanins [4] has attracted much attention to this species. Anthocyanins from sour cherry have been shown to possess strong antioxidant and anti-inflammatory activities [5] and to inhibit tumor development in Apc^{Min} mice and the growth of human colon cancer cell lines [6]. Moreover, cyanidin, the anthocyanin aglycon, has shown more efficient anti-inflammatory activity than aspirin [5]. Although cherry fruit tissue has recently been used in meat products for improved nutritional qualities (less rancidity and the inhibition of the formation of heterocyclic aromatic amines), the use of purified anthocyanins extracted from cherry cells cultured in vitro is an alternative to consider. The production of anthocyanins extracted from cherry fruit is restricted to their seasonal production; moreover, the fruit has too high a value as a fresh fruit to be used for anthocyanin extraction. Plant cells/tissue cultures offer an opportunity for continuous production of plant metabolites. Moreover, plant cell culture is an attractive production source, since it is scalable according to specific needs [7] and also gives greater potential for the manipulation of anthocyanin quality [8, 9].

Here we report the preliminary results of the induction of anthocyanin biosynthesis in sour cherry (*P cerasus* L) callus cell cultures. The evaluation and characterization of anthocyanin pigments produced in vitro as well as of those extracted from the whole fruits of several cultivars are reported. The anthocyanins pigment profiles in fruits and in callus cultures producing anthocyanins were characterized by reverse-phase high-performance liquid chromatography (RP-HPLC). This technique coupled with a photodiode array detector has become the method of choice for monitoring anthocyanin profiles [4, 10, 11]. Oxygen radical absorbance capacity (ORAC) assay and Trolox equivalent antioxidant capacity (TEAC) assay on fruit and callus extracts have also been performed in order to evaluate the antioxidant activity of the extracts.

MATERIALS AND METHODS

Chemicals

All reagents (from Carlo Erba, Milan, Italy, if not otherwise indicated) were of analytical reagent grade or

HPLC grade, as required. Anthocyanin standards were supplied by Extrasynthese (Lyon, France).

Plant material

Fruits of sour cherry (*P cerasus* L) cv Amarena Mattarello (AM), Visciola Ninno (VN), and Visciola Sannicandro (VS) (genotypes from the local germplasm) were picked up in June 2003 on a local experimental field (Bari, Italy). Cherries were flushed with nitrogen in freezer bags prior to storage at −20°C.

Callus induction and anthocyanin production

In vitro shoot culture of *P cerasus* L, cv AM, was previously set up [12].

Callus cultures were induced from leaf segments on a callus induction medium (CIM), containing Murashige and Skoog (MS) mineral salts and vitamins, 30 g L^{-1} sucrose, 1 mg L^{-1} α-naphthaleneacetic acid (NAA), and 0.1 mg L^{-1} N^6-benzyladenine (BA). Leaf explants were taken from plants grown in vitro on a plant multiplication medium (PMM) [12]. The explants were incubated in a growth chamber at 25 ± 2°C in the dark. Callus cultures were maintained on the same CIM in the dark and transferred to a fresh CIM every three weeks.

At the end of the growth cycle, callus cultures were transferred to several types of media, later referred to as anthocyanin induction media (AIM) [13], and then incubated under light (Philips TLD/83, 125 μmol $m^{-2}s^{-1}$), with a 16 hour photoperiod. Anthocyanin producing calli were harvested after two weeks of incubation under light, flushed with nitrogen, and stored at −20°C for further analysis.

Extraction of the anthocyanins

Pitted and frozen cherries (10 g) of each cv were ground twice for 30 second in a Waring blender (Waring, Conn, USA) in the presence of liquid N_2, thus providing a uniform powdered sample. Frozen calli were pulverized with a pestle and mortar and then treated as the homogenized fruits. A sample of the powder (1 g) was centrifuged (Allegra 21 R centrifuge, Beckman Coulter, Fullerton, Calif) at 10 000 g for 10 minutes at 4°C. The supernatant juices were stored at −20°C as stock solutions for the analysis of antioxidant activity. Another sample of the same powder (3 g) was extracted with a double volume of acidified methanol (0.01% HCl) (v/v), at 4°C, with stirring overnight. After extraction, the colored liquid was separated from the solid matrix and concentrated at 35°C in vacuo and then dissolved in the mobile phase used for HPLC analysis.

HPLC/DAD analysis

The profile of anthocyanins was determined using an HPLC system consisting of a Model SCL-10AVP system controller, equipped with a solvent delivery unit Model LC-10ADVP; an online vacuum membrane degasser, Model DGU-14A; a column oven, Model CTO-10ASVP; and a photodiode array detector UV-Vis, Model SPD-10AVP, in conjunction with an LC workstation Model Class VP 5.3 (all from Shimadzu, Milan, Italy). Analytical separation of anthocyanin compounds was carried out on a Polaris C18A column (150 × 2.0 mm, id 5 μm, Varian, Palo Alto, Calif) equipped with a C18 guard column. The samples were introduced onto the column via a Rheodyne Model 9125 nonmetal injection valve with a peak sample of 5 μL volume. The temperature of the column oven was set at 30 ± 0.1°C. Solvent A was water : formic acid (9 : 1 v/v); solvent B was acetonitrile : water : formic acid (5 : 4 : 1 v/v). The percentage of solvent B was increased linearly from 8% to 18% in 12 minutes, followed by elution under isocratic conditions for the next 5 minutes, and by a second linear gradient segment from 18% to 35% B in 13 minutes. The column was reconditioned with the initial eluent for about 20 minutes. The solvent flow rate was 0.2 mL/min. Acquisition range was set between 240 and 600 nm with a sampling period of 0.32 second and a time constant of 0.64 second. The chromatogram was monitored at 518 nm. The purity of the peaks was also monitored using the diode array purity test system included in the software.

Identification of anthocyanins

The anthocyanin identification in sour cherry extracts was made from matching UV-Vis spectra and retention times with authentic standards. The quantities of different anthocyanins were assessed from the peak areas and calculated as equivalents of cyanidin 3-glucoside. The standard curve of this compound showed excellent linearity over the concentration range of 4–50 mg/mL with correlation coefficient better than 0.9999 and nearly passed through the origin. Relative standard deviations were less than 2%. Sour cherry samples were analyzed in triplicate, and the mean peak areas of all anthocyanins were used to determine the quantities present in the different cultivars and in the callus cell cultures.

ORAC assay

ORAC assays for fruit and calli juices were carried out following the modified procedures of the method previously described by Ou et al [14]. This assay measures the ability of antioxidant components in test materials to inhibit the decline in disodium fluorescein (FL) (Sigma-Aldrich, St Louis, Mo) fluorescence that is induced by the peroxyl radical generator, 2′, 2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) (Wako Chemicals, Richmond, Va). The reaction mixture contained in the final assay mixture (0.7 mL total volume) FL (6.3×10^{-8} M) and AAPH (1.28×10^{-2} M). All reagents were prepared with 75 mM phosphate buffer, pH 7.4. The final volume was used in a 10 mm wide fluorometer cuvette. FL, phosphate buffer, and samples were preincubated at 37°C for 15 minutes. The reaction was started by the addition of AAPH. Fluorescence was measured and recorded every 1 minutes at the emission length of 515 nm

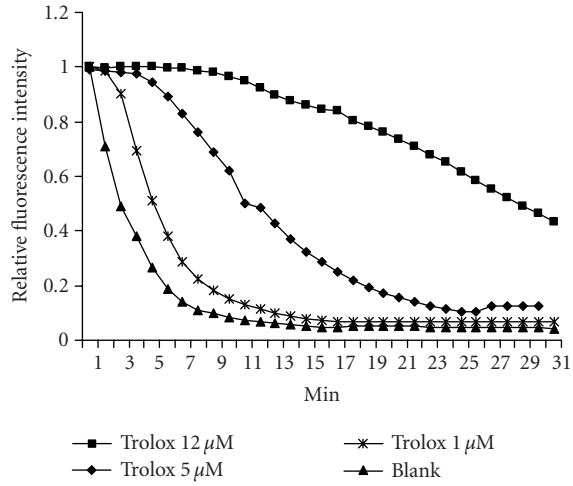

Figure 1. Trolox concentration effect on FL fluorescence decay curve. Data is pooled from two runs.

and excitation length of 493 nm using a Shimadzu RF-5301PC (Columbia, Md) until the fluorescence of the last reading declined to a value of less than or equal to 5% of the first reading. This usually took about 30 minutes. Phosphate buffer was used as the blank and 1, 5, 12.5 μM Trolox (Sigma-Aldrich, Steinheim, Germany) were used as the control standards. Samples and Trolox calibration solutions were analyzed in duplicate. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve (Figure 1) and were expressed as Trolox equivalents (TE) as micromole (μmol) per 100 g of fresh weight (FW).

ABTS radical cation decolorization assay

This TEAC assay for fruit and callus juices was carried out following the procedures previously described by Re et al [15]. 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; (ABTS) (Sigma-Aldrich, St Louis, Mo) was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 hours before use. For the study of fruit and callus juices, the $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 ($\pm$0.02) at 734 nm. Fruit and callus juices were diluted so that, after the introduction of a 10 μL aliquot of each extract into the assay, they produced between 20%–80% inhibition of the blank absorbance. After addition of 1.0 mL of diluted $ABTS^{\bullet+}$ solution to 10 μL of extracts or Trolox standards (final concentration 0–15 μM) in PBS, the absorbance reading was taken up to 6 minutes. Appropriate PBS blanks were run in each assay. All determinations were carried out twice. The final TEAC decolorization assay values were calculated by using a regression equation between the Trolox concentration and the percentage of inhibition of absorbance at 734 nm after 6 minutes and were expressed as TE as micromole (μmol) per 100 g of FW.

RESULTS AND DISCUSSION

Sour cherry (*P cerasus* L) is a temperate fruit with marginal importance, even though since the 1980s this crop has become more appreciated than the sweet cherry crop for reasons such as minor agrobiological needs, the greater ease of mechanical harvesting, and its numerous uses in the food industry. Sour cherry may acquire new interest, mainly due to the fact that it can be considered as a "functional food" because of its high content of antioxidant compounds. Recent studies have revealed that anthocyanins from sour cherry exhibit in vitro antioxidant activities comparable to those from commercial products, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and superior to vitamin E at 2 mM concentration [5]. In an anti-inflammatory assay, cyanidin (the aglycon of the main tart cherry anthocyanins) showed better anti-inflammatory activity than aspirin [5]. Thus, the production of a "natural aspirin" could be a pharmaceutical alternative for people with digestive tract ulcer or allergies to aspirin and to nonsteroidal anti-inflammatory compounds.

The interest in sour cherry anthocyanins has pushed us into a new research project concerned with the in vitro production of anthocyanin from the species and the characterization of the secondary metabolites, compared to the in vivo products from the fruits of different cultivars.

Leaf explants cultured in the dark produced actively growing callus cultures in a short time. The production of anthocyanins was observed at different levels in most of the AIM tested. Even in the CIM (control medium) anthocyanin production was noticeable, confirming that an important factor for anthocyanin induction is light. Takeda [16] reported that, in carrot cell cultures, light irradiation induced the expression of enzymes of the phenylpropanoid metabolic pathway, such as phenylalanine ammonia-lyase and chalcone synthase, at the transcription level.

During several subcultures (nearly ten), a stepwise selection of callus cultures allowed the isolation of a cell line with high and homogeneous anthocyanin production (Figure 2).

The pigmented callus cultures as well as the fruit extracts from different local cultivars were used for the evaluation and quantification of the anthocyanins.

Figure 3 shows the anthocyanin profiles found in AM cherry extract and in callus extract. The spectra of the separated anthocyanins in sour cherry samples were very similar to those of cyanidin 3-glucoside, the standard used for their quantification (Figure 4). Cyanidin 3-glucosylrutinoside, cyanidin 3-sophoroside, cyanidin 3-rutinoside, and cyanidin 3-glucoside were identified as

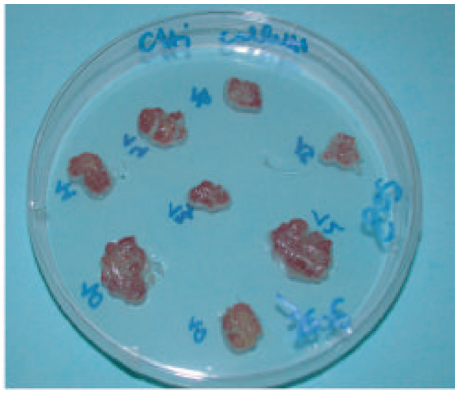

Figure 2. Callus culture of sour cherry (*P cerasus* L) cv Amarena Mattarello on callus induction medium, after ten days of light exposure.

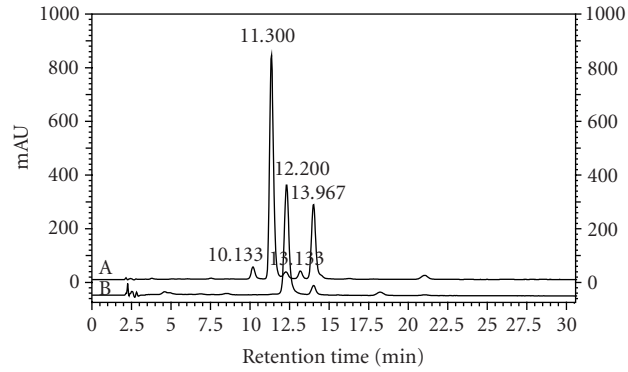

Figure 3. HPLC profile of sour cherry (*P cerasus* L) cv Amarena Mattarello fruit extract (A) and callus extract (B). Chromatographic conditions as reported in the test. Identification peaks: cyanidin 3-sophoroside (t_r 10.133); cyanidin 3-glucosylrutinoside (t_r 11.300), cyanidin 3-glucoside (t_r 12.200), cyanidin 3-rutinoside (t_r 13.967).

major components in the analyzed samples in agreement with the findings previously reported in the literature [4]. The chromatographic results showed that, in the analyzed sour cherry, the same compounds were present in all the cultivars but at different levels. The total anthocyanin content ranged from 27.8 to 80.4 mg/100 g (Table 1).

As reported in Table 1, the total anthocyanin content was much higher in the fruit extracts than in the callus extract. Our in vitro system is at a preliminary stage of development and it is important to set up the best possible microenvironmental conditions to improve the anthocyanin production. The callus cultures we selected are capable of producing 20-fold less anthocyanin than the fruit of the same field-grown cultivar (AM) (Table 1). Our aim is now to greatly improve the pigment production, to make the in vitro process economically viable and an alternative to the field-grown material. To raise the efficiency it is important, for example, to induce anthocyanin production not only at the surface but even inside the callus.

Although total anthocyanin content in our cultivars varied, depending on the genotype, each cultivar profile contained the same compounds in quite similar proportions. Cyanidin 3-glucosylrutinoside was the main anthocyanin found, followed by cyanidin 3-rutinoside, cyanidin 3-sophoroside, and cyanidin 3-glucoside. The anthocyanin profile found in fruit extracts was similar in all tested genotypes and was quite different from that found in the callus extract. In the callus extract only cyanidin 3-glucoside and cyanidin 3-rutinoside were present, the first one at a very high proportion (72.14%) (Table 1). This aspect reveals the ability of the in vitro cell to modulate the anthocyanin metabolism towards less evolved molecular structures. It is reported that the metabolic flux of in vitro systems is often simplified but could be driven towards the accumulation of specific compounds with interesting characteristics [9, 17], thus providing a powerful tool for biotechnological applications.

Since it has been shown that the role of fruit and vegetables in protection against cancer, cardiovascular disease, and cerebrovascular disease is to be attributed to the various antioxidant compounds contained in these foods, and that the biological activities of anthocyanins could also be due to their antioxidant properties, the evaluation of the antioxidant capacity of anthocyanin containing fruits or anthocyanin extracts is an important parameter for the suitable formulation of functional foods.

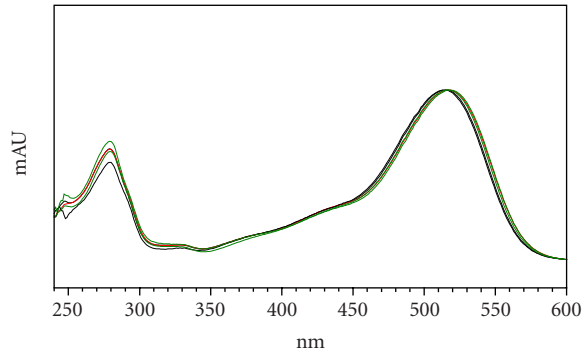

Figure 4. Overlapped spectra of cyanidin 3-glucoside chloride standard with the spectra of peaks eluting at times 10.133; 11.300; 12.200; 13.133; 13.967 minutes.

Several methods have been developed to measure the total antioxidant capacity of various biological samples. Among them, ORAC and TEAC are the most important.

We used an improved ORAC assay using FL as a fluorescent probe, since it has been shown that the previously used probe, the β-Phycoerythrin, gave problems in terms of photostability and reproducibility [18].

The total antioxidant capacity of fruit extracts, measured as $ORAC_{FL}$, ranged from a low 1145 to 1916 μmol TE/100 g of FW (Figure 5). These values are not comparable with those reported in several papers in which β-Phycoerythrin is used as the fluorescent probe, as

Table 1. Total anthocyanin content and composition of sour cherry (*P cerasus* L) fruit extracts of Visciola Ninno, Amarena Mattarello, and Visciola Sannicandro cultivars and of callus culture extract. Anthocyanin composition was determined as a percentage of total anthocyanins calculated from the peak area at 518 nm; nd = not detected.

Extracts	Total anthocyanin (mg/100 g)	Peak area at 518 nm (%)			
		Cyanidin 3-sophoroside	Cyanidin 3-glucosylrutinoside	Cyanidin 3-glucoside	Cyanidin 3-rutinoside
VN	27.8 ± 0.010	2.58	62.23	1.87	33.32
AM	80.4 ± 0.100	2.91	64.54	1.13	31.42
VS	74.6 ± 0.050	3.87	67.58	1.71	26.84
Callus	4.2 ± 0.001	nd	nd	72.14	27.86

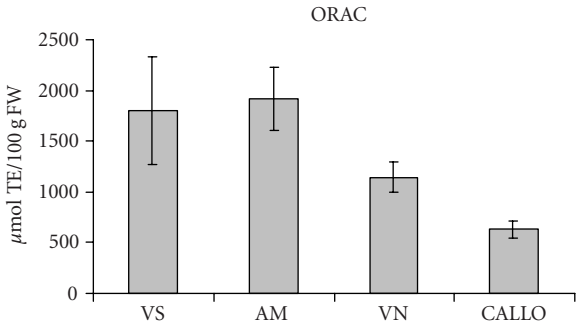

Figure 5. ORAC value of sour cherry (*P cerasus* L) fruit and callus extracts. The results are expressed as micromole Trolox equivalents per gram of fresh weight. Data is expressed as means ± SD of two assays per extract. Fruits are taken from the cultivars Amarena Mattarello, Visciola Ninno, and Visciola Sannicandro. Callus has been generated in vitro from the leaves of sour cherry cv Amarena Mattarello.

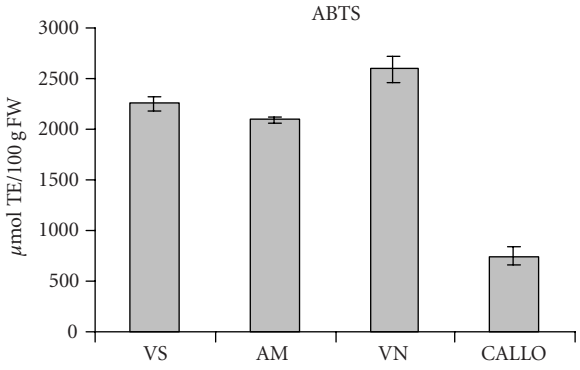

Figure 6. TEAC value of sour cherry (*P cerasus* L) fruit and callus extracts. The results are expressed as micromole Trolox equivalents per gram of fresh weight. Data is expressed as means ± SD of two assays per extract. Fruits are taken from the cultivars Amarena Mattarello, Visciola Ninno, and Visciola Sannicandro. Callus has been generated in vitro from the leaves of sour cherry cv Amarena Mattarello.

described by Ou et al [14]. In TEAC assay, values showed a range of 2000–2600 μmol TE/100 g of FW (Figure 6). Few reports deal with the evaluation of the antioxidant activity of fruit extracts using the TEAC procedure [19]. The values found in sour cherry fruits are comparable to those found in some berry fruits, for example, strawberry, and are higher than in apple and kiwiafruit [20]. The total antioxidant capacity of the callus extracts was lower than that of fruits, in both ORAC and TEAC assays (630–746 μmol TE/100 g of FW) (Figures 5 and 6). However, if we compare the total antioxidant capacity of the callus to that of the fruit, the value is nearly 4-fold less, whereas it was 20-fold less in terms of anthocyanin content. The antioxidant capacity can be supported even by other polyphenolic compounds contained in sour cherry fruits, as reported by Wang et al, [21]. These compounds could be at a high level in callus cultures, thus contributing to the total antioxidant capacity.

The values found for sour cherry in the ORAC and TEAC assays were quite similar, even though the two methods are based on different reaction mechanisms. TEAC is based on the inhibition of the absorbance of the radical cation of ABTS by antioxidants. The antioxidants are oxidized by the oxidant $ABTS^{\bullet+}$, with a single electron transfer (SET) reaction from the antioxidant molecule to the oxidant. The ORAC assay is based on a hydrogen atom transfer (HAT) reaction, with a peroxyl radical $ROO^{\bullet}$ that abstracts a hydrogen atom from the antioxidants, thus retarding or inhibiting the reaction between the $ROO^{\bullet}$ and the target molecule probe. The different reaction mechanisms of the two assays could explain the different rank order of the three cultivars. The ORAC value for cv VN was different from the TEAC value, whereas the values for the other materials were comparable.

The antioxidant activity assays used juice extracts while the anthocyanin analysis used methanolic extracts in order to perform both assays on samples extracted with the most suitable solvent for each method. Therefore, it is possible to compare the anthocyanin content to the values found in the antioxidant capacity assays. The trend shown by the ORAC assay fitted that of the anthocyanin content particularly for fruits. The higher anthocyanin contents were for cv AM and cv VS, which showed the higher ORAC values. For callus, we discussed above the possible contribution of antioxidant compounds other than anthocyanins. Moreover, the antioxidant melatonin was recently identified in two cultivars (Balaton and

Montmorency) of tart cherry [22]. This finding provides an interesting link with the anecdotal consumption of cherries as a health promoting food, and represents a further reason for studying this fruit species.

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RESEARCH ARTICLE

Effect of Light on Anthocyanin Levels in Submerged, Harvested Cranberry Fruit

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Anthocyanins are a group of plant antioxidants known for their therapeutic use. The effects of natural light, red light, and far-red light on individual as well as total anthocyanin content in cranberry fruit (*Vaccinium macrocarpon* Ait) were examined in an experimental setting designed to mimic water-harvesting conditions. The reversed-phase high-performance liquid chromatography (HPLC) method was used to separate and analyze the anthocyanins. In contrast to the case of the control sample that was kept in the dark, natural light increased the total anthocyanin level by 75.3% and 87.2% after 24 and 48 hours of water immersion, respectively. Red light and far-red light increased the total anthocyanin level by 41.5% and 34.7%, respectively. The amount of each individual anthocyanin increased differently under natural light, red light, and far-red light, suggesting that expressions of enzymes that catalyze the anthocyanin biosynthesis are regulated differently by environments.

INTRODUCTION

The quality and commercial value of American cranberry fruit (*Vaccinium macrocarpon* Ait) are determined by their color [1]. The red color of cranberry fruit is due to the presence of anthocyanins. Anthocyanins have important therapeutic values, including antitumor [2, 3], antiulcer [4], antioxidant, and anti-inflammatory activities [5]. Six anthocyanins have been reported in cranberries based on the high-performance liquid chromatography (HPLC) analysis of acid-alcohol fruit extracts on reversed-phase C_{18} column. These are cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, peonidin 3-galactoside, peonidin 3-glucoside, and peonidin 3-arabinoside [6, 7]. The proportions of individual anthocyanins in cranberry fruit may affect the color stability of cranberry products such as juice and sauce [8, 9]. Yan et al [10] reported that cyanidin 3-galactoside showed antioxidant activity superior to six other monoglycosides of quercetin and myricetin isolated from cranberry fruit as well as vitamin E by evaluating compounds for 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity and ability to inhibit low-density lipoprotein oxidation in vitro.

Light has been shown to be the most important environmental factor influencing anthocyanin biosynthesis in plants [11], although in some species, such as *Vitis vinifera* cv. Shiraz anthocyanin accumulation appears not to be light-sensitive [12]. Phytochromes are among the most extensively researched photoreceptors which sense light, and are known to be involved in anthocyanin biosynthesis [13, 14, 15]. Phytochromes respond to red (660 nm) and far-red (730 nm) light, and direct plant gene expression by switching between the red-absorbing form (Pr) and the far-red absorbing form (Pfr). Previously, we have examined the effect of various wavelengths of light on the development of the cranberry plant and anthocyanin biosynthesis in cranberries which were still attached to the plant. We have observed that red light stimulates flowering and anthocyanin biosynthesis in cranberry plant and fruit, respectively [16].

In general, leaves and stems decrease light exposure for berries lower on the plant. Preharvest anthocyanin content of cranberries at the bottom and the top of the plant varies significantly, primarily due to the differences in light accessibility [17].

Water-harvesting has become a common practice in the cranberry industry, and it is accomplished by flooding the cranberry bog with water to float the buoyant fruit for easy collection. However, potential effects on the berries due to the water-harvest technique have not been studied systematically. One study did show that prolonged fruit immersion increases fungal rot of the berries [18]. During the water-harvest, cranberries on the surface of the water receive the same or more light than the fruit still attached to the plant. In this paper, we evaluate the effects

of natural light, red light, and far-red light on individual as well as total anthocyanin levels in cranberry fruit under conditions that mimic water-harvesting.

MATERIALS AND METHODS

Plants

Cranberries (*Vaccinium macrocarpon* Ait, cv "Early Black") used in this study were obtained from the bog of the Cranberry Experiment Station, the University of Massachusetts, East Wareham, Mass, in October 1999.

Light sources

Red light, with a photon fluence rate of 12 μmole $m^{-2}s^{-1}$, was obtained from six 40-w fluorescent tubes (F48T12/R-660/HO, Red, General Electric Company, USA) filtered through a red plastic sheet (Roscolux color filter # 27, ROSCO Laboratories, Port Chester, NY). Far-red light, with a photon fluence rate of 5 μmole $m^{-2}s^{-1}$, was obtained from 500-w brilliant white light halogen double-ended quartz FCL bulbs (Osram Sylvania Products Inc, Winchester, Ky) filtered through 3 mm far-red plastic (type FRF700, West Lakes Plastics, Lenni, Pa). Light sources in each case were kept at a distance of 0.8 meter from the berries. All light measurements were made with a Model IL1400A Radiometer/Photometer (International Light, Inc, Newburyport, Mass).

Experimental setting

Cranberries were taken from a flooded bog after the harvest machine had knocked the fruit loose from the vines and selected in approximate same size and color for experiment in order to avoid variability in the anthocyanin content. The fruit were randomly divided into five groups and held in beakers containing water. Two sizes of beakers (1000 mL and 250 mL) were used. The 1000 mL beaker (diameter: 11.6 cm) contained 800 mL of water, and approximate 34 berries forming just one layer on the surface of the water were placed in the beaker. The 250 mL beaker (diameter: 7.5 cm) contained 200 mL of water, and approximate 17 berries forming just one layer on the surface of the water were placed in the beaker. Two groups in the 1000 mL beakers were placed in a nursery area outside the laboratory and received a cycle of daylight for 24 and 48 hours, respectively. The remaining three groups in the 250 mL beakers were placed in a temperature-controlled darkroom at 20°C. One of these 250 mL beakers was kept in the darkroom and was used as a control sample. The other two, also kept in the darkroom, received 30 minutes of red light or far-red light per day, respectively, for two days. The berries from the two groups placed outside were collected after 24 and 48 hours, respectively, and the fruit from the three groups placed in the temperature-monitored room were individually collected after 48 hours. Eight grams of the berries from each group were weighed and homogenized in 10 mL of ethanol: 1.5 N HCl (85 : 15, v/v) to extract the anthocyanins overnight at 4°C. The sample extracts were filtered through 0.2 μm filters before injection into the HPLC column.

Table 1. Effect of light on total anthocyanin level in submerged, harvested cranberry fruit.

Light treatment	Total anthocyanin (mg)/100 g fresh fruit
Natural light (48 h)[a]	35.47* ± 2.39
Natural light (24 h)[b]	33.24* ± 1.47
Dark	18.95 ± 0.88
Red light	26.82* ± 1.6
Far-red light	25.53* ± 2.89

Values are expressed as mean ± SE (n = 3).

a: water immersion time was 48 hours.

b: water immersion time was 24 hours.

*$P < .02$.

HPLC analyses

HPLC analyses of anthocyanins were carried out on a Waters 515 Dual Pump HPLC system, equipped with a 996-photodiode-array detector and a C_{18} column (4.6 × 150 mm) with 5 μm particle size (Waters Corporation, Milford, Mass). The software used to control the HPLC system and data analysis was Millennium 32 (Waters Corporation, Milford, Mass). Elution was carried out using a mobile phase formed by a linear gradient of (A) H_2O-acetic acid (10 : 1) and (B) methanol-acetic acid (10 : 1), with 100% (A) at 0 minute to 40% (A) and 60% (B) at 20 minutes. The flow rate was fixed at 0.2 mL/min. Anthocyanin separation and elution were detected by monitoring absorbance at 535 nm. Anthocyanin content was calculated in absolute quantities using the extinction coefficient ($\varepsilon^{1\%}_{1\,cm}$) at 535 nm as 982 [19].

RESULTS AND DISCUSSION

Composition of anthocyanins plays a role in their therapeutic effects [20]. Although six anthocyanins have been identified in cranberries [21, 22, 23], biosynthesis of those individual anthocyanins in response to environmental conditions such as light is not understood. In an attempt to elucidate anthocyanin biosynthesis, we measured total as well as individual anthocyanin content in cranberry fruit under different light conditions in an experimental setting designed to mimic water-harvesting conditions.

Statistical analysis (Student t test) was performed to detect the statistical difference between total anthocyanin content under natural light (48-hour and 24-hour), red light, and far-red light conditions and that under dark conditions. Table 1 shows that the total anthocyanin level varied significantly (> 98% confidence level ($P < .02$)) when the submerged, harvested cranberries were exposed to various light conditions. The total anthocyanin content of berries before exposure to any experimental light conditions was 18.95 ± 0.88, and was the same as the

Table 2. Percentage of anthocyanin increased in submerged, harvested cranberries exposed to different light conditions in comparison with the control, which was kept in the dark.

	Natural light (48 h)	Natural light (24 h)	Red light	Far-red light
Cyanidin 3-galactoside	89.3*	69.0*	29.1***	17.0***
Cyanidin 3-glucoside	53.8***	38.5***	71.8***	92.3***
Cyanidin 3-arabinoside	77.5*	68.2*	30.6**	30.3**
Peonidin 3-galactoside	99.6*	92.5*	43.5*	35.1*
Peonidin 3-glucoside	100.0*	80.7*	54.4*	45.6*
Peonidin 3-arabinoside	72.4*	72.4*	72.4*	69.8*
Total anthocyanins	87.2*	75.3*	41.5*	34.7*

*$P < .02$; **$.05 < P < .1$; ***$.1 < P < .5$

control that was kept in the dark. Compared to the control, cranberries exposed to one 24-hour day-night cycle had 75.3% higher anthocyanin content, and berries exposed to a 48-hour day-night cycle posted only a small further increase (87.2%). Red and far-red light had substantially less effect on total anthocyanin biosynthesis than natural light (75–87% vs. 35–42%). Red light increased total anthocyanin content (41.5%) more than far-red light (34.7%).

Separation of cranberry anthocyanins by reverse-phase HPLC revealed six anthocyanins which were assumed to be cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, peonidin 3-galactoside, peonidin 3-glucoside, and peonidin 3-arabinoside (Table 2) according to previous reports [6, 24]. The relative amounts of the six anthocyanins in the control samples which were kept in the dark (Figure 1) are consistent with earlier reports [6, 24]. Variation in different individual anthocyanins under different light conditions was also subjected to the statistical analysis (Student-test), which showed significant differences except for the cyanidin 3-glucoside under each light condition, and for cyanidin 3-galactoside under red light and far-red light conditions, as shown in Table 2. Compared with the dark conditions, the natural light conditions enhanced all the anthocyanins substantially, 72%–100% (in the 48-hour cycle), except for the cyanidin 3-glucoside, which increased by 54% (Table 2), whereas the red and far-red light had the most prominent effect on cyanidin 3-glucoside and peonidin 3-arabinoside, showing 70–92% increase (Table 2). The biosynthesis of cyanidin 3-galactoside was least affected by red and far-red light, showing only 29 and 17% increase, respectively (Table 2).

Light-dependent anthocyanin biosynthesis significantly depends on plant species and experimental conditions [13]. Although experimental conditions in our previous study [16] were different (30 minutes of light treatments per day for eight days), results had shown that red light and sunlight increased anthocyanin biosynthesis more than the far-red light did, consistently with the conclusions of the present study. However, in the above two cases—cranberry fruit that were still attached to the plant and cranberry fruit that were no longer attached to the plant, the effect of far-red illumination appeared to be close to the effect of red light, not similar to the dark control. Exposure of etiolated normal seedlings of *Brassica rapa* to red light and far-red light showed that far-red illumination enhanced more anthocyanin synthesis than red light [25]. Study of different phytochromes in Arabidopsis photomorphogenesis has shown that phytochrome A regulates plant responses to far-red light irradiation, whereas phytochrome B plays a predominant role in responses to red light irradiation [26]. Therefore, it is considered that coactions between different photoreceptors involved in the effects of red light and far-red light on anthocyanin content in cranberry fruit are as coactions between the photoreceptors involved in flavonoid biosynthesis [27].

In addition, anthocyanins contain two parts in their structures: aglycones and sugars. The biosynthesis of anthocyanins was catalyzed by several enzymes from PLA (phenylalanine ammonia-lyase), C4H (cinnamic acid 4-hydroxylase), 4CL (4-coumarate:CoA ligase), CHS (chalcone synthase), CHI (chalcone isomerase), F3H (flavanone 3 β-hydroxylase), DFR (dihydroflavonol 4-reductase), AS (anthocyanin synthase) through 3GT (UDP-glucose:flavonoid 3-O-glycosyl transferase). CHS is the first committed enzyme of flavonoid biosynthesis. AS is the first committed enzyme of anthocyanin biosynthesis. The expressions of the above enzymes are regulated differently by environments such as light and temperature. This results in the disproportional increase of different anthocyanins such as peonidin 3-glucoside compared to cyanidin 3-glucoside, due to the different aglycones although same sugar; or cyanidin 3-galactoside compared to cyanidin-3-glucoside, due to the different sugars although same aglycon.

This study demonstrates that during water-harvesting conditions, where the berries are no longer attached to the plant, exposure of the berries to light still results in increased anthocyanin levels. This study also shows that levels of individual anthocyanins increase differently following different light exposure such as natural light, red light, and far-red light. The variation in composition of anthocyanin may be manipulated to obtain a more valuable antioxidant product from cranberries. This study also contributes to our understanding of cranberry anthocyanin biosynthesis under water-harvesting conditions.

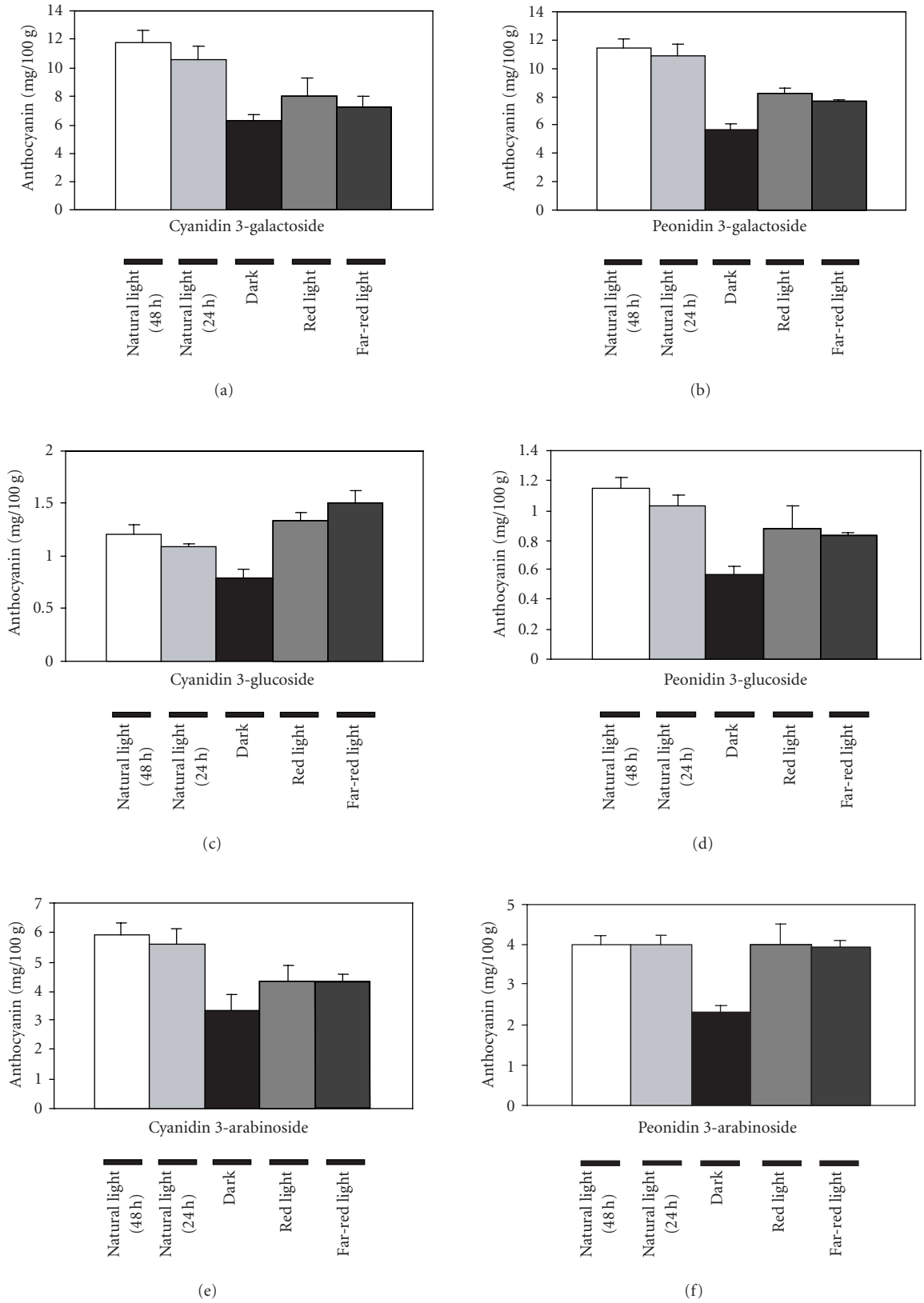

Figure 1. Effect of light on individual anthocyanin levels in submerged, harvested cranberry fruit. Cranberries were exposed to different light conditions and individual anthocyanin content was analyzed. Different light conditions, natural light (48 h), natural light (24 h), dark, red light, and far-red light, are indicated in the bottom. Values are mean from triplets with standard error bars.

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RESEARCH ARTICLE

To Stretch the Boundary of Secondary Metabolite Production in Plant Cell-Based Bioprocessing: Anthocyanin as a Case Study

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Plant cells and tissue cultures hold great promise for controlled production of a myriad of useful secondary metabolites on demand. The current yield and productivity cannot fulfill the commercial goal of a plant cell-based bioprocess for the production of most secondary metabolites. In order to stretch the boundary, recent advances, new directions and opportunities in plant cell-based bioprocessing have been critically examined for the 10 years from 1992 to 2002. A review of the literature indicated that most of the R&D work was devoted predominantly to studies at an empirical level. A rational approach to molecular plant cell bioprocessing based on the fundamental understanding of metabolic pathways and their regulations is urgently required to stimulate further advances; however, the strategies and technical framework are still being developed. It is the aim of this review to take a step forward in framing workable strategies and technologies for molecular plant cell-based bioprocessing. Using anthocyanin biosynthesis as a case study, an integrated postgenomic approach has been proposed. This combines the functional analysis of metabolic pathways for biosynthesis of a particular metabolite from profiling of gene expression and protein expression to metabolic profiling. A global correlation not only can thus be established at the three molecular levels, but also places emphasis on the interactions between primary metabolism and secondary metabolism; between competing and/or complimentary pathways; and between biosynthetic and post-biosynthetic events.

INTRODUCTION

Plants are probably the best cell factories on this planet from which more than 100 000 low molecular secondary metabolites have been discovered, with the estimated total number in plants exceeding 500 000 [1]. Plant metabolites not only are used for food purposes but also serve as an important historical source of medicines. Plant cell-based bioprocessing is the use of the biosynthetic pathways of plant cells/tissues for the production of valuable metabolites and for biotransformation. Despite worldwide effort over 40 years, "potential" is still the word most frequently used to describe this technology as it has met with very limited commercial success [2, 3]. To advance our knowledge and tools in translating "potential" into "commercial success," the present paper examines the recent advances in this area and intends to establish strategies and the technical framework for a rational molecular bioprocessing approach. To illustrate this approach, we present some data on the functional analysis of metabolic pathways for biosynthesis of anthocyanins: from profiling of gene expression and protein expression to metabolic profiling in *Vitis vinifera* cell culture as a model system. Emphasis was placed on a global correlation at three molecular levels—gene transcript, enzyme, and metabolite—as well as on the interactions between the biosynthetic pathway and post-biosynthetic events. The latter has been largely overlooked in the past.

Focusing on the fundamental understanding of the complex metabolic pathway and regulation of secondary metabolism in plant cell cultures, we are developing advanced knowledge of biosynthesis as well as post-biosynthesis pathways of anthocyanins from the genetic to the metabolite level [2, 4, 5, 6, 7]. The metabolic pathways were characterized dynamically using techniques such as precursor feeding, elicitation, metabolic inhibition and analysis of strains, mRNA expression, enzyme activities, and anthocyanin profiles. Information gained on identification, regulation, and manipulation of the limiting and key steps involved in the anthocyanin pathways is leading us to new avenues for a rational bioprocess engineering optimization of anthocyanin production at both the molecular and the cellular level. One of our current aims is to develop an integrated process that rationally combines different enhancement strategies for further productivity increases in anthocyanin production in

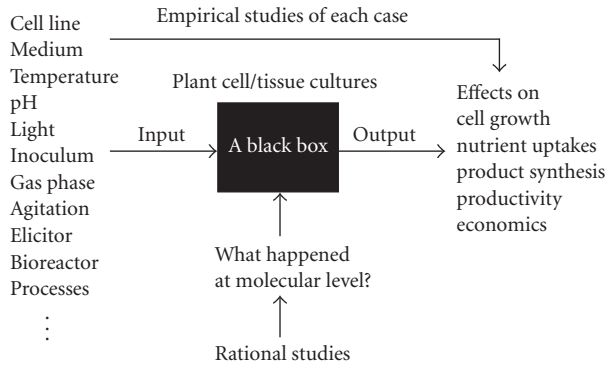

Figure 1. Moving from empirical to rational approaches for the understanding of plant cell-based bioprocessing at molecular level (genetic, enzymatic, and metabolic levels) which remains a black box at large.

V vinifera cell cultures [3]. Another interesting strategy is to elucidate the mechanism for anthocyanin transport and storage in plant cells with the aim of manipulating the transport and storage for enhancing anthocyanin production.

RATIONAL APPROACH: EXPECTATION AND REALITY

Empirical approaches have been employed for the development and optimization of plant cell-based bioprocesses since their onset. The typical feature of the empirical approaches is to optimize the plant cell culture system with regard to its input factors (cell line, medium, culture parameters, bioreactors, process operations, etc) and output factors (cell growth, nutrient uptake, productivity, yield, etc). As illustrated in Figure 1, what occurs at the cellular and molecular levels remains largely unknown, as a "black box."

This "brute-force" empirical approach has to be applied to every plant cell culture process, which is time-consuming, costly, and often suboptimal [8, 9]. As demonstrated by the limited commercial success to date, There may be little hope in stretching the biosynthetic capability of secondary metabolites in plant cell bioprocesses. Rational approaches, in contrast, which have been proposed since the mid-1980s, are directed to the understanding of the black box with the rapid development of modern genetic technology [10]. With the expectation of a radical improvement in biosynthetic capability through engineering secondary metabolism in plant cells, the major challenge for a rational approach is to obtain information on the identification and regulation of biosynthetic genes and pathways.

To obtain a clear picture of what has been done in the development of rational approaches, a literature survey on plant cell/tissue cultures was carried out from 1992 to 2002 using Biological Abstracts. Although it was not intended to be a thorough survey, its results presented in Figure 2 did reveal some interesting information. The work is considered to be a rational study when it is devoted to characterizing the pathway genes, enzymes, cell physiology, and metabolite profiling. Otherwise it is considered to be an empirical study. Of the nearly 600 publications during this period, an average of 37% contributed to the rational approach, leaving the majority still in the empirical study domain (Figure 2). The movement toward a rational understanding is rather consistent, with no increasing trend (Figure 2). Furthermore, most of the rational studies are focused on a small handful of secondary metabolites, such as alkaloids (ajmalicine, vincristine, and vinblastine) and flavonoids (anthocyanins). The question is therefore raised: why has the R&D effort toward a rational approach in plant cell bioprocesses remained so stagnant given the rapid entry into the postgenomic era during the past 10 years?

RATIONAL APPROACH: CHALLENGES AND POTENTIALS

The primary task of a rational approach for plant cell bioprocessing is to identify the biosynthetic genes, enzymes, and pathways for a specific secondary metabolite. This is essentially a functional analysis after the genome discovery. Though advocated since the mid-1980s, it is only recently that the large-scale functional analysis of secondary metabolism becomes feasible with the completion of the sequencing of the Arabidopsis and rice genomes [11]. Technically, the paradigm shift from empirical to rational was born during the past decade and is now at the early stages of expected rapid growth in the next decade. With the continuing success of numerous sequencing projects, we have entered a new era of plant cell-based bioprocessing. Firstly, we have at our disposal an enormous resource of genes, for the functional analysis of their roles, protein products, and pathways and biosynthesis of specific classes of secondary metabolites. Secondly, the accumulated information in empirical studies of plant cell bioprocesses will provide important leads/conditions for such a functional analysis. Thirdly, large-scale, global, and dynamic analysis of

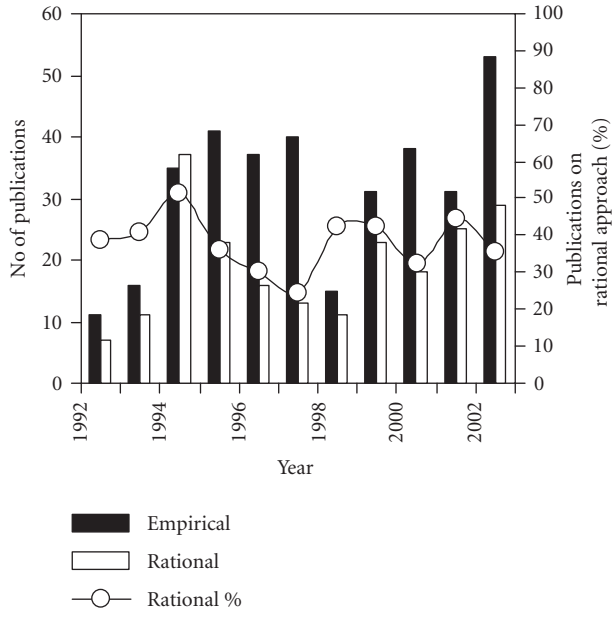

Figure 2. Number of publications on empirical approaches and rational studies of plant cell/tissue cultures, and the percentage of rational studies from 1992 to 2002. The search is based on the Biological Abstracts.

secondary metabolism will become feasible with the rapid advances in post-genomic tools, such as transcriptomics, proteomics, and metabolomics in this new era. This analysis will address the interactions at a dynamic and global level among the primary metabolism, the biosynthetic pathways for secondary metabolism, and the post-biosynthetic events of specific metabolites. As a result, plant cell bioprocess engineers will be empowered with greater capability to stretch the limits of natural biosynthetic pathways of plant cells by mobilizing the genes of interest into transgenic plant cells to perform valuable functions for industry, medicine, and the environment.

Although the future looks brilliant, the strategies and technical framework for a rational approach are still being developed. Below are several major challenges that must be faced:

(1) the extreme complexity of secondary metabolism will demand novel strategies and tools;

(2) the diversity, specificity, and variability of secondary metabolites within and among plant families or species have been the major impediment in the elucidation of many secondary pathways;

(3) regulatory properties in secondary metabolism, such as cell-type-specific localization and transient expression, may complicate the true biosynthetic potential of plant cells;

(4) many plant species have complex genomes; interactions among complex and diverse metabolisms may prevent efficient genetic and metabolic engineering manipulations.

RATIONAL APPROACH: STRATEGIES AND TECHNICAL FRAMEWORK

Rational manipulation of "linear primary pathways" such as starch biosynthesis is straightforward and has turned out to be successful [12]. However, the biosynthetic pathways of plant secondary metabolites are often extremely complicated as illustrated in Figure 3.

In formulating the strategies for a rational approach, the complexity will need to be considered in terms of linking primary metabolism with secondary metabolism, linking biosynthetic pathways with post-biosynthetic events, as well as linking targeted biosynthetic pathways with competing and/or complimentary pathways (Figure 3). Several strategies have been proposed recently to address the challenges and complexity [2, 11, 13].

The three main tasks of a rational approach are (i) characterization of all the genes involved, their protein products, and their metabolic products in a given biosynthetic pathway; (ii) characterization of their respective regulatory functions and roles; (iii) manipulation of the biosynthetic pathways for a given application via the engineering of metabolism. Here, we propose a technical framework to implement this approach.

Firstly, the tools of genomics, proteomics, and metabolomics have to be integrated to obtain a global understanding of secondary metabolism at three molecular levels. Any characterization at a single level may fail to be applied for rational bioprocess improvement. Oksman-Caldentey et al [11] have proposed an approach that combines metabolomics and transcriptomics. Using a genome-wide transcript cDNA-AFLP profiling [14] in

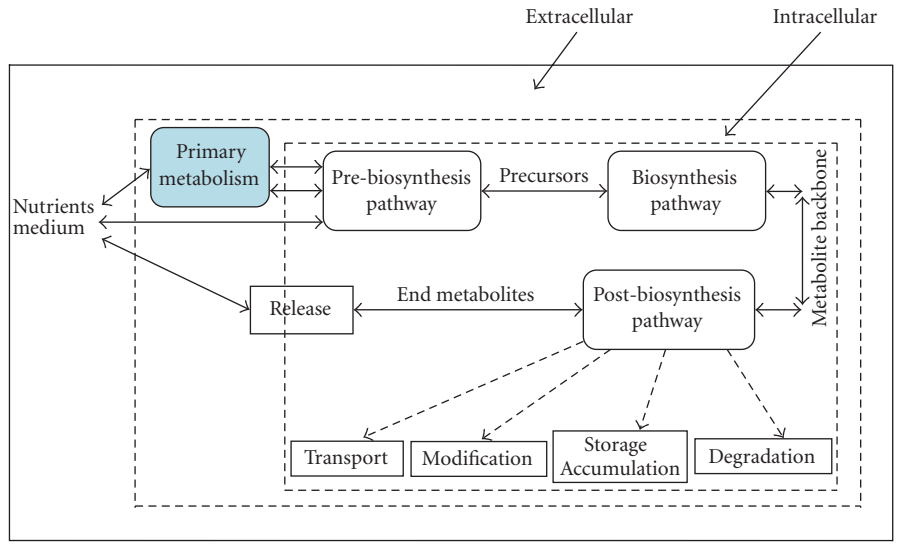

Figure 3. Pathway events involved in biosynthesis of a metabolite in plant cells: primary metabolism and secondary metabolism (pre-biosynthetic, biosynthetic, and post-biosynthetic pathways).

combination with a GC-MS-SIM alkaloid analysis, the simultaneous detection of the genetic onset of various secondary metabolic pathways and genetic reprogramming of primary metabolism to sustain secondary metabolism was achieved in a jasmonate-elicited BY-2 tobacco cell culture. The advantage of this approach is the capability of detecting the vast majority of transcripts without any prior sequence knowledge.

Secondly, genome-wide analysis is essential to understand the interactions between the biosynthetic pathways of targeted metabolites and their linked pathways (complimentary and competing pathways). The regulation and manipulation of complimentary pathways provides the means to sustain the targeted pathways, while the blocking of competing pathways is critical for redirecting metabolic flux. One example of these strategies was illustrated by the combination of activation tagging mutagenesis with high-throughput screening for biological active metabolites, which enabled the isolation of genetic material relevant to the synthesis of specific natural products [13]. This approach involved the generation of a callus library via plant cell mutagenesis prepared by activation T-DNA tagging [15], the development of a high-throughput screen assay for the targeted metabolites, and the functional genomics analysis of the overexpressing cell lines. The strategy has the advantage that no prior knowledge of the metabolic pathway is required—only a method of screening for the metabolite products.

Thirdly, the primary metabolism to sustain the respective secondary metabolism has to be characterized. The responses of plant secondary metabolism to any genetic/metabolic manipulation will largely depend on the cell physiological and nutritional state, as an indicator of primary metabolism. The empirical approach optimizes the growth environment with the cells as a black box, thereby bypassing the cell physiological state as an intermediate control objective. In a rational approach, this limitation must be addressed through explicit monitoring and control of cellular physiology. Konstantinov [16] has proposed a generic methodology for the design of systems capable of performing these advanced monitoring and control functions. The physiological state was quantified by a vector composed of several process variables that were selected among different classes, including specific metabolic rates, metabolic rate ratios, degrees of limitation, and others. This was demonstrated using plant cell culture of *Perilla frutescens* for anthocyanin production as an example. Lamboursain and Jolicoeur [17] highlighted the strong influence of plant cell nutritional status on cell growth and secondary metabolite production capacity of the cells. They defined the nutritional status as the intra- and extracellular concentrations in nutrients, total nitrogen, phosphate, and carbohydrate. The lack of information and control of cell nutritional status may be responsible for the poor reproducibility of plant cell bioprocesses.

Finally, the post-biosynthetic events will need to be characterized and targeted for engineering manipulation [2, 6]. As illustrated in Figure 3, secondary metabolism may include the pre-biosynthetic pathways to produce the precursors, the biosynthetic pathways to produce the core metabolite structures, and the post-biosynthetic events. These post-biosynthetic events—the chemical and enzymatic modifications, transport, storage/secretion, and catabolism/degradation—have been largely unexplored in the past. Zhang et al [2] have demonstrated the significance in characterization and manipulation of these post-biosynthetic events in stretching the biosynthetic capability of plant cells.

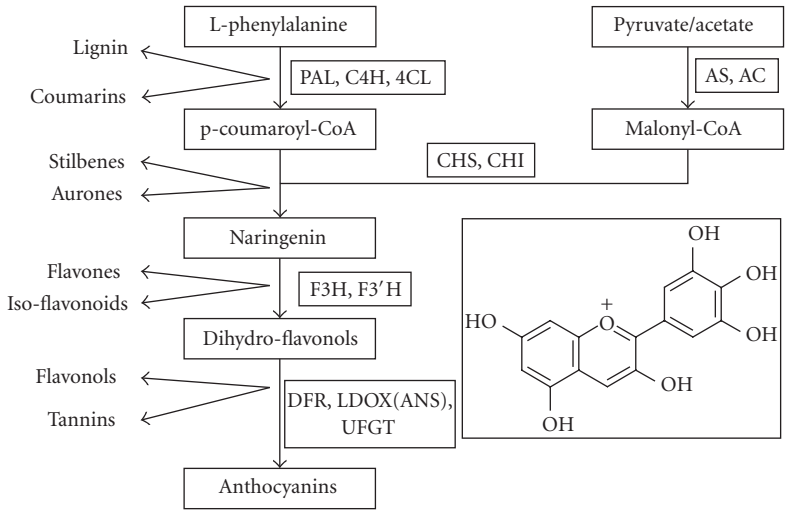

Figure 4. Schematic of anthocyanin biosynthetic pathways and the key enzymes involved. Branched pathways leading to other metabolites are also indicated. Phenylalanine ammonia lyase (PAL); cinnamate-4-hydroxylase (C4H); 4-coumarate-CoA ligase (4CL); acetyl-CoA synthetase (AS); acetyl-CoA carboxylase (AC); chalcone synthase (CHS); chalcone isomerase (CHI); flavanone 3β-hydroxylase (F3H); flavonoid 3′-hydroxylase (F3′H); dihydroflavonol 4-reductase (DFR); leucoanthocyanidin dioxygenase (LDOX (ANS)); UDP-glucose:cyanidin 3-O-glucosyltransferase (UFGT).

RATIONAL APPROACH: ANTHOCYANIN AS A CASE STUDY

In our laboratory, we are interested in the fundamental understanding of the biosynthetic pathways and regulation of secondary metabolism in plant cell cultures with the ultimate goal of rational development of commercial plant cell bioprocesses. The establishment of a rational approach is an ongoing effort toward an advanced knowledge of biosynthesis and post-biosynthesis pathways of anthocyanins in *V vinifera* cell culture as a model system from genetic to metabolite level [2, 3, 4, 5, 6, 7].

Anthocyanins, responsible for various attractive colors in plants, are becoming an important alternative to many synthetic colorants and have potential applications in nutraceutical developments [9]. There is a great deal of information available on the anthocyanin biosynthetic pathway as shown in Figure 4. As discussed above, our approach started with the establishment of integrated post-genomic tools to characterize anthocyanin metabolism at transcriptional, enzymatic, and metabolic levels. As the stilbene pathway competes directly for the precursors for anthocyanin biosynthesis, characterization of the stilbene biosynthetic pathway was carried out concurrently. In addition, anthocyanin transport and storage were initially targeted for the elucidation of post-biosynthetic events.

Transcriptional, enzymatic, and metabolic characterization of anthocyanin pathways

Using techniques such as precursor feeding, elicitation, metabolic inhibition, redirected transport, and analysis of strains, the dynamic profiles of mRNA expression, enzyme activities, and anthocyanin metabolites of the biosynthetic pathways in *V vinifera* cell culture were characterized. One example was the functional analysis of *V vinifera* cell cultures that were elicited with jasmonic acid, light, and sucrose alone and in combination [3, 4]. All these single conditions enhanced anthocyanin production and exhibited a synergistic improvement when combined [3, 4]. Early transcriptional studies were done by Northern blotting, and later quantitative RT-PCR, with a sample of results shown in Figure 5. Results indicated a strong correlation between transcriptional expression and improved anthocyanin biosynthesis and a role of jasmonic acid in upregulating DFR [4].

Metabolic profiling was also carried out substantively for these conditions. Results implicated the competition between anthocyanin and stilbene pathways, and the importance of methylated and acylated anthocyanin species in enhanced production [5]. Full characterization is in progress following the completion of the methodology development.

Characterization of anthocyanin post-biosynthetic events

Anthocyanins are synthesized in the cytoplasm and transported into the vacuole where they bind with a protein matrix and form anthocyanic vacuolar inclusions (AVIs) (Figure 6a) [6]. AVIs were considered to be the storage sites of anthocyanins. In recognition that the post-biosynthetic steps may play equally crucial roles in its yield improvement, we have been investigating the characteristics and roles of glutathione S-transferases (GSTs) and AVIs in anthocyanin transport and storage, respectively, in grape cells. We have isolated the AVIs and are

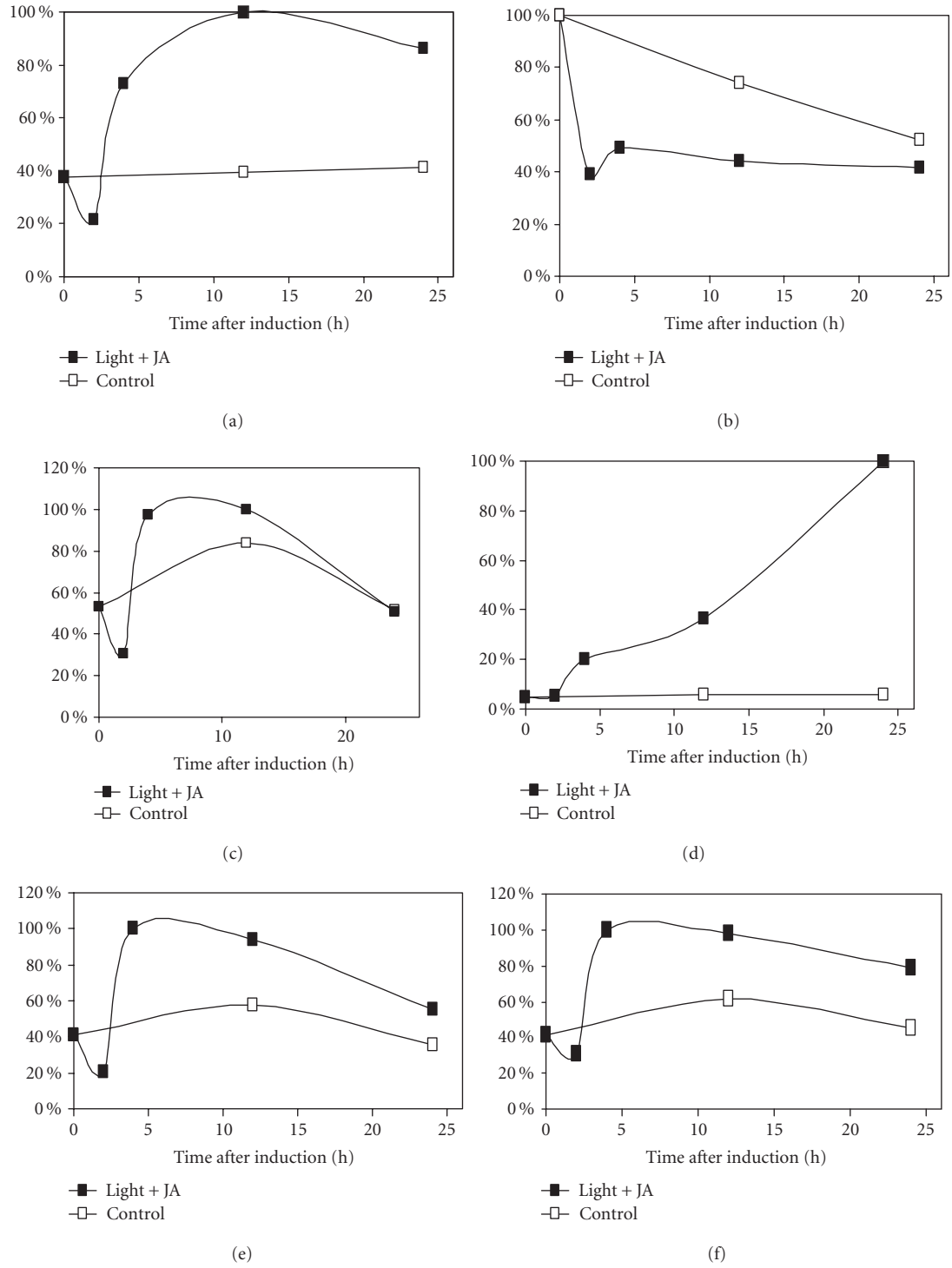

Figure 5. Quantification of mRNA level for six anthocyanin biosynthesis genes in *V vinifera* cell culture grown under darkness (control), and under light of 8000 Lux with JA elicitation on day 0. The RNA was probed with *V vinifera* cDNA clones for (a) CHS, (b) CHI, (c) F3H, (d) DFR, (e) LDOX, and (f) UFGT. Ribosomal RNA was used as an internal control. Images of Northern blot were visualized by Phosphorimager, corrected with respective rRNA, and quantified using ImageQuant software.

characterizing them using an integrated post-genomic approach as mentioned above. Initial results indicated AVIs may be composed of several protein species and have the selectivity for acylated anthocyanins (Figures 6b and 6c). It is expected that these studies will provide additional targets for rational metabolic engineering [2].

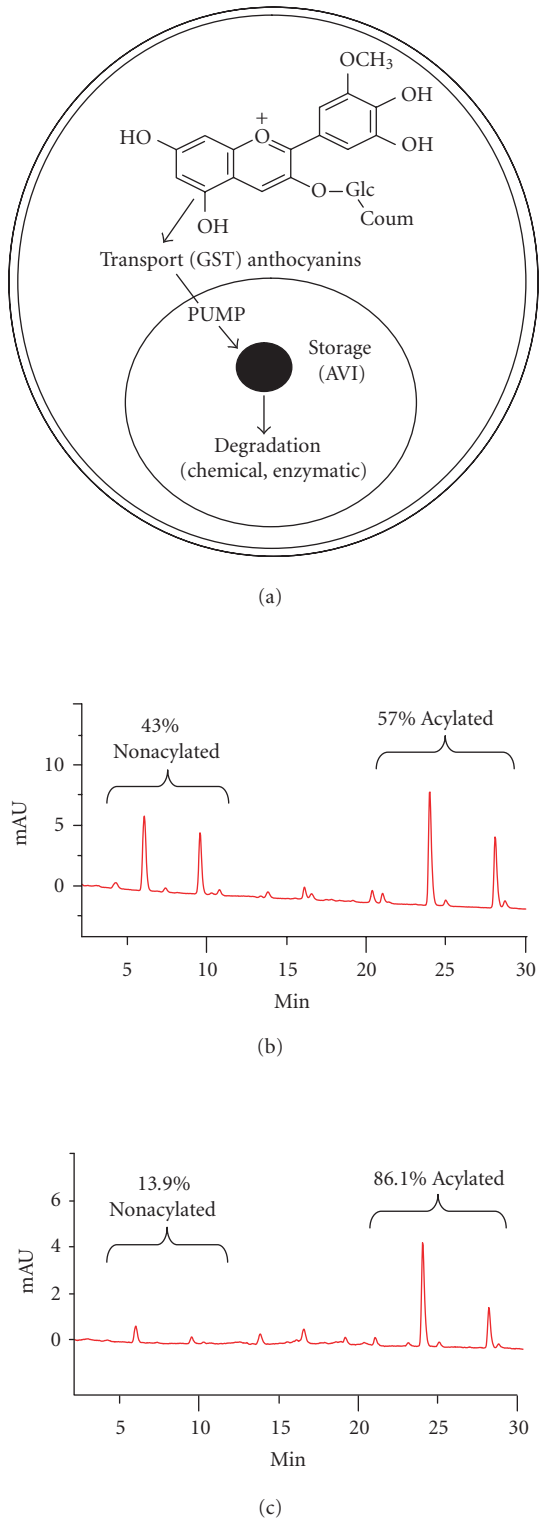

FIGURE 6. (a) A schematic of anthocyanin post-biosynthetic events. (b) HPLC profiles of whole cell. (c) HPLC profile of AVI extracts of the grape cell line at 520 nm. Nonacylated and acylated (p-coumaroylated) species are grouped and mean percentages of total peak area are shown ($n = 4$). mAU = milli absorbance units.

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RESEARCH ARTICLE

Effect of Grape Seed Extract and Quercetin on Cardiovascular and Endothelial Parameters in High-Risk Subjects

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Grape seed extract (GSE) has in vitro antioxidant activity but whether or not it works in vivo is not clear. In a fully randomised, crossover trial with 4-week treatment periods on 36 men and women with above-average vascular risk, we aimed to demonstrate that 2 g/day of GSE (1 g of polyphenols) alone, or with 1 g/day of added quercetin in yoghurt, favourably alters vascular function, endothelial function, and degree of oxidative damage in comparison to a control yoghurt. GSE alone improved flow-mediated dilatation determined ultrasonically by an absolute 1.1% compared with control. There was no effect of the combination of GSE with quercetin. No other blood or urine measure was altered. Thus sufficient polyphenols from GSE appear to be absorbed to influence endothelial nitric oxide production, and GSE has the potential to favourably influence vascular function.

BACKGROUND

Wine polyphenols have been postulated to have many favourable effects [1, 2, 3, 4] but most of this data has been obtained in vitro [5, 6]. Grape seed extracts (GSEs) contain a high concentration of many of the polyphenols in grape skins, in particular, the proanthocyanidins, which are also found in red wine. Green tea also contains polyphenols, in particular, the catechins, which are believed to mediate many of the cancer chemopreventive effects [7]. Green tea has been epidemiologically associated with protection from both cancer and heart disease [8]. Although there is abundant in vitro evidence that polyphenols have antioxidant and anticancer effects, there is a dearth of animal and human experiments [9]. CSIRO data (unpublished) indicates that GSEs inhibit low-density lipoprotein (LDL) oxidation and reduce aortic ring constriction in vitro. Extract of oligomeric proanthocyanidins from other sources such as Pycnogenol from pine bark enhances nitric oxide (NO) production from vascular endothelium in vitro [10]. Grape seed proanthocyanidin extract (GSPE, 0.1% level) has been shown to reduce atherosclerosis in cholesterol-fed rabbits by 30%–50% probably by inhibiting LDL oxidation as lipid levels were not altered while malondialdehyde levels in the aorta (an index of lipid oxidation) were reduced by 25% [11]. Plasma proanthocyanidins were not detectable, suggesting that absorption is very low. Rats given a dose as a single bolus do have low (0.5 μM) but detectable levels. The dose of GSPE was equivalent to a dose of 3.5 g for a 70 kg human [12]. Most over-the-counter forms of GPSE are in doses of 50–100 mg with a recommendation to take 1 capsule/day. This material has been available for many years and is regarded as safe and nontoxic.

Quercetin, a flavanoid prominent in onions and apples, has been epidemiologically associated with protection from coronary artery disease and cancer [13, 14] and is now available over the counter in 300–500 mg dose forms, with daily doses of up to 1500 mg. No clinical trials in the cardiovascular area have been performed with quercetin although it has been shown to inhibit monocyte adhesion to endothelial cells [15]. This is believed to be the first step in the process of atherosclerosis. One trial using 4 g/day has shown no effects on lipids, blood pressure, or platelet activation in normal volunteers [16].

In this study we hypothesised that 2 g/day of GSE would improve flow-mediated dilatation (FMD) and this might be mediated by changes in the production of NO. We also hypothesised that GSE and quercetin would function as antioxidants in plasma, reduce the level of F2 isoprostane in urine, and possibly influence the level of oxidized LDL in plasma and, secondary to this, reduce the activation of the endothelium. This would be assessed by changes in adhesion, clotting, and fibrinolytic molecules.

METHODS

Subjects

Forty-three men and women with above-average vascular risk due to high cholesterol, smoking, or high blood pressure were recruited by public advertisement

and screened at the Clinical Research Unit, CSIRO Health Sciences and Nutrition, Adelaide. There were no exclusion criteria on the basis of medication or consumption of alcohol. Subjects were excluded if their body mass index (BMI) was greater than 35 or if they suffered from diabetes mellitus, untreated metabolic disorders such as thyroid or adrenal disease, liver or kidney disease, or unstable coronary artery disease. All subjects gave written, informed consent and the protocol was approved by the Human Ethics Committee of CSIRO.

The trial was 12 weeks long and consisted of 3 four-week periods of a double-blind randomised crossover with control and active ingredients (1 g GSE from Tarac +/− 0.5 g quercetin) in 240 g of yoghurt taken twice daily. Blood samples and vascular compliance measures were taken at baseline and at the end of each period. The background diet was a low-polyphenol, low-quercetin diet. This was achieved by restricting tea and coffee to a maximum of 2 cups per day, restricting apples to one per day, and forbidding red wine and onions throughout the 12 weeks. Measures included FMD using ultrasound, vascular compliance using radial pulse analysis (Hypertension Diagnostics Inc/PulseWave CR-2000), fasting lipids, oxidized LDL, nitrates (to assess the antioxidant effects), C reactive protein (CRP), von Willebrand factor (VWF), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-1), vascular cell adhesion molecule (VCAM1), and intercellular adhesion molecule 1 (ICAM-1). Twenty-four-hour urine was collected to measure the oxidized lipid isoprostane $F_{2\alpha}$.

FMD was assessed in the brachial artery after blockage of blood flow in the forearm with a blood pressure cuff at 200 mmHg for 5 minutes. The response of the vessel 5 minutes after administration of 100 μg of glyceryl trinitrate (GTN) sublingually was also assessed [17].

Serum lipids

Serum lipids (total cholesterol, triglyceride, HDL cholesterol) were measured on 2 consecutive days at baseline and at the end of each 3-week intervention period. Venous blood samples (20 mL) were taken into plain tubes after an overnight fast of 12 hour. Serum was separated by low-speed centrifugation at 600 g for 10 minutes at 5°C (GS-6R centrifuge; Beckman, Fullerton, Calif) and frozen at −20°C. At the end of the study, all samples from each subject were analysed within the same analytic run. Total cholesterol and triacylglycerol were measured on a Cobas-Bio centrifugal analyzer (Roche Diagnostica, Basel, Switzerland) using enzymatic kits (Hofmann-La Roche Diagnostica, Basel, Switzerland) and standard control sera. Plasma HDL-cholesterol concentrations were measured after precipitation of apoB containing lipoproteins by PEG 6000. The coefficients of variation for the individual lipids were all less than 5%. The following modification of the Friedewald equation for molar concentrations was used to calculate LDL cholesterol in mmol/L: total cholesterol—(triacylglycerol/2.18)—HDL cholesterol.

All other tests were enzyme linked immunosorbent assays (ELISAs): VWF (Helena Laboratories, Melbourne Australia), Coaliza tPA (Chromogenix, Sweden), Coaset tPA (Chromogenix), Coaliza PAI-1 (Chromogenix), Coatest PAI-1 (Chromogenix), oxidised LDL: Mercodia oxidised LDL ELISA (Mercodia, Sweden), SVCAM1 (Immunokontact, Sweden), ICAM1 (Immunokontact), CRP (Alpha Diagnostic International, Texas), 8-isoprostane (8-iso PGF2α) (Cayman Chemical), nitrate/nitrite assay kit (Cayman Chemical).

Statistical analysis

Repeated measures analysis of variance was calculated with type of yoghurt as the within-subject factor and with sex and order as the between-subject factors. Where there was a significant treatment effect detected by repeated measures, paired Student t tests were used to locate differences. Bivariate correlation was conducted using Pearson's correlation coefficient. Analyses were performed with SPSS 10.0 for Windows (SPSS Inc, Chicago, Ill). Significance was set at $P < .05$.

RESULTS AND DISCUSSION

Twelve women and twenty-four men completed the study and one additional woman missed the last phase of treatment. Six subjects withdrew after commencement and 6 withdrew prior to commencement.

The risk profile of subjects was as follows: 6 subjects had high blood pressure (5 on medication), 3 were smokers, and 31 had high cholesterol (greater than 5 mmol/L on finger prick). Two volunteers on atorvastatin to lower cholesterol stopped the medication prior to beginning the trial. The average cholesterol was 6.5 mmol/L (range 4.68 to 8.63), average age 58 years (range 34–70), weight 83.1 Kg (63.1 kg to 118.7 kg), BMI 28.4 (19.8–37.5). Mean blood pressure was 127 mm Hg systolic and 74 mm Hg diastolic.

Blood pressure/vascular compliance

There was a weak ($P < .05$) trend to a lowering of systolic blood pressure over the duration of the trial with a fall from 127 mm Hg at baseline to 124 mm Hg at week 12. This is quite usual in clinical trials in which blood pressure is measured. There were no changes in any vascular parameter with treatment (see Table 1).

Flow-mediated dilatation after compression release and GTN dilatation

GSE alone produced an absolute 1.1% greater dilatation compared with control ($P < .05$) but the addition of quercetin apparently nullified this completely. GTN-induced dilatation was not influenced by GSE but quercetin again appeared to diminish the response compared with baseline ($P < .05$), but not compared with control.

Table 1. Cardiovascular measures produced by the HDI compliance instrument; mean of 35 complete measures ± SD.

	Baseline	GSE	GSE/quercetin	Control
Systolic BP (mmHg)	127 ± 15	124 ± 14	125 ± 11	124 ± 13
Diastolic BP (mmHg)	74 ± 9	73 ± 8	73 ± 10	73 ± 9
Mean BP (mmHg)	94 ± 13	91 ± 18	94 ± 12	91 ± 12
Pulse pressure (mmHg)	53 ± 9	51 ± 8	51 ± 7	51 ± 8
Pulse rate (beats/min)	58 ± 8	59 ± 8	58 ± 7	57 ± 7
Estimated cardiac ejection time (ms)	335 ± 25	337 ± 24	336 ± 23	333 ± 36
Estimated stroke volume (mL)	93 ± 13	92 ± 12	93 ± 12	94 ± 12
Estimated stroke volume index (mL/m^2)	47 ± 5	47 ± 4	47 ± 4	47 ± 6
Estimated cardiac output (L/min)	5.4 ± 0.7	5.5 ± 0.7	5.5 ± 0.7	5.4 ± 0.7
Estimated cardiac output index (L/min/m^2)	2.8 ± 0.2	2.7 ± 0.3	2.8 ± 0.3	2.7 ± 0.2
Large artery elasticity index	17.5 ± 4.6	18.4 ± 4.8	18.7 ± 6.1	18.2 ± 4.7
Small artery elasticity index	7.4 ± 3.8	7.4 ± 3.4	7.9 ± 3.9	7.4 ± 3.2
Systemic vascular resistance	1364 ± 275	1345 ± 229	1364 ± 221	1352 ± 209
Total vascular impedance	131 ± 32	124 ± 33	125 ± 35	127 ± 35

Table 2. Flow-mediated dilatation as measured by ultrasound. $N = 35$, mean SD. Treatments with different superscripts are different at $P < .05$.

	Baseline	GSE	GSE/quercetin	Control
Precompression cm^{-2}	44.3 ± 6.3	45.1 ± 6.4	45.9 ± 7.2	45.5 ± 7.3
Postcompression	46.2 ± 5.8	47.4 ± 6.5	47.6 ± 7.5	47.3 ± 7.3
	($n = 30$)	($n = 35$)	($n = 32$)	($n = 36$)
Change	$1.9^{1,2}$ ± 1.3	2.3^{1} ± 1.4	1.7^{2} ± 1.0	1.8^{2} ± 1.3
	(4.3%)	(5.1%)	(3.7%)	(4.0%)
Pre-GTN	44.8 ± 7.1	45.8 ± 7.1	46.9 ± 7.5	46.2 ± 7.1
Post-GTN	52.1 ± 6.8	52.7 ± 6.9	52.9 ± 7.3	52.8 ± 7.0
	($n = 38$)	($n = 30$)	($n = 29$)	($n = 31$)
Change	7.3^{1} ± 2.4	$6.9^{1,2}$ ± 2.3	6.0^{2} ± 3.0	$6.5^{1,2}$ ± 1.8
	(16.3%)	(15.1%)	(12.8%)	(14.1%)

Table 3. Effect of GSE and GSE/quercetin on serum lipids mean (mmol/L) ± SD.

	Period 1 baseline	Period 2	Period 3	Period 4	GSE	GSE/quercetin	Control
Total cholesterol	6.57 ± 1.07	6.63 ± 1.06	6.59 ± 0.99	6.58 ± 1.06	6.63 ± 0.93	6.64 ± 1.10	6.64 ± 1.05
Triglyceride	1.80 ± 0.80	1.98 ± 1.06	1.90 ± 0.83	1.73 ± 0.88	1.88 ± 0.92	1.88 ± 0.85	1.92 ± 1.03
HDL cholesterol	1.18 ± 0.31	1.18 ± 0.31	1.19 ± 0.34	1.16 ± 0.33	1.18 ± 0.33	1.16 ± 0.34	1.15 ± 0.31
LDL cholesterol	4.59 ± 0.98	4.56 ± 0.93	4.55 ± 0.93	4.65 ± 0.98	4.61 ± 0.83	4.62 ± 1.00	4.63 ± 0.99

This indicates that GSE favourably influences the endothelium enhancing NO production, release or slowing down oxidative destruction of it, but quercetin appears to interfere with this. It is known that quercetin inhibits LPS-induced NO release in RAW 264.7 macrophages [18] and can act as a prooxidant in other systems [19, 20, 21] at both low and high levels (see Table 2).

Serum lipids

No changes were noted but none were expected. Subjects were at high risk of cardiovascular disease by virtue of the high average cholesterol (see Table 3).

C reactive protein

CRP is an acute-phase protein produced by the liver in response to tissue damage or inflammation and may increase 500 fold acutely [22]. It is also elevated but to low levels by low-grade inflammatory conditions such as atherosclerosis and can be used to predict clinical events [23]. Statins which lower cholesterol by inhibiting synthesis in the liver also lower CRP and the mechanism appears to be unrelated to the degree of cholesterol lowering [24]. It may be related to their antioxidant activity or a direct anti-inflammatory activity. Thus in this study it was used to check both for potential toxic effects and to

Table 4. Effect of GSE and GSE/quercetin on plasma CRP, nitrate/nitrite, and adhesion molecules. $N = 35$, mean SD.

	Period 1 baseline	Period 2	Period 3	Period 4	GSE	GSE/quercetin	Control
CRP (mg/L)	—	3.63 ± 5.01	3.48 ± 4.19	3.69 ± 4.17	3.40 ± 3.53	3.63 ± 5.10	3.73 ± 4.64
Nitrate (μmol/L)	31.1 ± 12.7	28.0 ± 11.0	36.9 ± 36.7	30.0 ± 13.2	35.6 ± 36.7	30.5 ± 12.4	28.4 ± 12.7
ICAM1 (μg/mL)	—	0.49 ± 0.10	0.49 ± 0.11	0.48 ± 0.11	0.49 ± 0.11	0.48 ± 0.11	0.49 ± 0.11
VCAM1 (μg/mL)	—	0.99 ± 0.28	0.99 ± 0.22	1.00 ± 0.24	0.98 ± 0.20	0.98 ± 0.26	1.02 ± 0.27

Table 5. Effect of GSE and GSE/quercetin on clotting and fibrinolytic factors. $N = 35$, mean SD.

	Baseline	Period 2	Period 3	Period 4	GSE	GSE/quercetin	Control
VWF (%)	—	115.58 ± 39.12	101.66 ± 35.82	102.37 ± 35.55	109.11 ± 36.06	104.50 ± 39.58	106.00 ± 36.40
PAI-1 ng/mL	—	50.47 ± 33.51	45.22 ± 21.41	49.27 ± 31.72	50.47 ± 35.77	48.58 ± 29.24	45.90 ± 21.41
PAI-1 activity	16.45 ± 9.72	16.27 ± 9.08	16.28 ± 8.05	16.28 ± 10.37	15.39 ± 9.30	16.78 ± 10.05	16.76 ± 8.13
tPA (ng/mL)	—	7.235 ± 3.396	6.636 ± 2.350	6.857 ± 2.378	6.929 ± 2.686	6.908 ± 2.835	6.891 ± 2.766
tPA activity	1.432 ± 1.204	1.295 ± 0.865	1.140 ± 0.715	1.269 ± 1.011	1.261 ± 0.822	1.146 ± 0.764	1.297 ± 1.011
tPA/PAI-1 activity	20.37 ± 21.63	20.91 ± 21.15	24.46 ± 24.06	27.11 ± 32.45	24.12 ± 27.75	26.05 ± 29.90	23.04 ± 21.3
tPA/PAI-1 mass	—	0.205 ± 0.348	0.188 ± 0.483	0.194 ± 0.648	0.187 ± 0.119	0.206 ± 0.179	0.195 ± 0.138

demonstrate a potential of GSE to act like a statin in the vessel wall. No differences were found between periods or treatments. One person was excluded as he had a respiratory infection requiring antibiotics which caused a sharp rise in CRP levels (over a 100-fold rise) (see Table 4).

Nitrate/Nitrite

Plasma nitrate was measured as a surrogate index of NO production [25, 26]. NO is an endogenous vasodilator produced by endothelial cells. Red wine polyphenols enhance vasorelaxation and NO production in vitro [27, 28]. Ethanol itself also enhances NO production [29]. No differences were found either by period or by treatment with or without an outlier whose value rose 6-fold in the GSE period. However, dietary nitrites and nitrates can confound this measure quite easily so the absence of change does not mean that NO production did not rise (see Table 4).

Adhesion molecules

ICAM1 and VCAM1 are molecules which bind white cells to the endothelium and reflect the state of the health of the endothelium, particularly in relationship to atherosclerosis [30, 31]. If the endothelium is damaged by oxidized lipid, cigarette smoke, or high blood pressure, these markers increase [32]. An antioxidant might be expected to lower the level of these markers (vitamin E does in some studies [33]) as does a statin which lowers plasma lipid level [34]. Polyphenols from blue and red berries reduce adhesion molecules in endothelial cells in vitro [35]. GSE and GSE/quercetin had no effect, nor were there any time effects (see Table 4).

Clotting and fibrinolytic factors

VWF mediates the binding of platelets to injured vessels and protects coagulation factor VIII. It is produced in the endothelium and is released when the endothelium is damaged by atherosclerosis, diabetes, insulin resistance, or hypertension [36, 37, 38]. Tissue-type plasminogen activator is released from endothelial cells to initiate the process of breaking down clots in the vessel by activating plasminogen to plasmin which then breaks down fibrin. Endothelial dysfunction impairs the release of active tPA [39] and is associated with an enhanced release of PAI-1 [40]. GSE and GSE/quercetin had no effect on VWF, tPA, or PAI-1 and there were no time effects. In [41] de Maat et al found no effect of black or green tea on any of the markers we measured, but wine polyphenolics have been shown in cultured human endothelial cells [42] to increase production of tPA (see Table 5).

Urine isoprostanes

8-Isoprostane (8-iso PGF2α) is a stable end product formed from arachidonic acid by free radical action and is measurable in plasma and urine. The level is believed to represent the degree of oxidative stress in lipids [43, 44].

In this experiment, we saw no changes in urinary isoprostanes either absolute or expressed in relation to creatinine to adjust for incomplete urinary collections (see Table 6). The values measured in this study were in the range described for subjects with type 2 diabetes. Vitamin E has been shown to reduce plasma and urine isoprostanes in some studies [45, 46] but not in others [47]. Subjects with type 2 diabetes have elevated (double) level of urinary isoprostane compared with controls and it falls by 32% with treatment with vitamin E [48]. Ide et al [49] found that in healthy young men vitamin E could reduce urine isoprostane levels by 48%. Whole grains have been found to lower isoprostane by 28% in one study [50], as did fruit and vegetables in another study [51], although van den Berg found no effects of fruit and vegetables [52]. Tea polyphenols did not alter isoprostanes [53] while dealcoholised red wine reduced the levels in plasma with a trend in urine [54].

Table 6. Effect of GSE and GSE/quercetin on urine isoprostane (iso PGF2α). $N = 35$, mean SD.

	Period 2	Period 3	Period 4	GSE	GSE/quercetin	Control
Isoprostane (pg/mL)	617 ± 348	713 ± 507	709 ± 578	668 ± 407	741 ± 644	630 ± 362
Creatinine (mmol/L)	11.59 ± 4.92	11.37 ± 5.51	12.72 ± 5.46	11.59 ± 4.92	11.69 ± 5.23	12.72 ± 5.46
Isoprostane/creatinine pg/mg	529 ± 392	561 ± 327	519 ± 328	534 ± 362	562 ± 416	513 ± 254
Isoprostane excretion ng/d	1178 ± 986	1243 ± 1220	1107 ± 743	1147 ± 407	1270 ± 1348	1112 ± 694

Table 7. Effect of GSE and GSE/quercetin on oxidised LDL levels U/L; $N = 35$, mean SD.

Period 2	Period 3	Period 4	GSE	GSE/quercetin	Control
94973±26906	94637±24862	94359±31705	95284 ± 24563	99142 ± 22728	98383 ± 22973

Although the results are negative except for the FMD changes, they are not incompatible with the current literature which is not uniform in its results. They are also compatible with the observed lack of change in the levels of oxidized LDL (see Table 7) in this study. There is no published data on measurement of oxidized LDL in plasma using this method.

There were no changes in urine chemistry, haematology, clotting, or biochemistry with GSE or GSE/quercetin. There were some time-related changes in urea and creatinine chloride and bicarbonate which may have been due to warmer weather (data not shown).

CONCLUSIONS

We have demonstrated that sufficient antioxidant polyphenols from GSE were absorbed to influence FMD but no other endothelial functions were affected. It is known from rat and rabbit studies that the absorption of proanthycyanidins from GSE is very limited. In one rat study [55], after feeding 0.25 g/kg of GSE (equivalent to feeding over 20 g to humans), a level of 18 μg/mL of dimer was achieved after 1 hour. In the rabbits, despite an equivalent dose spread over the day, no proanthocyanidins were detected, even though in this model lipid peroxidation and aortic atherosclerosis were reduced [11]. In vitro studies often use levels of 10–50 μg/mL [6] which is 10–20 times higher than what might be achieved in human studies. Quercetin is known to be absorbed and 1 g/day can produce levels 23 times higher than control capsules. Despite this level and its demonstrable in vitro antioxidant action, it has no effects on platelets or lipids [56]. Although red wine extract inhibits endothelin (ET-1) production, neither isolated red wine polyphenols (quercetin, resveratrol, D-,L-catechin, D-,L-epicatechin) nor the anthocyanins delphinidin, pelargonidin, cyanidin, peonidin, petunidin, or malvidin affect ET-1 production [6].

Although there is no data yet relating impaired FMD to cardiac events in subjects without coronary disease, those patients with coronary disease who have very impaired FMD have more events [57]. Subjects with impaired FMD are more likely to have coronary disease on angiography [58]. Statins improve mortality and one mechanism may be via their improvement of FMD [59]. Subjects with low acetylcholine induced forearm vasodilatation are more likely to have acute events [60].

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RESEARCH ARTICLE

Characterization of Acylated Anthocyanins in Callus Induced From Storage Root of Purple-Fleshed Sweet Potato, *Ipomoea batatas* L

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Four anthocyanins were isolated from a highly pigmented callus induced from the storage root of purple-fleshed sweet potato (*Ipomoea batatas* L) cultivar Ayamurasaki. The anthocyanins were respectively identified as cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, and peonidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside by chemical and spectroscopic analyses. These anthocyanins were examined with respect to the stability in neutral aqueous solution as well as the radical scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. These acylated anthocyanins exhibited both higher stability and higher DPPH radical scavenging activity than corresponding nonacylated cyanidin and peonidin 3-*O*-sophoroside-5-*O*-glucosides.

INTRODUCTION

A purple-fleshed sweet potato, *Ipomoea batatas* L cultivar Ayamurasaki, accumulates high levels of anthocyanin pigments in the storage root [1]. The major anthocyanins are cyanidin and peonidin 3-*O*-sophoroside-5-*O*-glucosides acylated with caffeic, ferulic, or *p*-hydroxybenzoic acids [2, 3, 4]. The sweet potato anthocyanins possess not only moderate stability in neutral aqueous solution [5] and high thermostability [6] but also various health benefits like antioxidative activity [7, 8, 9], antimutagenicity [10, 11], and antidiabetic action [12, 13, 14]. Therefore, the Ayamurasaki pigment is utilized as a high-quality natural food colorant with potentially preventive function against life-style-related diseases.

Recently, from the storage root of the cultivar Ayamurasaki we have established a high-anthocyanin accumulating cell line. In the crude pigment extract, some cell-line-specific anthocyanins have been detected which were different from those in the original storage root [15]. Previously, we have isolated and determined the molecular structures of the two anthocyanins, in which one was a known anthocyanin, cyanidin 3-sophoroside-5-glucoside, and the other was a new one, cyanidin 3-(*p*-coumaroyl) sophoroside-5-glucoside [5]. In sequence, we have isolated four more callus anthocyanins. The purpose of this study is to characterize the structures and the stability in a neutral aqueous solution and the antioxidative activity to utilize the pigment for food or other material.

MATERIALS AND METHODS

Chemicals

All the reagents and solvents employed were of analytical grade and used without further purification. 1,1-diphenyl-2-picrylhydrazyl (DPPH, Wako Pure Chemicals, Japan), *α*-tocopherol (Kishida Chemicals, Japan), and 2,6-di-*tert*-butyl-4-methylphenol (BHT, Wako Pure Chemicals) were used for DPPH radical scavenging assay. Open column chromatographies were carried out on Amberlite XAD-2000 (Rohm and Haas, USA) and ODS (Silica Gel Chromatorex, Fuji Silysia Chemical Ltd, Japan) resins.

HPLC methods

Solvent A (1.5% H_3PO_4 in H_2O) and solvent B (1.5% H_3PO_4, 20% CH_3COOH, 25% MeCN in H_2O) were used for analytical high-performance liquid chromatography (HPLC), and solvent A (15% CH_3COOH in H_2O) and solvent B (15% CH_3COOH, 30% MeCN in H_2O) were used for preparative HPLC. Analytical HPLC was run on LC-10AD intelligent pump (Shimadzu, Japan) with a linear gradient elution of solvent A : B = 75 : 25 to 15 : 85 for 40 minutes on Luna column (4.6 id × 100 mm, Phenomenex, USA) at 35°C with a flow rate of 1.0 mL/min monitoring at 520 nm equipped with SPD-10A diode array detector (Shimadzu). For purity check of anthocyanins, other analytical HPLC was performed on L-6200 intelligent pump system (Hitachi, Japan) with a linear gradient elution of solvent A : B = 75 : 25 to 45 : 55 for 60 minutes on Inertsil ODS-3 column (4.6 id × 250 mm, GL Sciences, Japan) at 30°C with a flow rate of 1.0 mL/min monitoring at 520, 310, or 280 nm equipped with MD-1510 multiwavelength detector (Jasco, Japan). Preparative HPLC was run on L-6200 intelligent pump system with isocratic elutions of solvent A : B = 80 : 20 for **1**, 75 : 25 for **2**, 60 : 40 for **3**, and 50 : 50 for **4** on Inertsil ODS column (20 id × 250 mm, GL Sciences) at room temperature with a flow rate of 7.0 mL/min monitoring at 310 nm with L-4200 UV-Vis detector and D-2000 integrator (Hitachi).

Spectral analyses

UV-Vis spectra were recorded on V-550 spectrophotometer (Jasco) in 0.01% HCl-MeOH for structural determination and for colorimetric measurements of antioxidative activity assay. The bathochromic shift test was carried out by the addition of 5% $AlCl_3$-MeOH. Electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) spectra were recorded on Mariner Workstation system (Applied Biosystems, USA) in 1% CH_3COOH-50% MeCN with a positive mode by an infusion-injection. High-resolutional electrospray ionization Fourier-transform ion-cyclotron resonance mass spectrometry (ESI/FT-ICRMS) spectra were recorded on APEX II 70e (Bruker Daltonics, Germany) in aqueous MeOH containing CH_3COOH with a positive mode. ^1H (800 MHz) and ^{13}C (200 MHz) NMR spectra were measured on Avance 800 spectrometer (Bruker BioSpin, Germany) in DMSO-d_6 : TFA-d_1 = 9 : 1 with tetramethylsilane (TMS) as an internal standard at 30°C.

Plant materials

A previously established high-anthocyanin-accumulating callus culture generated from the storage root of sweet potato cultivar Ayamurasaki has been used for this study [15]. Suspended cell cultures were initiated by transferring about 1.0 g (fresh weight) of callus to 50 mL of liquid medium in 250 mL Erlenmeyer flasks. Basal Murashige and Skoog (MS) medium [16] supplemented with 1.0 mg/L 2,4-D, and 3% sucrose has been used as a maintenance medium (MM). Medium pH has been adjusted to 5.8 before autoclaving. Subcultures have been done in 7-day intervals. The cultures were incubated on a rotary shaker (130 rpm) at 25°C in the dark. For the purpose of present analysis, suspension cultures have been produced under two different medium conditions: MM and a high-anthocyanin producing medium (APM).

The APM was a modified MS medium with 9.4 mM KNO_3, without NH_4NO_3, with 5% sucrose and nil growth regulators [17]. Medium pH has been adjusted to 5.8 before autoclaving and suspension cultures were incubated in 250 mL flasks. Five hundred mg of cell aggregates were placed in flasks containing 50 mL medium. The cultures were harvested after 7 days' growth on MM (200 g) and after 14 days on APM (199 g).

Extraction and isolation of anthocyanins

The aggregates were removed from the culture media, rinsed with distilled water, separated from the liquid by vacuum filtration, and weighed. A total amount of 399 g of fresh tissue was steeped in 15% CH_3COOH (1 L) for one day and filtered. This operation was repeated three more times (1, 1, and 0.5 L). The combined crude extract (3.5 L) contained nineteen or more anthocyanins [18]. The extract solution was applied on two XAD-2000 resin columns (30 id × 385 mm), the columns were washed with water (3 L), eluted stepwise with 10%, 20%, 30%, 40%, 60%, or 70% EtOH all with 1% CH_3COOH (1 L). Pigments containing 30%, 40%, 60%, and 70% EtOH fractions were combined and evaporated to dryness under reduced pressure. Subsequently, the residue was separated using ODS column (60 id × 320 mm) with 10%, 20%, 30%, 40%, 60%, or 70% EtOH all with 1% CH_3COOH (1 L). HPLC analysis confirmed that **1** was contained in 20% EtOH fraction, **2** and **3** in 60% EtOH fraction, and **4** in 70% EtOH fraction, and each fraction was evaporated to dryness under reduced pressure. Finally, **1**, **2**, **3**, and **4** were isolated on purification of the individual fractions by preparative HPLC monitoring at 310 nm. The elutions were evaporated to dryness, dissolved in minimum amount of TFA, precipitated with excess ether, and dried in a silica gel desiccator under reduced pressure.

Chemical analysis

Alkaline hydrolysis of isolated pigment was performed as follows. The pigment powder (3 mg) was dissolved in 2N NaOH, left for 15 minutes with a sealed cap, and then acidified with CH_3COOH. The components in the reaction mixture were identified by analytical HPLC.

Cyanidin 3-O-(2-O-(6-O-(E)-caffeoyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-O-β-D-glucopyranoside(1)

UV-Vis λ_{max} (0.01% HCl-MeOH) nm: 526 (bathochromic shift with $AlCl_3$ into 39 nm), 331, 281, $E_{440}/E_{vis.max} = E_{440}/E_{526} = 0.14$, $E_{acyl.max}/E_{vis.max} =$

E_{331}/E_{526} = 0.49; ESI-TOFMS: m/z 935 (M^+ = $C_{42}H_{47}O_{24}^+$), 287 (Cy^+ = $C_{15}H_{11}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 935.24620 (calculated 935.24518 for M^+ = $C_{42}H_{47}O_{24}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Cyanidin 3-O-(2-O-(6-O-(E)-p-coumaroyl-β-D-glucopyranosyl)-6-O-(E)-caffeoyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside (2)

UV-Vis λ_{max} (0.01% HCl–MeOH) nm: 528 (bathochromic shift with $AlCl_3$ into 44 nm), 320, 297, 283, $E_{440}/E_{vis.max}$ = E_{440}/E_{528} = 0.14, $E_{acyl.max}/E_{vis.max}$ = E_{330}/E_{528} = 1.02; ESI-TOFMS: m/z 1081 (M^+ = $C_{51}H_{53}O_{26}^+$), 287 (Cy^+ = $C_{15}H_{11}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 1081.28241 (calculated 1081.28196 for M^+ = $C_{51}H_{53}O_{26}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Cyanidin 3-O-(2-O-(6-O-(E)-p-coumaroyl-β-D-glucopyranosyl)-6-O-(E)-p-coumaroyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside (3)

UV-Vis λ_{max} (0.01% HCl–MeOH) nm: 529 (bathochromic shift with $AlCl_3$ into 48 nm), 315, 299, $E_{440}/E_{vis.max}$ = E_{440}/E_{529} = 0.15, $E_{acyl.max}/E_{vis.max}$ = E_{315}/E_{529} = 1.21; ESI-TOFMS: m/z 1065 (M^+ = $C_{51}H_{53}O_{25}^+$), 287 (Cy^+ = $C_{15}H_{11}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 1065.28613 (calculated 1065.28704 for M^+ = $C_{51}H_{53}O_{25}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Peonidin 3-O-(2-O-(6-O-(E)-p-coumaroyl-β-D-glucopyranosyl)-6-O-(E)-p-coumaroyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside (4)

UV-Vis λ_{max} (0.01% HCl–MeOH) nm: 528 (no bathochromic shift with $AlCl_3$), 315, 297, $E_{440}/E_{vis.max}$ = E_{440}/E_{528} = 0.13, $E_{acyl.max}/E_{vis.max}$ = E_{315}/E_{528} = 1.25; ESI-TOFMS: m/z 1079 (M^+ = $C_{52}H_{55}O_{25}^+$), 301 (Pn^+ = $C_{16}H_{16}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 1079.30256 (calculated 1079.30269 for M^+ = $C_{52}H_{55}O_{25}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Stability test

Stability of anthocyanins **1**, **2**, **3**, and **4** in neutral aqueous solution was compared with that of nonacylated anthocyanins cyanidin 3-*O*-sophoroside-5-*O*-glucoside (Cy3S5G) and peonidin 3-*O*-sophoroside-5-*O*-glucoside (Pn3S5G) isolated from original storage root according to a previously reported method [19]. Each anthocyanin TFA salt was dissolved in McIlvaine (pH 7.0, 0.1 M citrate–0.2 M phosphate) buffer solution to make 50 μM test solution, and the Vis spectra (400–700 nm) were measured automatically at appropriate time intervals. Based on the absorbance at $\lambda_{vis.max}$ of each spectrum, the residual color (%) was calculated as percent of the initial absorbance (= 100%). The stability of the anthocyanin could be evaluated on the basis of the half-life ($t_{1/2}$), defined as the time required to reach 50% residual color.

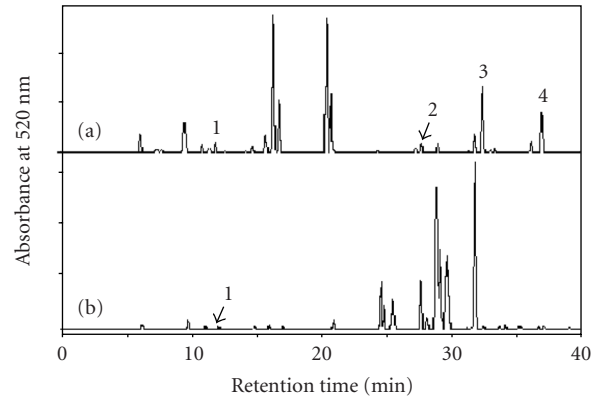


Figure 1. (a) HPLC chromatograms of the crude pigments of the purple sweet potato callus, and (b) storage root of sweet potato (*Ipomoea batatas* L), cv Ayamurasaki. Analytical HPLC was run with a linear gradient elution of solvent A : B = 85 : 15 to 65 : 35 for 100 minutes on Luna column at 35°C with a flow rate of 1.0 mLmin^{-1} monitoring at 520 nm.

DPPH radical scavenging activity assay

Radical scavenging activity of **1**, **2**, **3**, and **4** was tested according to the DPPH-colorimetric method developed by Yamaguchi et al [20] and compared with Cy3S5G, Pn3S5G, and α-tocopherol as natural antioxidants and BHT as a synthetic antioxidant. Each sample was dissolved in EtOH to 500 μM concentration. Sample solution (25 μL) was added 375 μL of EtOH, 350 μL of Tris-HCl buffer solution (pH 7.4, 0.1 M), and 250 μL of 500 μM DPPH-EtOH solution (to obtain a final sample concentration was 12.5 μM), and immediately shaken and then kept standing for 20 minutes in the dark at room temperature. The absorbance of residual DPPH in sample solution was measured at 520 nm. Initial and blank were measured without substrate and without DPPH, respectively. The DPPH radical scavenging activity (RS%) was calculated as RS% = $100(A_i - A_s + A_b)/A_i$, in which A_i, A_s, and A_b were the absorbances at 520 nm of initial, sample, and blank solutions, respectively. The experiment was conducted with four replicates.

RESULTS AND DISCUSSION

The deep-purple callus induced from the storage root of purple-fleshed sweet potato cultivar Ayamurasaki was extracted with 15% CH_3COOH. The crude extract contained 19 or more anthocyanins as detected by analytical HPLC (Figure 1(a)) [18]. The crude extract was successively purified by an absorbing resin and ODS column chromatographies and then preparative HPLC to afford four anthocyanins **1**, **2**, **3**, and **4** (named as YGM-0d, -3′, -7a, and -7e, resp) as red powders of TFA salts which were respectively obtained in amounts of 46, 28, 114, and 22 mg (yield of 0.0115%, 0.007%, 0.0286%, and 0.006%, resp).

Table 1. NMR chemical shifts purple-fleshed sweet potato callus anthocyanins **1**, **2**, **3**, and **4** from tetramethylsilane in DMSO-d_6/TFA-d_1 (9/1), 800 MHz.

Position	**1**		**2**		**3**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Aglycon								
2	162.57	—	162.58	—	162.55	—	162.69	—
3	144.59	—	144.36	—	144.28	—	144.39	—
4	133.65	8.92 s	133.35	8.27 s	133.35	8.84 s	135.62	8.95 s
5	155.31	—	155.41	—	155.37	—	155.63	—
6	104.32	6.99 d (1.8)	105.02	6.95 d (2.0)	105.12	6.95 d (1.6)	104.89	6.98 d (1.9)
7	167.80	—	167.92	—	167.91	—	168.24	—
8	96.22	7.06 d (1.8)	96.36	6.97 d (2.0)	96.38	6.93 d (1.6)	96.70	7.05 d (1.9)
9	111.74	—	111.88	—	111.87	—	112.00	—
10	155.20	—	155.24	—	155.29	—	155.54	—
1′	119.66	—	119.64	—	119.63	—	119.42	—
2′	117.73	8.08 d (2.3)	117.76	8.03 d (2.3)	117.74	8.03 d (2.3)	114.11	7.98 d (2.1)
3′	146.41	—	146.46	—	146.48	—	148.60	—
4′	155.48	—	155.41	—	155.42	—	155.99	—
5′	117.18	7.13 d (8.7)	117.16	7.11 d (8.7)	117.15	7.11 d (8.7)	116.84	7.10 d (8.7)
6′	127.87	8.31 dd (2.3, 8.7)	127.79	8.27 dd (2.3, 8.7)	127.80	8.27 dd (2.3, 8.7)	129.40	8.36 dd (2.1, 8.7)
OMe	—	—	—	—	—	—	56.19	3.90 s
Glucose-a								
1	99.85	5.59 d (7.6)	99.67	5.67 d (7.4)	99.50	5.68 d (7.4)	100.65	5.64 d (7.3)
2	81.54	4.02 t (8.3)	81.86	4.05 t (8.1)	81.84	4.06 t (8.0)	81.74	3.95 t (7.6)
3	76.56	3.69 t (8.9)	76.06	3.76 t (8.8)	76.05	3.76 t (8.8)	75.95	3.75 t (8.7)
4	69.43	3.39 t (8.9)	69.94	3.49 t (9.3)	69.98	3.48 t (9.3)	69.83	3.47 t (9.2)
5	77.65	3.56 m	74.27	3.93 m	74.26	3.94 m	74.44	3.87 m
6a	60.65	3.57 m	63.20	4.32 dd (6.8, 12.2)	63.24	4.31 dd (7.1, 12.1)	63.16	4.27 dd (6.8, 12.6)
6*b*	60.65	3.74 brd (10.1)	63.20	4.42 brd (10.0)	63.24	4.42 brd (12.1)	63.16	4.40 brd (11.3)
Glucose-b								
1	104.44	4.84 d (7.8)	104.71	4.81 d (7.8)	104.69	4.82 d (7.8)	104.47	4.78 d (7.8)
2	74.75	3.14 t (8.4)	74.85	3.15 t (8.7)	74.86	3.15 t (8.4)	74.65	3.19 t (8.4)
3	76.26	3.27 t (9.1)	76.23	3.26 t (8.7)	76.24	3.27 t (8.7)	76.28	3.29 t (8.9)
4	69.55	3.24 t (8.8)	69.61	3.25 t (8.7)	69.66	3.26 t (8.8)	69.77	3.26 t (9.4)
5	74.18	3.14 m	74.20	3.18 m	74.21	3.19 m	74.30	3.30 m
6*a*	62.58	3.99 dd (6.2, 12.5)	62.66	3.99 dd (4.3, 11.8)	62.67	4.00 dd (4.3, 11.7)	63.23	4.03 dd (5.4, 11.6)
6b	62.58	3.92 brd (10.1)	62.66	3.95 brd (10.0)	62.67	3.96 brd (10.4)	63.23	4.13 brd (10.1)
Glucose-c								
1	101.59	5.12 d (7.7)	102.24	5.07 d (7.7)	102.35	5.05 d (7.7)	102.01	5.10 d (7.7)
2	73.19	3.51 t (8.5)	73.39	3.56 t (8.5)	73.41	3.56 t (8.9)	73.35	3.56 t (8.5)
3	76.02	3.41 t (8.9)	76.29	3.41 t (9.0)	76.30	3.41 t (9.0)	76.37	3.42 t (9.0)
4	69.70	3.31 t (9.2)	69.88	3.29 t (9.2)	69.94	3.30 t (9.3)	69.83	3.31 t (9.4)
5	77.65	3.49 m	77.86	3.50 m	77.85	3.48 m	77.82	3.50 m
6a	60.74	3.58 dd (5.7, 12.3)	61.08	3.59 dd (6.2, 12.3)	61.04	3.58 dd (5.8, 12.0)	60.93	3.59 dd (5.7, 12.2)
6b	60.74	3.78 brd (11.8)	61.08	3.83 brd (10.1)	61.04	3.81 dd (1.9, 11.9)	60.93	3.80 dd (2.0, 10.0)

Table 1. Continued.

G_a-Acid I								
1	—	—	125.74	—	125.20	—	125.19	—
2	—	—	115.40	6.94 d (2.0)	130.35	7.33 d (8.6)	130.30	7.28 d (8.7)
3	—	—	145.70	—	115.98	6.78 d (8.7)	115.95	6.76 d (8.7)
4	—	—	148.53	—	160.08	—	160.05	—
5	—	—	115.88	6.77 d (8.7)	115.98	6.78 d (8.7)	115.95	6.76 d (8.7)
6	—	—	121.61	6.86 dd (2.0, 8.7)	130.35	7.33 d (8.6)	130.30	7.28 d (8.7)
α	—	—	113.98	6.02 d (15.8)	113.96	6.026 d (15.9)	113.79	5.96 d (15.9)
β	—	—	145.53	7.24 d (15.8)	144.82	7.24 d (15.8)	144.76	7.23 d (15.8)
Carbonyl	—	—	166.77	—	166.78	—	166.68	—
G_b-Acid II								
1	125.72	—	125.19	—	125.17	—	125.12	—
2	115.15	6.99 d (2.0)	130.36	7.33 d (8.6)	130.58	7.38 d (8.6)	130.52	7.38 d (8.7)
3	145.25	—	116.03	6.78 d (8.7)	116.03	6.79 d (8.7)	115.97	6.80 d (8.7)
4	148.48	—	160.08	—	160.08	—	160.08	—
5	116.01	6.77 d (8.3)	116.03	6.78 d (8.7)	116.03	6.79 d (8.7)	115.97	6.80 d (8.7)
6	121.39	6.83 dd (2.0, 8.3)	130.36	7.33 d (8.6)	130.58	7.38 d (8.6)	130.52	7.38 d (8.7)
α	113.94	6.00 d (15.8)	113.86	6.17 d (15.8)	113.86	6.23 d (15.9)	113.79	6.18 d (15.9)
β	145.65	7.22 d (15.8)	144.83	7.32 d (15.8)	145.30	7.37 d (15.8)	145.24	7.36 d (15.7)
Carbonyl	166.46	—	166.48	—	166.49	—	166.47	—

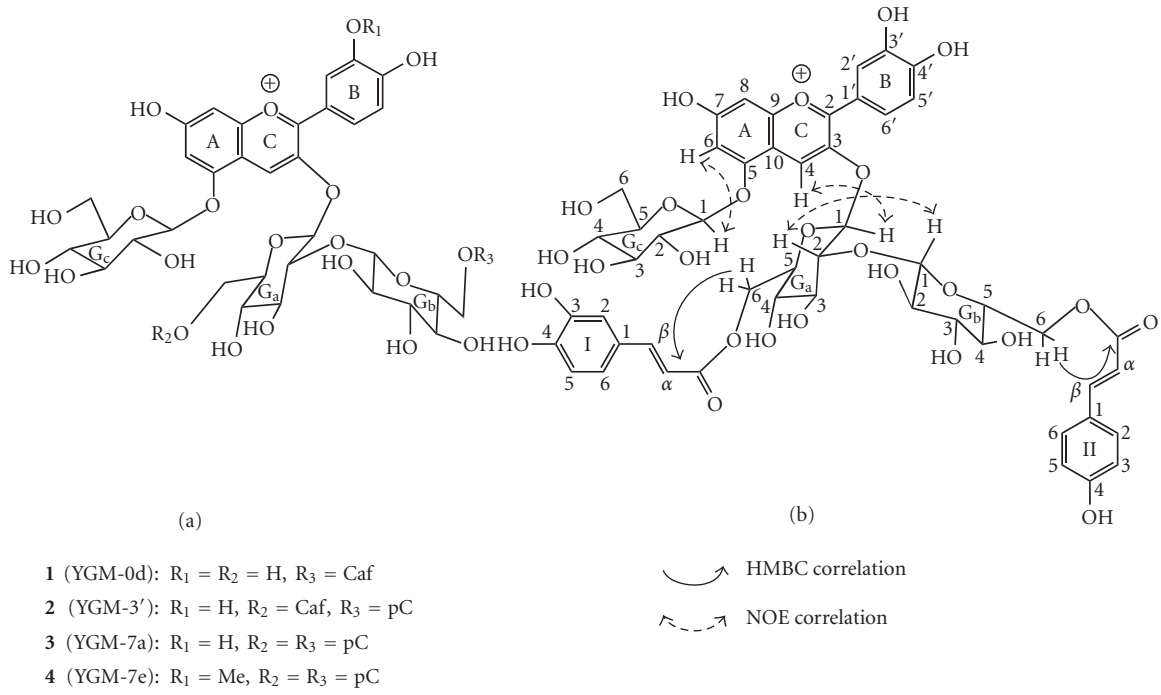
*Values in parentheses indicate coupling constants (J in Hz).

**Abbreviations: s = singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet, and brd = broad doublet.

On alkaline hydrolysis, **1**, **2**, and **3** gave cyanidin 3-*O*-sophoroside-5-*O*-glucoside (Cy3S5G) [19], while only **4** gave peonidin 3-*O*-sophoroside-5-*O*-glucoside (Pn3S5G). Moreover, in the hydrolysates of **1**, **2**, **3**, and **4**, caffeic acid from **1**, caffeic acid and *p*-coumaric acid from **2**, *p*-coumaric acid from **3**, and *p*-coumaric acid from **4** were detected as acylating acid(s) by HPLC cochromatographic analysis with authentic cinnamic acids. In the UV-Vis spectra, the absorptions around 331 and 320 nm of **1** and **2**, 315 nm of **3** and **4** also respectively supported the presence of caffeoyl and *p*-coumaroyl residues in their molecules. Numbers of the aromatic acids were respectively estimated one for **1**, and two for **2**, **3**, and **4**, on the basis of $E_{acyl.max}/E_{vis.max}$ (absorbance at $\lambda_{acyl.max}$/absorbance at $\lambda_{vis.max}$) values of 0.49 for **1**, and 1.02–1.25 for **2**, **3**, and **4**, respectively.

Since absorption maxima ($\lambda_{vis.max}$) of **1**, **2**, and **3** showed bathochromic shift by addition of $AlCl_3$, but not in **4**, **1**, **2**, and **3** could be elucidated as cyanidin-(Cy)-based anthocyanins and **4** as a peonidin-(Pn)-based one. ESI–TOFMS measurement of **1**, **2**, **3**, and **4** showed molecular ion peaks at m/z 935, 1081, 1065, and 1079 corresponding to $C_{42}H_{47}O_{24}^+$, $C_{51}H_{53}O_{26}^+$, $C_{51}H_{53}O_{25}^+$, and $C_{52}H_{55}O_{25}^+$, respectively, and also showed fragment ion peaks of Cy^+ at m/z 287 in **1**, **2**, and **3** and of Pn^+ at m/z 301 in **4**. The molecular formulas of **1**, **2**, **3**, and **4** were confirmed by high-resolutional ESI/FT–ICRMS at m/z 935.24620, 1081.28241, 1065.28613, and 1079.30256, respectively. All these findings indicated **1** as mono-caffeoyl Cy3S5G, **2** as caffeoyl-*p*-coumaroyl Cy3S5G, **3** as di-*p*-coumaroyl Cy3S5G, and **4** as di-*p*-coumaroyl Pn3S5G.

The complete structures of **1**, **2**, **3**, and **4** were established by ^{13}C and ^1H NMR analyses containing 2D pulse experiments such as a homonuclear double quantum filtered correlation spectroscopy (DQF-COSY), a total correlation spectroscopy (TOCSY), a heteronuclear single-quantum correlation (HSQC), a nuclear Overhauser and exchange spectroscopy (NOESY), and a heteronuclear multiple bond correlation spectroscopy (HMBC) technique. Assignment of ^{13}C- and ^1H-signals was summarized in Table 1. ^1H-signals in low magnetic field (δ_H 6–9 ppm) shows characteristic aglycons and cinnamic acids. Anthocyanins **1**, **2**, and **3** and **4** have Cy and Pn moieties, respectively, due to the corresponding signals of benzopyrylium nucleus and 1, 3, 4-trisubstituted aromatic B-ring. Only **4** has an additional methoxyl signal at high magnetic field (δ_H 3.90 ppm, δ_C 56.19 ppm). The presence of *trans* (*E*)-caffeoyl residue in **1** and **2** spectra and (*E*)-*p*-coumaroyl residue in **2**, **3**, and **4** spectra is confirmed with the 1, 3, 4-trisubstituted and 1, 4-disubstituted benzenes, respectively having the (*E*)-olefinic proton signals with large coupling constant (about $J_{\alpha,\beta}$ = 16 Hz). In high magnetic field (δ_H 3–6 ppm), the spectra also show all sugars of **1**, **2**, **3**, and **4** to be β-D-glucopyranosyl configuration because of the resonances at lower magnetic field (δ_H 4.78–5.68 ppm) of all anomeric protons and the large J values (J = 7.2–9.4 Hz) of the anomeric protons and the ring protons. As shown in Table 1,



1 (YGM-0d): $R_1 = R_2 = H$, R_3 = Caf
2 (YGM-3′): $R_1 = H$, R_2 = Caf, R_3 = pC
3 (YGM-7a): $R_1 = H$, $R_2 = R_3$ = pC
4 (YGM-7e): R_1 = Me, $R_2 = R_3$ = pC

Figure 2. Purple sweet potato callus anthocyanins. (a) Structures of **1**, **2**, **3**, and **4**; Caf = caffeic acid and pC = *p*-coumaric acid, (b) typical NOE and HMBC correlations of caffeoyl-*p*-coumaroylated anthocyanin **2**.

G_a-2H (at δ H 3.95–4.06 ppm) and G_a-2C (δ C 81.54–81.86 ppm) clearly shift to downfield more than G_b-2H (at δ H 3.14–3.19 ppm) and G_b-2C (δ C 74.65–74.86 ppm) or G_c − 2H (at δ H 3.51–3.56 ppm) and G_c-2C (δ C 73.19–73.39 ppm). On the basis of this data, we concluded that glucose b (G_b) links to glucose a (G_a)-20H. On the other hand, NOESY and HMBC spectra gave more direct and certain data on the presence of β-D-G_b (1 → 2) G_a bond of the sophorosyl residue.

The connecting relations among an aglycon, three sugars, and acyl groups in **1**, **2**, **3**, and **4** were confirmed by NOESY and HMBC measurements, for example, anthocyanin **2** shown in Figure 2(b). In the NOESY spectra of **1**, **2**, **3**, and **4**, three intensive NOE signals between aglycon-4H and G_a-1H (aglycon-4H/G_a-1H), G_a-2H/G_b-1H, and aglycon-6H/G_c-1H indicated that G_a, G_b, and G_c connected at aglycon-3-OH, at G_a-2OH, and at aglycon-5OH through glycosyl bond, respectively. In the HMBC spectra of **1**, **2**, **3**, and **4**, the clear ^{1}H-^{13}C cross peaks between G_a-1H and aglycon-3-carbon signals (G_a-1H/aglycon-3C), G_b-1H/G_a-2C, G_a-2H/G_b-1C, and G_c-1H/aglycon-5C verified the connections of G_a/aglycon-3OH, G_b/G_a-2OH, and G_c/aglycon-5OH, respectively. Moreover, the distinct correlation peaks between G_b-6H and acyl-carbonyl carbon signals (about δ_C 166.5 ppm) provided decisive proof that acylating acids were linked at G_b-6OH. In **2**, **3**, and **4**, the cross peak between G_c-6H and acyl-carbonyl carbon signals (about δ_C 166.8 ppm) also showed directly links aromatic acids and G_c-6OH [3]. In conclusion, **1**, **2**, **3**, and **4** were unambiguously determined as cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, and peonidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, respectively, by chemical and spectroscopic analyses (Figure 2(a)). Only anthocyanin **4** is a new compound, while **1**, **2**, and **3** are known ones. Anthocyanins **1** and **2** have already been identified in *Ipomoea cairica* flowers [21], but the exact binding sites of the acyl residues were not established. Similarly, anthocyanins **2** and **3** have been identified in the pigment of *Ipomoea asarifolia* flower [22], and in the pigment of *Ajuga reptans* flower and the corresponding cell cultures [23], respectively. Pigment **1** is also found in original purple-fleshed sweet potato storage root (Figure 1(b)) or sweet potato leaf pigment as a minor component, while *p*-coumaroylated **2**, **3**, and **4** are confirmed to be cell-line-specific pigments.

The stability of **1**, **2**, **3**, and **4** was compared with nonacylated Cy3S5G and Pn3S5G in neutral aqueous solution at room temperature. The stability was evaluated on the basis of the half-life ($t_{1/2}$) that was defined as the time required to reach 50% residual color. As shown in

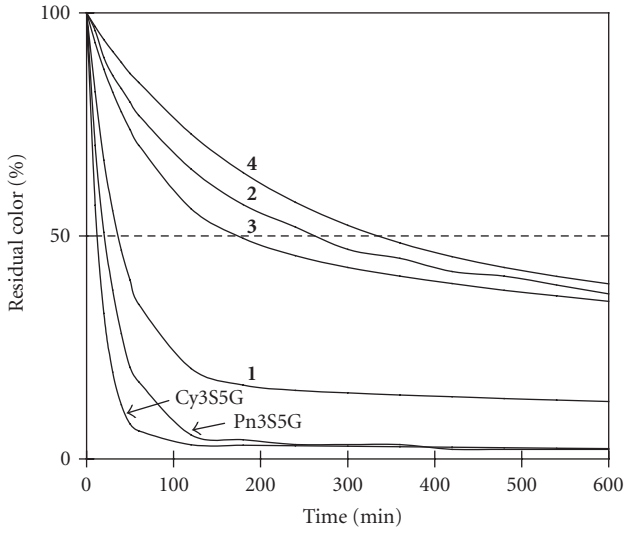


FIGURE 3. Stability of purple sweet potato callus anthocyanins **1**, **2**, **3**, and **4** in neutral aqueous solution at room temperature.

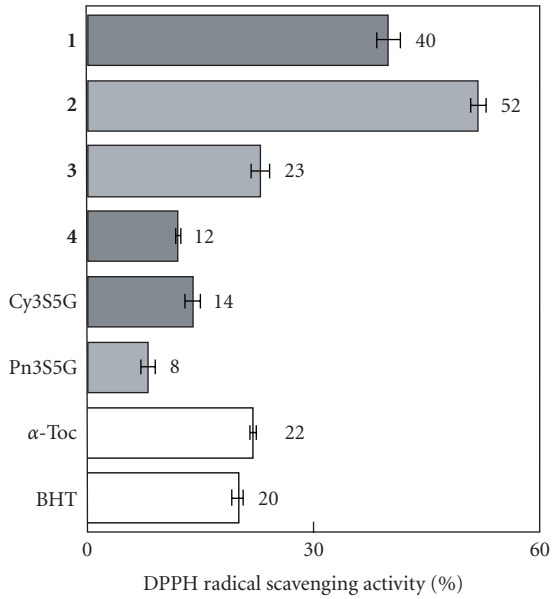


FIGURE 4. DPPH radical scavenging activity (%) of purple sweet potato callus anthocyanins **1**, **2**, **3**, and **4** (pH 7.4 at room temperature). α-Toc = α-tocopherol and BHT = 2,6-di-*tert*-butyl-4-methylphenol. Each value is the mean (RS%) ± standard deviation (n= 4).

Figure 3, diacylated anthocyanins ($t_{1/2}$ of **2**: 266 minutes, **3**: 180 minutes, and **4**: 337 minutes) were more stable than monoacylated **1** ($t_{1/2}$ = 37 minutes), and nonacylated Cy3S5G ($t_{1/2}$ = 15 minutes) and Pn3S5G ($t_{1/2}$ = 19 minutes). Effect of the structures on the stabilities of the anthocyanins suggested that the two aromatic acids protect the aglycon nucleus by a sandwich-type hydrophobic stacking mechanism and inhibit the attack of a water molecule that leads to a loss of color [24, 25].

These anthocyanins were evaluated for the antioxidant activity as scavenging ability against DPPH radical. As shown in Figure 4, activity of identically substituted but Cy-based **3** exhibited higher scavenging activity than Pn-based **4**. The caffeoyl-*p*-coumaroylated **2** and monocaffeoylated **1** showed higher activity than di-*p*-coumaroylated **3**. Therefore, anthocyanins with catechol group(s) in the aglycon and/or the acyl group(s) were thought to be very effective in radical scavenging. Moreover, since hydroxycinnamic acid acylation enhanced the activity (**1**, **2**, **3** > Cy3S5G, and **4** > Pn3S5G), the acylation might intramolecularly and synergistically achieve the radical scavenging activity of aglycon in addition to their own activity [26].

The cell line generated from Ayamurasaki storage root accumulates high levels of anthocyanin pigments. Depending on medium conditions under which the tissue is produced, the total amount of anthocyanins is equal to that of the original storage root on an MM [15] or 2.5-fold higher on a high-anthocyanin producing medium [27].

Thus the callus pigment has a potential to be utilized as a high-quality natural food colorant/natural food ingredient with protective action against oxidative damage [18].

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RESEARCH ARTICLE

Caffeoylquinic Acids Generated In Vitro in a High-Anthocyanin-Accumulating Sweet Potato Cell Line

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Accumulation of phenolic compounds has been monitored in a suspension culture of anthocyanin-accumulating sweet potato cell line grown under the conditions of modified Murashige and Skoog high-anthocyanin production medium (APM) over a period of 24 days. Tissue samples extracted with 15% acetic acid were analysed using HPLC at a detection wavelength of 326 nm. Among others, the following derivatives of caffeoylquinic acids were detected: 4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 3,4,5-tricaffeoylquinic acid. Their total amount reached a maximum of 110 mg/gFW between the 4th and the 15th day of culture growth on APM. The major compound of the phenolic mixture was 3,5-dicaffeoylquinic acid with maximum accumulation level of 80 mg/100 gFW. The potential effects of targeted phenolic compounds on the nutraceutical qualities of in vitro produced anthocyanin-rich extracts are discussed.

INTRODUCTION

Free radicals are generated continuously in the human body as a byproduct of normal metabolism [1]. They are implicated in some diseases such as Alzheimer's disease [2], cancer [3], and vascular disorders (atherosclerosis, diabetes, hypertension) [4] and accelerate the aging process [5]. Increasing the level in foods of highly active radical scavenging compounds is important for maintaining a balance between oxidants and antioxidants in the body and for eliminating oxidative stress. The ability of phenolic compounds to scavenge free radicals and to contribute significantly towards antioxidant activities of vegetable and/or fruit extracts is well documented [6, 7] and a rapid screening method for relative antioxidant activities of flavonoids and phenolics has been developed [8]. Application of anthocyanin-based natural food colorants has been proposed [9] for increasing the antiradical activity of foodstuffs.

An extract prepared from the storage root of purple-fleshed sweet potato displayed an array of health-beneficial properties in in vitro studies such as radical scavenging [10, 11] and antimutagenic [12] activities and reduction of carbon tetrachloride-induced liver injury [13]. Phenolic acids and anthocyanins were identified to play a major role in these properties of sweet potato extract. Antihyperglycemic (antidiabetic) effect of sweet potato anthocyanins included into rat diet has also been reported [14].

Plant cell cultures have been successfully applied to produce secondary metabolites of interest, among them anthocyanins [15, 16]. We have established an anthocyanin-accumulating cell line (purple line (PL)) from the storage root of purple-fleshed sweet potato (*Ipomoea batatas* Lam cv Ayamurasaki, Convolvulaceae) [17]. Under the condition of a high-anthocyanin producing medium (APM) the sweet potato cell line accumulates 3 times more pigments than the field-grown storage root of Ayamurasaki cultivar [18] and is considered for a commercial production of natural food colorants. Evaluation of the potential chemopreventive properties of the cell lines anthocyanin-rich aqueous extracts in in vitro assays revealed enhanced radical scavenging, antimutagenic, and antiproliferation activities in comparison to the extract of field-grown Ayamurasaki storage root [19]. Enhanced accumulation of anthocyanins in the PL cell culture was suggested to contribute towards these activities. The biosynthetic pathway of phenolics is closely related to that of anthocyanins [20] and in an anthocyanin-accumulating cell line the presence of other phenolic compounds can be expected. Accumulation of two common phenolics, caffeic acid (CA) and chlorogenic acid (caffeoylquinic, CH), at low levels of maximum 10 mg/100 gFW in the PL cell line culture has been detected [21]. We also have observed that

degradation of CH is concomitant with the appearance of new major compounds in the phenolic mixture [21]. Therefore the objective of the present study was to monitor the generation and degradation of caffeoylquinic acid derivatives induced under the conditions of high-APM. Cochromatography with standards has been employed to identify the major phenolic compounds. Their possible contributions toward chemopreventive properties of the PL cell lines extracts have been discussed.

MATERIALS AND METHODS

Plant material and culture conditions

Callus culture has been developed from the sweet potato storage root, cv Ayamurasaki, as described previously [17]. Suspended cell cultures were initiated by transferring about 1 g (fresh weight) of callus to 25 mL of liquid medium in 100 mL Erlenmeyer flasks. Basal Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) was used as a multiplication medium (MM). The cultures were incubated on a rotary shaker (130 rpm) at 25°C in the dark. The medium was changed weekly.

For the experiment, seven-day-old subcultures were transferred into a liquid high-APM which was a modified MS with 9.4 mM KNO_3, without NH_4NO_3, with 5% sucrose and no growth regulators [18]. One hundred mg of cell aggregates were placed in 50 mL Erlenmeyer flasks containing 10 mL medium (pH 5.8 before autoclaving). The samples (6 replications per each sampling day) were harvested in 2–3 day intervals for a period of 24 days (growth period).

Extraction of phenolics and anthocyanins

Cell aggregates separated from the culture medium by vacuum filtration were ground and steeped in 15% acetic acid for 16 hours. The volume of acetic acid solution was calculated in the proportion of 20 mL/gfw of tissue. The samples were centrifuged at 10 000 rpm for 10 minutes and the phenolic compound in the supernatant identified.

Identification of phenolics and HPLC analysis

The supernatants were filtered through a 0.2 μm filter membrane (DISMIC-13cp, Advantec, Japan), injected (10 μL) into a YMC-Pack ODS-AM AM-302 column (150 $\times$ 4.6 mm, 5 μm; YMC, Kyoto, Japan) at 40°C. The HPLC system consisted of two LC-10AT pumps, an SIL-10AXL autoinjector, a CTO-10AC column oven, and an SPD-M10AVP photodiode array UV-VIS detector (Shimadzu, Kyoto, Japan) controlled by a CLASS-LC10 workstation (Shimadzu). The mobile phase consisted of mixtures of 0.2% (v/v) formic acid in water (solvent A) and methanol (solvent B). The elution profile was a linear gradient starting with 2% B from 0 to 15 minutes, 2% to 45% B from 15 to 50 minutes, and 45% B from 50 to 65 minutes. The flow rate was 1 mL/min [21]. CH and CA standards were purchased from Wako Pure Chemical Industries (Osaka, Japan). Because derivatives of caffeoylquinic acids are not commercially available, 4,5-dicaffeoylquinic acid (4,5-DCQA), 3,5-dicaffeoylquinic acid (3,5-DCQA), 3,4-dicaffeoylquinic acid (3,4-DCQA), and 3,4,5-tricaffeoylquinic acid (3,4,5-TCQA) standards were purified from the sweet potato leaves as described earlier [22]. Standard curves were prepared for calculations of the absolute concentration of compounds based on the peak areas. Peaks were detected at 326 nm wavelength and identified by co-chromatography.

RESULTS AND DISCUSSION

The extract of the PL cell line suspension culture, multiplied on standard MS enriched with 1.0 mg/L 2,4-D (multiplication medium), is rich in anthocyanins [17]. Tissue transfer into a high-APM resulted in further increase in accumulation of anthocyanins concomitant with a 3-fold increase in the total phenolics level [21]. Monitoring the generation of phenolics in the cell lines tissue over one growth period of 24 days exhibited a mixture of about 30 different compounds (Figure 1). The reversed-phase HPLC chromatograms indicated dynamic changes in the phenolic pattern over time (Figure 1).

Further to the previous detection of two minor components, CA and CH, whose accumulation sharply increased from traces amounts to 10 mg/100 gFW during the first two days of culture growth and was followed by a gradual decrease over time [21], we have monitored an enhanced accumulation of a major compound represented by a peak that exhibited a retention time of 47.2 minutes (peak 4, Figure 1). Through comparison and cochromatography with standards of CH derivatives (caffeoylquinic acids) isolated from the sweet potato leaves, this peak was identified as 3,5-DCQA. Accumulation of 3,5-DCQA was concomitant with the decrease of CH level (Table 1). Between the 2nd and the 4th day of culture after transfer to high-APM the relative concentration of 3,5-DCQA in the mixture increased from 0.1% to 15.8% and remained constant till day 15 (Table 1). The total amount of 3,5-DCQA increased during the first 4 days after transfer to high-APM to 80 mg/100 gFW and decreased between the 15th and the 18th day to 20 mg/100 gFW (Figure 2a). 3,5-DCQA was the major compound of the phenolics mixture produced by the PL cell line and analysed under the described conditions (Table 1).

The time course of accumulation for three other compounds represented by peaks with retention times of 46.7, 50.4, and 57.1 minutes (Figure 1) was also monitored. Through cochromatography with standards of caffeoylquinic acids, these compounds were identified as 4,5-DCQA, 3,4-DCQA, and 3,4,5-TCQA, respectively (Figure 3). The data suggests that active accumulation of both 4,5-DCQA and 3,4-DCQA was delayed compared to 3,5-DCQA and was at significantly lower levels (Figures 2b and 2c). The maximum concentration of 4,5-DCQA

Table 1. Relative concentrations* (%) of selected phenolic compounds in the extract of the PL cell line tissue grown on a high-anthocyanin producing medium during 24 days. (T: concentration less than 0.1%; ND: not detected.)

Day	CA	CH	3,5-DCQA	4,5-DCQA	3,4-DCQA	3,4,5-TCQA	Total conc	Total amount (mg/gFW)
0	4.7 ± 0.0♦	0.6 ± 0.0	8.7 ± 0.0	1.2 ± 0.0	0.4 ± 0.0	ND	15.2	20.2 ± 0.0
2	6.0 ± 1.4	3.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	ND	ND	9.4	21 ± 3.8
4	3.8 ± 1.3	1.2 ± 0.4	15.8 ± 5.2	1.8 ± 0.1	0.6 ± 0.1	ND	23.2	117 ± 17.6
6	5.1 ± 2.6	1.1 ± 0.5	13.0 ± 7.4	1.7 ± 0.6	0.7 ± 0.1	T	21.6	109 ± 29.5
15	4.1 ± 1.6	1.5 ± 0.7	13.5 ± 5.9	2.9 ± 0.8	1.4 ± 0.1	0.1 ± 0.01	23.5	102 ± 36.6
18	6.7 ± 3.0	0.9 ± 0.1	3.0 ± 0.2	1.6 ± 0.6	—	0.1 ± 0.01	12.3	40 ± 9.4
21	8.2 ± 5.4	1.1 ± 0.4	4.4 ± 1.5	2.0 ± 0.9	1.2 ± 0.1	0.12 ± 0.01	17.0	52 ± 11.2
24	9.1 ± 1.7	0.8 ± 0.1	3.9 ± 0.6	1.3 ± 0.2	1.1 ± 0.1	0.13 ± 0.01	16.33	48 ± 8.5

*The relative concentrations calculated based on the total peak area of phenolics detected at 326 nm (100%) and the area of the peaks of interest (%).
♦Standard deviations of 6 independent determinations for each sampling point.

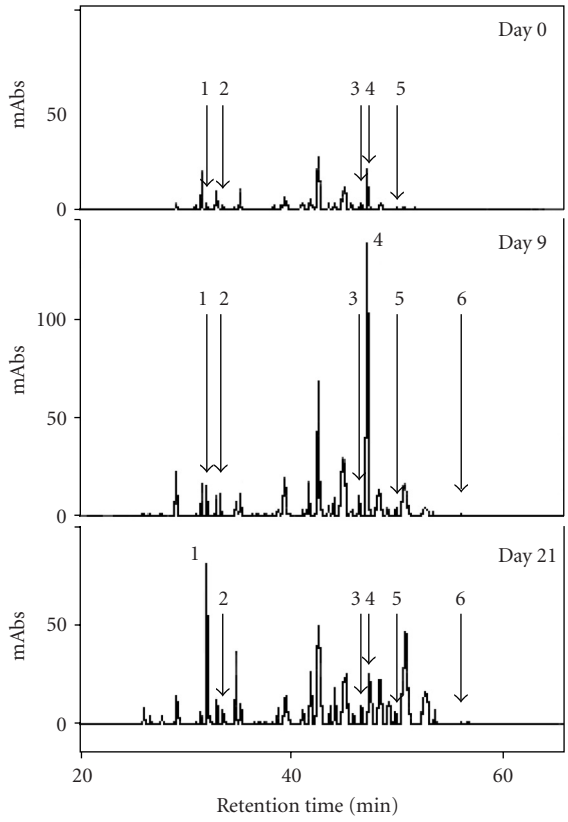


Figure 1. HPLC chromatogram of phenolic compounds monitored in crude extract of the PL cell line suspension culture over one growth period of 24 days in a high-anthocyanin production medium. 1: caffeic acid (CA); 2: chlorogenic acid (CH); 3: 3,4-dicaffeoylquinic acid (3,4-DCQA); 4: 3,5-dicaffeoylquinic acid (3,5-DCQA); 5: 4,5-dicaffeoylquinic acid (4,5-DCQA); 6: 3,4,5-tricaffeoylquinic acid (3,4,5-TCQA).

was 14.7 mg/100 gFW at the 15th day of culture. The highest level of 3,4-DCQA of 4.0 mg/100 gFW was observed at the stationary phase of growth period (day 21). Traces of the CH derivative with the most evolved molecular structure, 3,4,5-TCQA, were detected among the phenolic compounds at the 6th day of the growth period followed by a steady increase over time (Figure 2d). The highest level of accumulation (1.2 mg/100 gFW) was reached at a stationary phase. According to the relative concentrations calculated based on the peak area, the levels of accumulation of these selected phenolics in the tissue of PL cell line grown on APM were 3,5-DCQA > CA > 4,5-DCQA > CH > 3,4-DCQA > 3,4,5-TCQA.

Son et al [23] reported that the main phenolic acids identified in various sweet potato cultivars are CA, CH, and two isomers of DCQA. Detailed identification of the isomers of DCQA in extracts from sweet potato leaves of over 1389 varieties has been conducted by Islam et al [22]. These authors identified three isomers of DCQA: 4,5-DCQA, 3,5-DCQA, 3,4-DCQA, and 3,4,5-TCQA. Di- and tricaffeoylquinic acids are biosynthesised through conversion of CH, and isolation of an enzyme which catalyses the conversion of CH to 3,5-DCQA from sweet potato root has been reported [24]. Identification of the derivatives of CH in the callus culture established from the storage root of purple-fleshed sweet potato indicates that the biosynthetic pathway for the generation of these compounds is active in the callus culture and that their biosynthesis can be enhanced through modification of the cultures medium. The highest level of the CH and derivatives in the PL cell line culture of about 110 mg/100 gFW was obtained between the 4th and the 15th day of growth on APM (Table1). The content of DCQA isomers alone was 93.0 mg/100 gFW with the contribution of CA and CH being 10.0 and 6.0 mg/gFW, respectively. Walter et al [25] reported that total phenolic content of sweet potato storage root (peels removed) ranged among cultivars from 14 to 51 mg/100 g FW, which is 8- to 2-fold lower than that obtained in our experiment. However, phenolics in sweet potato storage root are not equally distributed but localised in several tissues, including the periderm and tissue beneath it as the selected sides of accumulation [26]. A study of the chemical composition of the outer cortex of storage root revealed that within the external 3- mm layer the concentration of DCQA isomers varies between cultivars from 168 to 638 mg/100 gFW, that of CA from 33 to 138 mg/gFW, and CH from 22 to 173 mg/gFW [27].

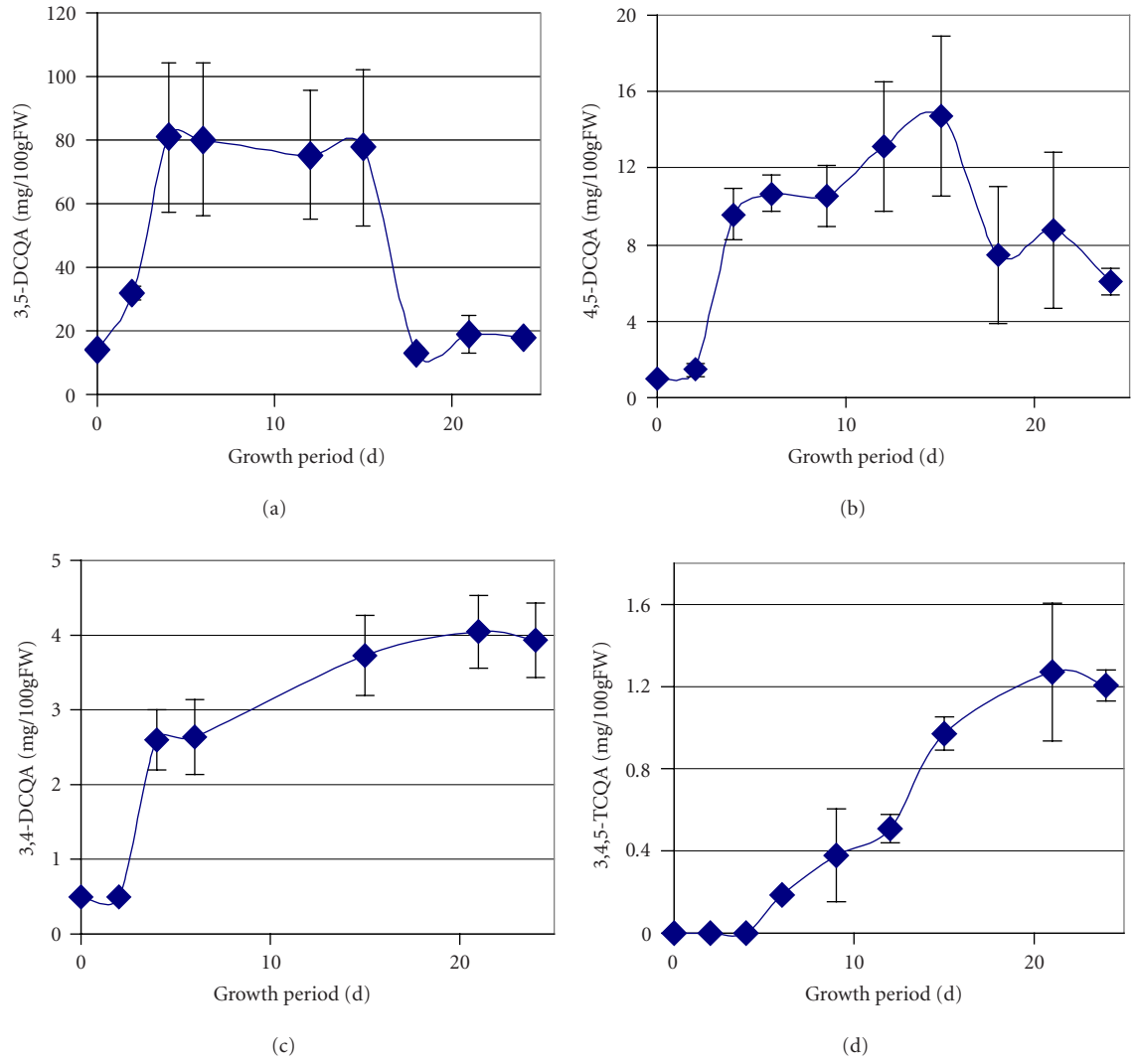
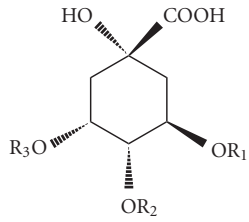


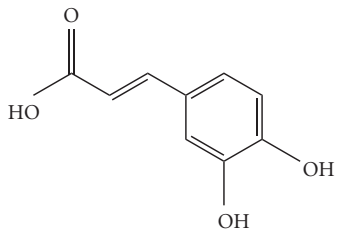
FIGURE 2. Accumulation of (a) 3,5-dicaffeoylquinic acid (3,5-DCQA), (b) 4,5- dicaffeoylquinic acid (4,5-DCQA), (c) 3,4-dicaffeoylquinic acid (3,4-DCQA), (d) 3,4,5-tricaffeoylquinic acid (3,4,5-TCQA) in the PL suspension culture over one growth period in a high-anthocyanin producing medium (APM). Bars represent standard deviations of six replications.

The function of caffeoylquinic acid derivatives present in the outer cortex of sweet potato storage root is the protection against fungal diseases [27]. Yoshimoto et al [28] found that 3,4,5-TCQA, followed by the DCQA isomers, displayed superior antimutagenic activities to CH against cooked food mutagen Trp-P-1. We have evaluated antimutagenic activity of anthocyanin-rich aqueous extract of the PL cell line produced under the conditions of MM and high-APM medium and observed that the extract produced under high-APM conditions displayed stronger antimutagenic activity (73% inhibition of Trp-P-1-induced reverse mutation of *Salmonella typhimurium* TA98) compared to the cell line extract produced under the conditions of MM medium (54%) and the extract of field-grown sweet potato storage root, which was selected as the donor tissue for cell line development (36%) [19]. As described in the present paper, the accumulation of caffeoylquinic acids by the PL suspension culture under the conditions of APM might have been responsible for this result.

Caffeoylquinic acids exhibited some medical properties: Zhu et al [29] reported that DCQA specifically and irreversibly inhibited the replication of the human immunodeficiency virus, HIV-1. In contrary to the DCQA, their likely precursors, CH, CA, and quinic acid, were not active against HIV-1. The authors suggested that the DCQAs are promising lead compounds for developing new antiretroviral drugs. Under the conditions of high-APM the sweet potato cell line accumulates relatively high levels of DCQAs and therefore it might be considered as a source of their continuous production through plant cell culture-based technology.



Quinic acid



Caffeic acid

Phenolic compound	R_1	R_2	R_3
Chlorogenic acid	Caffeic	H	H
3,5-DCQA	Caffeic	H	Caffeic
3,4-DCQA	Caffeic	Caffeic	H
4,5-DCQA	H	Caffeic	Caffeic
3,4,5-TCQA	Caffeic	Caffeic	Caffeic

Figure 3. Molecular structures of phenolic compounds in the PL cell line generated from the purple-fleshed sweet potato, cv Ayamurasaki.

Reports on the strong antioxidative and antimutagenic properties of the targeted phenolic compounds, CA, CH, and derivatives, by other researchers [28, 30] and our previous data on evaluation of the chemopreventive properties of the PL cell line extracts [19] suggest that inducing accumulation of caffeoylquinic acids in the sweet potato cell line may increase the nutraceutical properties of the anthocyanin-phenolic acid complex produced by the tissue of PL cell line. However the nutraceutical quality of this complex will entirely depend on the bioavailability and/or degradation during the digestion process. It has been reported that both CA and CH are absorbed at high levels in the thin intestine of humans: 97% and 33%, respectively [31]. It is of our further interest to evaluate the stability of generated phenolic compounds in food systems and their bioavailability in humans.

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Founding of this project through JSPS postdoctoral fellowship to Izabela Konczak by the Japan International Science and Technology Exchange Centre is thankfully acknowledged.

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RESEARCH ARTICLE

Bioavailability and Biokinetics of Anthocyanins From Red Grape Juice and Red Wine

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In a comparative study, 9 healthy volunteers ingested a single oral dose of 400 mL red grape juice or red wine with dose-adjusted anthocyanin content (283.5 mg or 279.6 mg, resp) in crossover. The content of anthocyanin glucosides was detected in plasma and urinary excretion. Additionally, the plasmatic antioxidant activity was assessed after intake. Based on the plasma content, biokinetic criteria of the single anthocyanins were calculated, such as AUC, c_{max}, t_{max}, and the elimination rate $t_{1/2}$. The urinary excretion of total anthocyanins differed significantly and amounted to 0.18% (red wine) and 0.23% (red grape juice) of the administered dose. Additionally, the plasmatic antioxidant activity increased to higher levels after juice ingestion compared to wine. The intestinal absorption of the anthocyanins of red grape juice seemed to be improved compared to red wine, suggesting a possible synergistic effect of the glucose content of the juice. The improved absorption resulted in an enhanced plasmatic bioactivity.

INTRODUCTION

The health protecting effects of anthocyanins are well known as is revealed from epidemiological studies [1, 2, 3]. The in vitro proved antioxidative activity is the predominant characteristic of anthocyanins as for other plant phenolics and may be in relation to health benefits, protecting thus the body tissues against the oxidative damage by oxygen free radicals, which may play a role in chronic diseases.

Despite the indications of their biological activity, there is up to now only sparse information on the in vivo bioavailability and bioactivity. Moreover, biokinetic data of their absorption, distribution, metabolism, and elimination in human beings are lacking so far but are a precondition for the assessment of their efficacy in the organism. Additionally, it is controversially debated whether alcohol may affect the bioavailability and renal excretion of this group of plant phenolics.

The objective of this study is the comparative assessment of the bioavailability and bioactivity of anthocyanins of red grape juice versus red wine after ingestion.

MATERIALS AND METHODS

We recruited for the study 9 healthy volunteers (4 males and 5 females) in the age range 24 to 34 years with body mass indices from 19.7 to 26.3 kg/m^2. As beverages, a German red wine (Lemberger) and a commercial red grape juice concentrate were provided. In an open, single-center study under controlled conditions, the volunteers ingested after an overnight fasting a single portion of either 400 mL red wine (variety Lemberger, Germany) or the same volume of diluted commercially obtained red grape juice concentrate with identical total anthocyanin doses of 283.5 mg (red grape juice) or 279.6 mg (red wine). The test phases were separated by a 4-week interval. Each person served as his or her own control. The order of the beverage ingestion was not randomized. The wine and the juice were ingested together with white rolls and 30 g of cheese. The volunteers were instructed not to consume any anthocyanin-containing foods or juices or alcohol 24 hours before and during the study.

Venous blood samples were taken initially (predose as baseline) and at 0.25, 0.50, 1.0, 1.5, 2, and 3 hours after intake of juice or wine, respectively. Urine samples were additionally collected predose and quantitatively in hourly intervals over a period of 7 hours. The study design was approved by the Ethical Commission of the University of Giessen, Germany.

In plasma and urine, the anthocyanin content was analyzed by the usual HPLC methods [4, 5, 6, 7]. In brief description, after extraction from plasma by using an ODS solid phase extraction cartridge and elution with 5 mL 0.44 M trifluoruacetic acid (TFA) in methanol, the anthocyanins were separated using a Prontosil Eurobond RP-18 column protected by a LiChrospher 100 RP-18 guard column and isocratic elution with water/acetonitrile/formic acid (81/10/9, pH 1.6) as mobile phase. Single substances were detected at 520 nm with a photodiode array detector

Table 1. Ingested bioactive compounds.

Component/parameter	Red grape juice conc. diluted (400 mL)	Red wine (400 mL)
Antioxidant capacity by TRAP assay (mmol)	6.0	8.6
Total anthocyanins (mg)	283.5	279.6
Flavan-3-ols (mg)	15.2	74.8
Flavonols (mg)	5.6	2.4
Resveratrols (mg)	9.2	6.8
Phenolic acids (mg)	16.8	23.2
Total phenolics (mg)	330.3	386.8

and a UV-VIS detector. Identity of the separated substances was ensured by comparing their retention time and UV-VIS spectra with those of commercially available standards. The detection limits of the analyzed anthocyanins were between 0.65 ng/mL (cyanidin-3-glucoside) and 5.2 ng/mL (malvidin-3-glucoside) in all matrices. For calibration (average r^2 value 0.998), blank urine and plasma samples were spiked with known concentrations of standard solutions.

The chromatographic conditions were identical for beverages, plasma, and urine. Juice and red wine samples were diluted 1 : 50 with HPLC mobile phase and centrifuged before injection into the HPLC column. The analyzed anthocyanin profile in grape juice differed slightly from that in wine in a lower content of glucosides of petunidin (42 versus 91 μg/mL) and malvidin (327 versus 461 μg/mL) and a higher glucoside content of delphinidin (124 versus 95 μg/mL) and peonidin (208 versus 45 μg/mL). In predose plasma and urine samples of the volunteers, the anthocyanin levels were below the detection limit.

Total polyphenolics were determined according to [8] by a modification of the Folin-Ciocalteau method. Conjugated polyphenols were hydrolyzed with 1 M hydrochloric acid (HCl) and polyphenol-lipid links broken with 2 M NaOH in 75% methanol followed by precipitation of plasma proteins with 0.75 M metaphosphoric acid. The polyphenols were, after centrifugation, extracted from the supernatant with acetone/water (1/1) and assayed with the Folin-Ciocalteau reagent. Results were given as milligram gallic acid equivalents (GAE) per liter. The anthocyanin glucoside values in plasma and urine were based for the calculation of the pharmacocinetics/biokinetics, such as the maximal concentration c_{max}, its peaking time t_{max}, the area under the curve AUC, and the elimination half-life $t_{1/2}$ [4]. Noncompartmental pharmacokinetic evaluation according to standard methods was performed [9] by using the WinNonlin Professional software (version 3.3, Pharsight Co, Mountain View, Calif). The extent and the relative bioavailability (wine versus juice) was tested for equivalence by calculating the 90% confidence interval (CI) on the basis of a one-sample *t* test of the log-transformed intraindividual differences in dose-normalized data. Distributional assumptions were confirmed by the Shapiro-Wilk test prior to performing the *t* test, which was based on the assumption of log-normally distributed intraindividual differences. Significant differences were accepted with *P* values less than or equal to .05.

The bioactivity of the phenolics in juice and wine was estimated by measuring the antioxidant activity in plasma samples with the aid of the commonly used TRAP assay according to [10]. The total phenolics ingested are shown in Table 1.

RESULTS

The pharmacokinetics of the single anthocyanins in plasma after red grape juice and red wine intake are shown in Table 2. It is noteworthy that the anthocyanins in plasma as well as in urine were nearly exclusively detected as glucosides, as was also demonstrated by others [11, 12, 13, 14]. No other conjugated or free forms could be identified in plasma; merely, in the urinary excretion, two additional small peaks of unidentified metabolites appeared after juice and wine intake. The following data are therefore related to the anthocyanin glucosides. After grape juice ingestion the geometric mean c_{max} of total anthocyanins of about 100 ng/mL was estimated at a peaking time t_{max} of half an hour, whereas after red wine intake a lower c_{max} of about 43 ng/mL was reached only at a peaking time t_{max} of 1.5 hours on average. The geometric mean plasmatic AUC attained, after wine intake, only 60% of the level after grape juice. But because of the higher interindividual variability of the AUC and c_{max} after red grape juice, no statistically significant differences of the plasmatic total anthocyanin levels between the two drinks could be ascertained. Only the values of cyanidin and delphinidin glucosides were without any doubt enhanced after grape juice drinking because the concentration of both anthocyanins after wine consumption was below the detection limit.

The plasma profile of the absorbed substances was also reflected in the urinary excretion pattern of the

Table 2. Plasma pharmacokinetic parameters of anthocyanins following administration of red grape juice and red wine.

	Anthocyanin	c_{max} (ng/mL)	t_{max} (h)[a]	AUC(0–3) (ng*h*mL^{-1})	$t_{1/2}$ (h)
Red grape juice	Cya-3-gluc	0.42 (118)	0.5 (0.5–0.5)	0.60 (82.9)	1.61 (22.7)
	Del-3-gluc	6.12 (66.7)	0.5 (0.5–1.0)	11.9 (55.0)	1.72 (26.6)
	Malv-3-gluc	48.8 (87.6)	0.5 (0.5–1.0)	71.7 (60.4)	1.50 (34.4)
	Peo-3-gluc	27.3 (51.0)	0.5 (0.5–0.5)	49.7 (36.2)	1.63 (19.4)
	Pet-3-gluc	16.1 (40.5)	0.5 (0.5–0.5)	31.5 (30.5)	1.68 (48.8)
	Total anthocyanins[g]	100.1 (64.2)	0.5 (0.5–1.0)	168.4 (42.3)	1.83 (28.5)
Red wine	Cya-3-gluc	NA	NA	NA	NA
	Del-3-gluc	NA	NA	NA	NA
	Malv-3-gluc	18.5 (24.0)	1.5 (1.0–1.5)	40.4 (21.2)	1.80 (31.9)
	Peo-3-gluc	12.6 (16.1)	1.5 (1.0–1.5)	30.7 (17.7)	1.83 (40.1)
	Pet-3-gluc	12.3 (16.2)	1.5 (0.5–1.5)	29.1 (15.1)	2.15 (22.9)
	Total anthocyanins[g]	42.9 (16.0)	1.5 (1.0– 1.5)	100.8 (14.4)	1.99 (28.1)

[a]Median (range).

[g]Sum of cyanidin 3-glucoside, delphinidin 3-glucoside, malvidin 3-glucoside, peonidin 3-glucoside, and petunidin 3-glucoside.

NA = not applicable (concentrations below LOQ).

Data are geometric means (with geometric coefficients of variation (%) in parentheses).

anthocyanin glucosides (Table 3). The maximal excretion rate R_{max} could be detected between 1.5 and 2.5 hours after intake. This is consistent with observations of Miyazawa et al [5] and Matsumoto et al [11]. Despite the high variation also seen with the grape juice anthocyanin excretion pattern, differences between both beverages in the excreted total amount were statistically significant, reaching 0.23% of the administered dose after grape juice and 0.18% after wine ingestion with petunidin and peonidin glucoside as the highest rates and cyanidin glucoside as the lowest-percentage excretion rate in both cases. It should however be mentioned that the cumulative excreted amounts do not correspond exactly to the absorption rate. Biliary secretion, for example, may also contribute to the elimination process of some substances.

The content of total polyphenolics in plasma increased after intake of both beverages with a c_{max} after about 30 minutes (Figure 1). In contrast to plasmatic anthocyanins, the c_{max} value after grape juice intake significantly exceeded the analogous value after wine intake, indicating that flavonoids other than anthocyanins may be better absorbed. It should be noted that this increase occurred on the basis of a relatively high GAE fasting level that may be accounted for by other reducing substances (SH-compounds, ascorbic and uric acids, etc) reacting also with the modified Folin-Ciocalteau method as was described by other authors [8].

Besides the anthocyanin levels in plasma and urine, we measured the plasmatic antioxidative capacity as biomarker for the physiological efficiency of all polyphenolics that were absorbed after beverage consumption. The assessed TRAP values were adjusted to the ascorbic acid and uric acid content of the plasma to avoid any misinterpretation. As is evident from Figure 2 and Table 4, both of the beverages were able to enhance the plasmatic antioxidant activity, peaking between 50 and 63 minutes and dropping to initial values again after 2 hours. As with the plasmatic polyphenols, the TRAP value biokinetics over the time after grape juice intake surpassed the level after wine intake with statistical significance, suggesting a superior bioactivity of the anthocyanins and other polyphenolics and/or their metabolites from red grape juice compared to red wine.

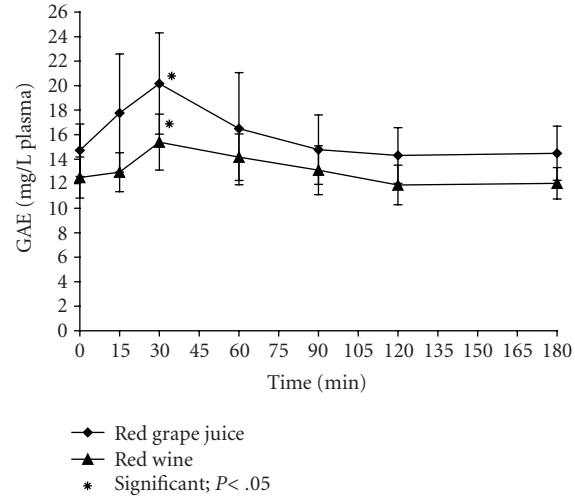

Figure 1. Total phenolics in plasma.

DISCUSSION

In this comparative study, we tested the bioavailability of anthocyanins as the valuable components in red grape juice and red wine in human beings. From the

Table 3. Urinary pharmacokinetic parameters of anthocyanins following administration of red grape juice and red wine. (R_{max}= maximal observed excretion rate; Ae (0–7) = total amount of excreted anthocyanins; Xe (0–7) = percentage of excreted dose.)

	Anthocyanin	R_{max} (μg/h)	t_{max}, R (h)[a]	Ae (0-7) (μg)	Xe (0-7) (%)
Red grape juice	Cya-3-gluc	1.26 (75.8)	0.5 (0.5–1.5)	2.88 (72.1)	0.09
	Del-3-gluc	39.6 (91.1)	0.5 (0.5–1.5)	101.9 (72.5)	0.20
	Mal-3-gluc	86.7 (126)	0.5 (0.5–1.5)	236.3 (95.7)	0.18
	Peo-3-gluc	86.0 (79.1)	0.5 (0.5–1.5)	240.5 (65.8)	0.29
	Pet-3-gluc	20.2 (45.8)	0.5 (0.5–1.5)	53.4 (31.1)	0.32
	Total anthocyanins	241.4 (82.3)	0.5 (0.5-1.5)	653.6 (66.6)	0.23
Red wine	Cya-3-gluc	0.66 (117)	2.5 (1.5–4.5)	1.45 (67.3)	0.06
	Del-3-gluc	14.9 (51.5)	0.5 (0.5–1.5)	47.7 (26.9)	0.12
	Mal-3-gluc	60.2 (33.4)	1.5 (1.5–2.5)	206.8 (31.7)	0.11
	Peo-3-gluc	44.1 (43.7)	0.5 (0.5–1.5)	151.5 (31.5)	0.84
	Pet-3-gluc	20.5 (52.5)	1.5 (1.5–1.5)	66.4 (47.5)	0.18
	Total anthocyanins	137.6 (29.2)	1.5 (1.5–2.5)	491.0 (19.8)	0.18

[a]Median (range).

Data are geometric means (with geometric coefficients of variation (%) in parentheses).

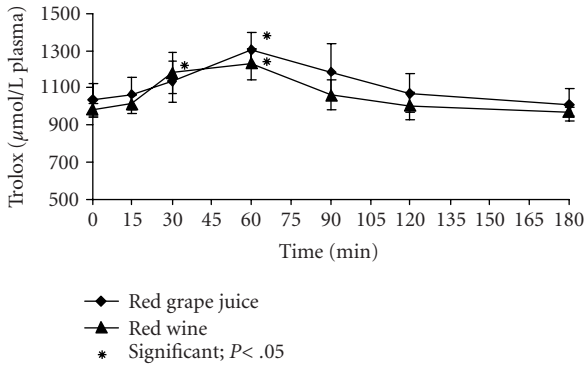

Figure 2. Plasmatic TRAP values after ingestion of red grape juice and red wine.

well-known French paradox, a health protective effect of the polyphenol-rich red wine had been suggested, assuming that the intestinal absorption of anthocyanins may be relieved by the alcohol content of the wine [15]. But exact information on the bioavailability of anthocyanins from alcoholic and nonalcoholic beverages is lacking so far. Moreover, kinetic data on the absorption and elimination rates of those polyphenolics may be of relevance, too, in terms of the suggested health protection.

A first proof that anthocyanins appear in plasma as intact glucosides after oral ingestion had been furnished by Tsuda et al in animals [13]. In the meantime, it could be demonstrated by our group and other authors that besides monoglucosides, higher condensed glycosylated forms in fruit juices were also incorporated from the intestine into the blood of rats and humans and are excreted in urine as such [4, 5, 6, 11, 12, 14, 16, 17, 18, 19, 20].

The generally low plasma and urine concentrations in this study suggest that the small absorbed anthocyanin dose is subjected to a rapid metabolism. Also a decompostion of anthocyanins cannot be excluded, since Tsuda et al [13] detected protocatechuic acid in the plasma of rats fed cyanidin-3-glucoside but in a 100-fold higher dose per kg body weight than in the present study. As the percentage of the excreted cyanidin glucoside in the present study was by far lower than for the other anthocyanins, this may indicate that cyanidin in vivo serves as precursor and is methylated to peonidin as was already proved in rats [13] (Table 3, Xe (0–7)). The excreted total anthocyanin amount is in contrast to the values of Lapidot et al [21] who assessed a more than 10-fold excretion rate after red wine intake with a comparable anthocyanin dose but within 12 hours. Bub et al [20], on the other hand, found less than 0.03% of the ingested dose in the urine.

Even though the plasma biokinetics revealed no statistical differences between the beverages, the analogous values in the urinary excretion (Table 3, R_{max}, Ae (0–7) and Xe (0–7)) suggest a higher bioavailability of anthocyanins from red grape juice compared to red wine. Perhaps the observation period was relatively short to recognize bioequivalence in plasma kinetics, too. Nevertheless, it has also been considered that due to absorption on plasma proteins, the analytical recovery rate of anthocyanins may be lowered. Murkovic et al [14] calculated, from spiked plasma samples, a recovery rate of elderberry anthocyanins to only 20%.

The identical elimination rates ($t_{1/2}$) from plasma corresponding with a (nonstatistical) tendency to lower c_{max}- and higher t_{max}-values after red wine intake lead to the conclusion that either the anthocyanin absorption from red wine was inhibited or the intestinal uptake from red grape juice was enhanced (Table 2). Up to now, there are contradictory findings about the effect of alcohol on the gastrointestinal function and absorption of flavonoids.

Table 4. TRAP biokinetics in plasma of 9 volunteers.

	c_{max} μmol* L^{-1}	c_{max}/Dose μmol* L^{-1}* μmol^{-1}	t_{max} min	AUC (0-180) mmol* min^{-1}* L^{-1}	AUC (0-180)/Dose mmol* min^{-1}* $mmol^{-1}$
Red wine	1233 ± 85	0.143 ± 0.010	50 ± 15	192 ± 8	22.31 ± 0.95
Red grape juice	1333 ± 62	0.222 ± 0.010	63 ± 10	202 ± 11	33.72 ± 1.86
S	$P = .013$	$P < .0001$	—	$P = .016$	$P < .0001$

Data are arithmetic means ± SD.

Levanon et al [22] suggested that white wine may inhibit the postprandial contractions and thus delay the absorption of sugars and glucosides. Bub et al [20] found no difference in the absorption of malvidin-3-glucoside between red wine and dealcoholized red wine, but found a delayed bioavailability after red grape juice intake. The latter finding is in contrast to our results. Gee et al [23] and Hollman [24] could demonstrate that quercetin glucosides may interact with the intestinal glucose transporter SGLT-1, resulting in an elevated intestinal uptake. More recently, it has been shown that the intestinal lactase-phlorizin hydrolase (LPH) in the apical membrane of intestine epithelial cells and the cytosolic β-glucosidase seems to play an important role in the absorption and metabolism of flavonoids [25, 26]. Considering the sugar content of the administered red grape juice and the similar structural features to quercetin, it is conceivable that the anthocyanins are absorbed via cotransport with the intestinal brush border SGLT-1, elevating thus the anthocyanin uptake compared to red wine [27].

This assumption is corroborated by the significantly enhanced biokinetics of the plasmatic polyphenolics after grape juice intake (Figure 1). The superior bioactivity of red grape juice compared to red wine is not at least visible in the antioxidant capacity of the plasma. The c_{max} as well as the AUC of the measured TRAP values in plasma after grape juice ingestion significantly exceeded the values after wine intake (Table 4).

CONCLUSION

The results of the present study suggest that red grape anthocyanins are absorbed in small amounts and renally excreted as intact glucosides. Red grape juice may be quite equivalent or even superior to red wine with a comparable content of bioactive flavonoids/anthocyanins. Against past opinion, the strongly antioxidative anthocyanins are obviously better absorbed from grape juice than from wine. Consequently, the plasmatic antioxidative capacity (TRAP assay) in man is stronger elevated after grape juice consumption compared to wine. Finally, from the results it is hypothesized that the anthocyanin absorption may be improved by the glucose content of the grape juice; a potential alcohol effect on the intestinal uptake and metabolism remains to be elucidated. Nevertheless, further components evolving during wine processing may interfere with the anthocyanin absorption. In future studies, more exact data on the in vivo metabolism of glucosidically bound anthocyanins and other conjugates may substantiate their health protective effects.

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MINIREVIEW ARTICLE

New Family of Bluish Pyranoanthocyanins

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The use of anthocyanins has been investigated for the preparation of food and beverage natural colorants as they seem to have nontoxic effects. In this context, vinylpyranoanthocyanins were recently found to naturally occur in ageing red wine. This new family of anthocyanin-derived pigments may be obtained directly through the reaction between anthocyanin derivatives and other compounds. Some of these newly formed pigments have been found to exhibit a bluish color at acidic pH. The formation of bluish pigment was obtained through reaction between anthocyanin-pyruvic-acid adducts and flavanols in the presence of acetaldehyde. The formation of similar bluish pigments was attempted using other different precursors. The chromatic features of this kind of pigments bring promising expectations concerning the use of these naturally occurring blue pigments in the food industry.

VITIS VINIFERA ANTHOCYANINS AND DERIVATIVE PIGMENTS IN RED WINE

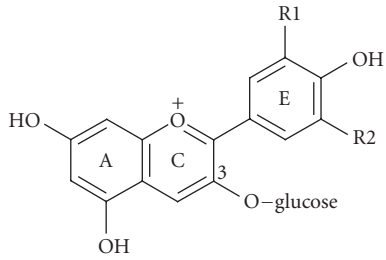
Anthocyanins are the most important group of water-soluble plant pigments visible to the human eye. These pigments are responsible for a great variety of colours of several fruits, vegetables, and plants. The colour of red wine is manly due to the presence of these polyphenolic compounds extracted from grape berries during the wine-making process. The general structure of *Vitis vinifera* anthocyanidin monoglucosides is represented in Figure 1. These compounds differ in their hydroxylation and methoxylation patterns of ring B yielding a wide range of colours from orange-red to violet at very acidic pH [1]. The glucosyl moiety linked at the 3-O position of ring C may also be acylated with acetic acid, coumaric acid, or caffeic acid.

These compounds undergo chemical transformations during wine ageing yielding new pigments that become responsible for the changing colour and its longevity [2]. These new pigments were first thought to result mainly from condensation reactions between anthocyanins and flavanols directly or mediated by acetaldehyde [3, 4, 5, 6, 7, 8, 9]. Nevertheless, over the last decade, reactions involving anthocyanins with other compounds such as pyruvic acid [10, 11, 12, 13, 14, 15], vinylphenol [16, 17], vinylcatechol [18], α-ketoglutaric acid [19], acetone [19, 20, 21], and 4-vinylguaiacol [21] have been demonstrated yielding new families of anthocyanin-derived pigments, namely, pyranoanthocyanins, with spectroscopic features that may somehow contribute to a more orange-red colour. This family of pyranoanthocyanins has been extensively investigated over the last years and several compounds have been recently evidenced in aged Port red wines [22, 23, 24] (see Table 1 and Figure 2).

It is interesting to notice that the anthocyanins-catechin pigments revealed the same λ_{max} as the pyruvate derivatives, which is hypsochromically shifted from that of original anthocyanins. Strikingly, pigments 9 and 13 which contain a procyanidin dimer unit in their structure revealed an important bathochromic shift (9 nm) from that of their counterparts with a single flavanol monomeric unit ((+)-catechin or (−)-epicatechin). This outcome highlights the importance of the type of flavanol moiety on the color characteristics of the pigments (and it suggests that some kind of intramolecular copigmentation between the flavanol residue and the flavylium chromophore may somehow occur).

PORTISINS—A NEW GROUP OF VINYLPYRANOANTHOCYANINS DETECTED IN PORT WINE

More recently, two new pigments with unique spectroscopic features exhibiting a bluish colour in acidic solution were found to occur in aged Port red wines [25] (see Figure 3, pigments 22 and 23). Indeed, these two pigments with maximum absorption in the visible region at 583 nm were detected by HPLC in two-year-old Port wine samples. These newly formed pigment structures in which anthocyanins are linked to flavanols by a vinyl linkage were named as portisins. Likewise, similar pigments arising from different anthocyanins and flavanols were tentatively detected by LC-DAD-MS in Port wine samples (Table 2).



Anthocyanidin	R1	R2
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

Figure 1. Structures of *V vinifera* anthocyanidin monoglucosides (flavylium form).

Table 1. Pyranoanthocyanins and detected in Port wine fractions. (Mv = malvidin; Dp = delphinidin; Pt = petunidin; Pn = peonidin; py = pyruvic acid derivative; gluc = glucoside; cat = (+)-catechin or (−)-epicatechin; PC = procyanidin dimer.)

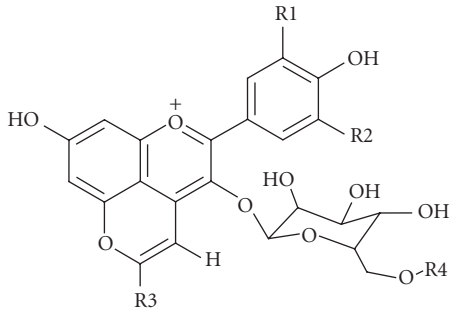
Pigment	Pyranoanthocyanin	λ_{max} (nm)	Structural elucidation
1	Mv 3-gluc-py	511	NMR, MS, UV-Vis
2	Pt 3-(acetyl)gluc-py		MS
3	Mv 3-(acetyl)gluc-py	511	NMR, MS, UV-Vis
4	Pt 3-(coumaroyl)gluc-py		MS
5	Mv 3-(coumaroyl)gluc-py	511	NMR, MS, UV-Vis
6	Pn 3-(coumaroyl)gluc-py		MS
7	Dp 3-gluc-py		MS
8	Dp 3-acetylgluc-py		MS
9	Mv 3-gluc-vinyl-(+)-cat-(+)-cat	520	NMR, MS, UV-Vis
10	Mv 3-(acetyl)gluc-vinyl-PC dimer		MS
11	Mv 3-gluc-vinyl-(+)-cat	511	NMR, MS, UV-Vis
12	Mv 3-(acetyl)gluc-vinyl-cat		MS
13	Mv 3-(coumaroyl)gluc-vinyl-(−)-epi-(+)-cat	520	NMR, MS, UV-Vis
14	Mv 3-gluc-vinyl-(−)-epi	511	NMR, MS, UV-Vis
15	Mv 3-(coumaroyl)gluc-vinyl-(+)-cat	511	NMR, MS, UV-Vis
16	Mv 3-(coumaroyl)gluc-vinyl-(−)-epi	511	NMR, MS, UV-Vis
17	Mv 3-gluc-vinylphenol		MS
18	Mv 3-(caffeoyl)gluc-vinylphenol		MS
19	Pn 3-(coumaroyl)gluc-vinylphenol		MS
20	Mv 3-(coumaroyl)gluc-vinylphenol		MS
21	Mv 3-(acetyl)gluc-vinylphenol		MS

Furthermore, a portisin with a phenol group replacing the flavanol moiety (pigment 26) has also recently been found to occur in aged Port wine (Table 2) (N. Mateus et al, unpublished data). However, the maximum absorption of this pigment in the visible region (538 nm) was found to be quite hypsochromically shifted from that of portisins with a flavanol moiety (Figure 4). The small hydroxylation pattern of the phenol ring probably contributes to this hypsochromic shift more significantly compared to the phloroglucinol ring of flavanols. Effectively, a similar pigment with a phloroglucinol moiety replacing the phenol group resulting from the reaction between malvidin 3-O-glucoside and phloroglucinol in the presence of acetaldehyde was shown to have a λ_{max} of 565 nm (unpublished data).

Concerning their formation, this new class of anthocyanin-derived pigments may be obtained through a reaction between anthocyanin-pyruvic-acid adducts and other compounds such as flavanols (eg, catechins, procyanidins) or phloroglucinol in the presence of acetaldehyde (Figure 5) [26], or directly by reaction with *p*-vinylphenol. The last step of their formation is thought to include decarboxylation, dehydration, and oxidation yielding a structure with extended conjugation of the π electrons, which is likely to confer a higher stability of the molecule and is probably at the origin of its blue color. Similar vinylpyranoanthocyanins had previously been synthesized using starting chemicals not found in grapes or in the yeasts [27].

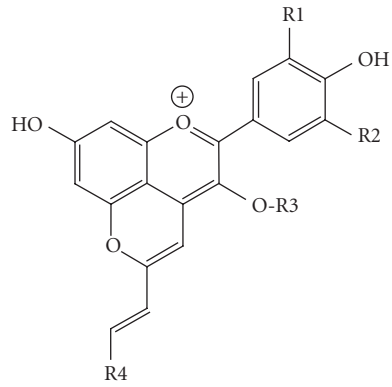
INTEREST AND POSSIBLE APPLICATION OF PORTISINS IN THE FOOD INDUSTRY

The chromatic features of this kind of pigments bring promising expectations concerning the use of these naturally occurring blue pigments in the food industry.



Pigment	R1	R2	R3	R4
1	OMe	OMe	COOH	H
2	OMe	OH	COOH	Acetyl
3	OMe	OMe	COOH	Acetyl
4	OMe	OH	COOH	Coumaroyl
5	OMe	OMe	COOH	Coumaroyl
6	OMe	H	COOH	Coumaroyl
7	OH	OH	COOH	H
8	OH	OH	COOH	Acetyl
9	OMe	OMe	Vinyl-(+)-cat-(+)-cat	H
10	OMe	OMe	Vinyl-PC dimer	Acetyl
11	OMe	OMe	Vinyl-(+)-cat	H
12	OMe	OMe	Vinyl-(+)-cat	Acetyl
13	OMe	OMe	Vinyl-(+)-epi-(+)-cat	Coumaroyl
14	OMe	OMe	Vinyl-(+)-epi	H
15	OMe	OMe	Vinyl-(+)-cat	Coumaroyl
16	OMe	OMe	Vinyl-(+)-epi	Coumaroyl
17	OMe	OMe	Vinylphenol	H
18	OMe	OMe	Vinylphenol	Caffeoyl
19	OMe	H	Vinylphenol	Coumaroyl
20	OMe	OMe	Vinylphenol	Coumaroyl
21	OMe	OMe	Vinylphenol	Acetyl

Figure 2. Pyranoanthocyanin structures detected in wine fractions (Table 1); cat = catechin; epi = epicatechin.



Pigment	R1	R2	R3	R4
22	OMe	OMe	Glucose	PC
23	OMe	OMe	Coumaroylglucose	PC
24	OMe	OMe	Glucose	Cat
25	OMe	OMe	Coumaroylglucose	Cat
26	OMe	OMe	Glucose	Phenol
27	OMe	OH	Glucose	Cat
28	OMe	H	Glucose	Cat
29	OMe	OMe	Acetylglucose	Cat

Figure 3. Portisin structures detected in Port red wine fractions (Table 2); cat = catechin; PC = procyanidin dimer.

Indeed, despite the extensive colour palette available in nature, pigments exhibiting blue colours are very scarce. For instance, the blue colours displayed by some flowers are mainly due to copigmentation phenomena [28, 29, 30, 31, 32]. Moreover, bluish hues may be obtained by the presence of quinonoidal forms of anthocyanins in high pH media [33, 34].

Therefore, the food industry has been searching for new alternative ways to produce products (foodstuffs and beverages) with bluish colours. Bearing this in mind, the production of bluish pigments was attempted in the laboratory using different precursors. Firstly, the formation of such pigments requires anthocyanins, which can be obtained using several red fruit extracts. Sweet cherry, bilberry, red apple, plum, blackberry, and elderberry extracts were used as anthocyanin sources for the synthesis of anthocyanin-derived pigments. Following this, the formation of the anthocyanin-pyruvic-acid

Table 2. Portisins detected in Port wine fractions. (Mv = malvidin; Pn = peonidin; Pt = petunidin; gluc = glucoside; cat = catechin; PC = procyanidin dimer.)

Pigment	Portisin	λ_{max} (nm)	Structural elucidation
22	VinylpyranoMv-3-gluc-PC	583	NMR, MS, UV-Vis
23	VinylpyranoMv-3-coumaroylgluc-PC	583	NMR, MS, UV-Vis
24	VinylpyranoMv-3-gluc-cat	572	MS, NMR, UV-Vis
25	VinylpyranoMv-3-coumaroylgluc-cat	577	NMR, MS, UV-Vis
26	VinylpyranoMv-3-phenol	538	MS, NMR, UV-Vis
27	VinylpyranoPt-3-gluc-cat	570	MS
28	VinylpyranoPn-3-gluc-cat	569	MS
29	VinylpyranoMv-3-acetylgluc-cat	577	MS

Table 3. Some anthocyanins respective pyruvic acid adducts and portisins obtained from different red fruit extracts. (Cy = cyanidin; Mv = malvidin; py = pyruvic acid derivative; gluc = glucoside; samb = sambubiose; ara = arabinose; rut = rutinose; cat = catechin.)

Source	Anthocyanin	λ_{max} (nm)	Pyruvic acid adduct	λ_{max} (nm)	Portisin	λ_{max} (nm)
Blackberries	Cy-3-gluc	516	Cy-3-gluc-py	505	VinylpyranoCy-3-gluc-cat	567
Sweet cherries	Cy-3-rut	516	Cy-3-rut-py	505	VinylpyranoCy-3-rut-cat	567
Elderberries	Cy-3-samb	516	Cy-3-samb-py	505	VinylpyranoCy-3-samb-cat	567
Red apple	Cy-3-gal	516	Cy-3-gal-py	505	VinylpyranoCy-3-gal-cat	567
Bilberries	Cy-3-ara	516	Cy-3-ara-py	505	VinylpyranoCy-3-ara-cat	567
Bilberries	Mv-3-ara	528	Mv-3-ara-py	511	VinylpyranoMv-3-ara-cat	572
Bilberries	Mv-3-gluc	528	Mv-3-gluc-py	511	VinylpyranoMv-3-gluc-cat	572
Grape berries	Mv-3-coumaroylgluc	532	Mv-3-coumaroylgluc-py	516	VinylpyranoMv-3-coumaroylgluc-cat	578

adduct was achieved through a reaction with pyruvic acid, as previously developed for grape malvidin 3-O-glucoside-pyruvic-acid adduct. The different anthocyanins from the red fruit extracts yielded pyruvic acid adducts with a λ_{max} hypsochromically shifted from that of genuine anthocyanins, some of which are indicated in Table 3. Consequently, the colour of all the extracts turned to a more orange-like hue. These anthocyanin-pyruvic-acid adducts were used as precursors for the formation of portisins, which was attempted using (+)-catechin in the presence of acetaldehyde.

As an example, Figure 6 shows the anthocyanin profile of an elderberry extract (*Sambucus nigra*) after two days of reaction with pyruvic acid. The anthocyanins of elderberries are two cyanidin monoglucosides (2) (cyanidin 3-O-glucoside and cyanidin 3-O-sambubioside) and a cyanidin 3,5-diglucoside (1) (3-O-sambubioside, 5-O-glucoside). This latter is not likely to react with pyruvic acid as position 5-O of the anthocyanin must be free from any substitution. Therefore, the only two pigments formed are the pyruvic acid adducts of the cyanidin monoglucosides ((3) and (4)), as seen from the respective HPLC chromatogram recorded at 520 nm (Figure 6a). Moreover, the HPLC chromatogram recorded at 570 nm of the purified pyruvate extract further treated with catechin in the presence of acetaldehyde is shown in Figure 6b. This portisin profile of the elderberry extract was obtained when practically all the pyruvic acid derivatives had reacted. The two portisins obtained correspond to the vinylpyranoanthocyanins of cyanidin 3-O-glucoside (5) and cyanidin 3-O-sambubioside (6), as confirmed by LC-DAD-MS (data not shown).

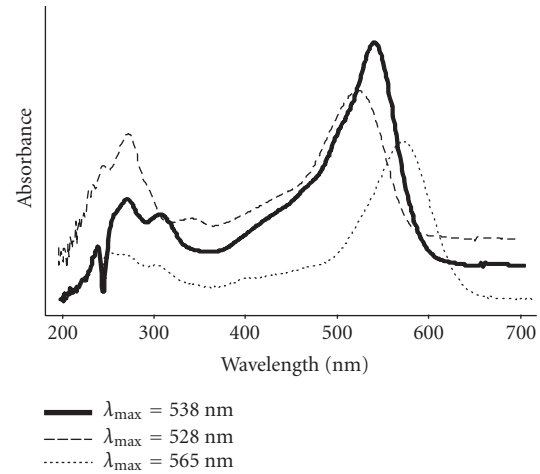


Figure 4. (a) UV-Vis spectra of malvidin 3-O-glucoside (solid), (b) vinylpyranoMv-3-gluc-phenol (dashed), and (c) vinylpyranoMv-3-gluc-phloroglucinol (dotted) recorded from the HPLC diode array detector (pH = 1.5).

Overall, the malvidin monoglucosides and derivatives appeared to be the anthocyanins with the highest λ_{max} in the UV-Vis spectrum when compared with cyanidin

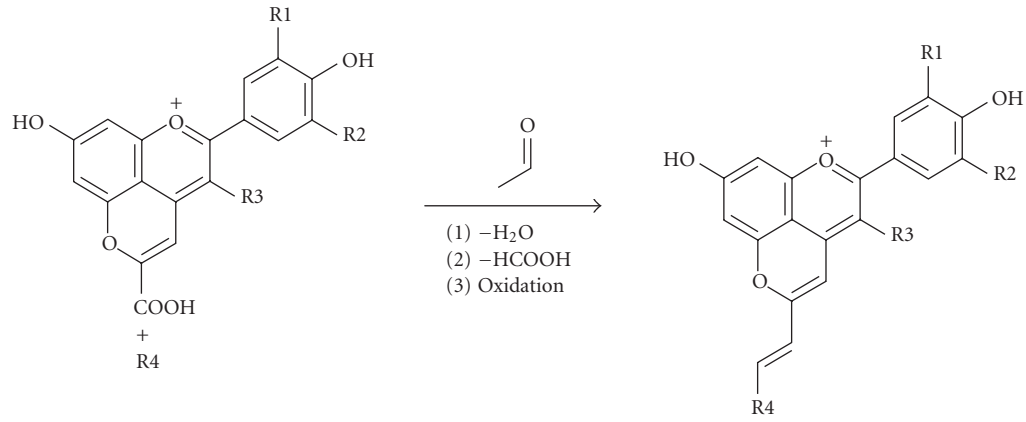


Figure 5. Formation reaction of portisins. R_1 and R_2 are H, OH, or OMe, R_3 is an (-O-glycosyl) group which is substituted with one, or more acyl groups, and R_4 is an aryl.

mono- or diglucosides, as seen from Table 3. The type of sugar moiety and the presence of a mono- or disaccharide in the anthocyanin structure did not seem to induce any influence on its λ_{max}. This behaviour was also observed with regard to the anthocyanin-pyruvic-acid adducts and the respective portisins.

Additionally, acylation of the sugar moiety of malvidin monoglucosides with *p*-coumaric acid yielded in a λ_{max} higher than its nonacylated counterpart. It has already been reported that, in the case of anthocyanins, acylation of the sugar moiety with hydroxycinnamic acids induces a bathochromic shift, as well as an intensification and stabilization of the colour, probably through intramolecular copigmentation phenomena, as reported elsewhere [35]. This bathochromic shift arising from the acylation of the sugar moiety was also observed for the portisins reported in aged Port red wine (Table 3).

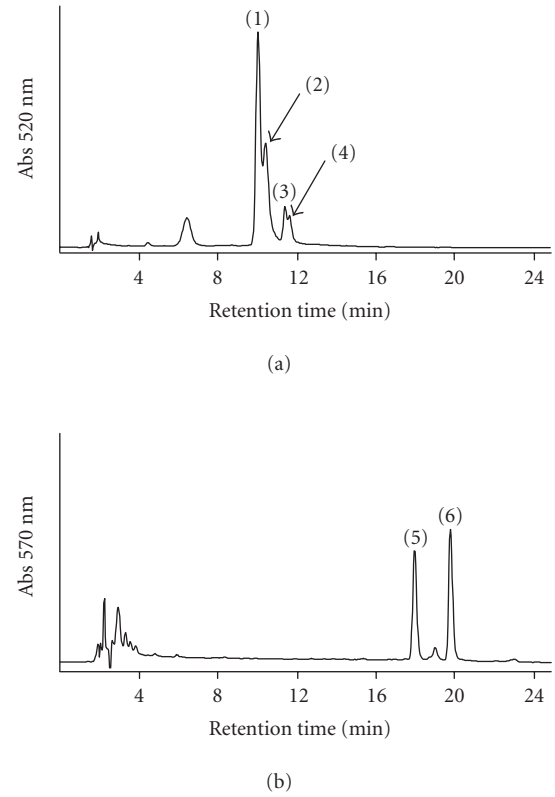


Figure 6. HPLC chromatograms of an elderberry extract. (a) anthocyanins after 2 days of reaction with pyruvic acid: Cy-3-(samb)-5-gluc (1), Cy-3-samb + Cy-3-gluc (2), Cy-3-samb-py (3), Cy-3-gluc-py (4); (b) portisins: vinylpyranoCy-3-samb (5), vinylpyranoCy-3-gluc (6).

CONCLUSION

The search for new natural food colourings has attracted the interest of several manufacturers over the last years. From the organoleptic point of view and considering the available colours widespread in nature, it can be seen that blue pigments are rare. Therefore, the production of new natural blue colourings for the food industry appears to be a priority. Concerning the food quality and safety, the natural colourings present significant benefits compared to the synthetic ones, even if it may be due to psychological concerns of the consumer. In fact, nature-derived pigments are easily accepted as being healthy and are thus a major issue for the food industry.

ACKNOWLEDGMENTS

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MINIREVIEW ARTICLE

Anthocyanins and Human Health: An In Vitro Investigative Approach

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Anthocyanin pigments and associated flavonoids have demonstrated ability to protect against a myriad of human diseases, yet they have been notoriously difficult to study with regard to human health. Anthocyanins frequently interact with other phytochemicals to *potentiate* biological effects, thus contributions from individual components are difficult to decipher. The complex, multicomponent structure of compounds in a bioactive mixture and the degradation of flavonoids during harsh extraction procedures obscure the precise assignment of bioactivity to individual pigments. Extensive metabolic breakdown after ingestion complicates tracking of anthocyanins to assess absorption, bioavailability, and accumulation in various organs. Anthocyanin pigments and other flavonoids that are uniformly, predictably produced in rigorously controlled plant cell culture systems can be a great advantage for health and nutrition research because they are quickly, easily isolated, lack interferences found in whole fruits, can be elicited to provoke rapid and prolific accumulation, and are amenable to biolabeling so that metabolic fate can be investigated after ingestion.

ANTHOCYANINS AND BIOMEDICINAL PROPERTIES

Anthocyanins are members of the flavonoid group of phytochemicals, a group predominant in teas, honey, wines, fruits, vegetables, nuts, olive oil, cocoa, and cereals. The flavonoids, perhaps the most important single group of phenolics in foods, comprise a group of over 4000 C_{15} aromatic plant compounds with multiple substitution patterns (www.nal.usda.gov/fnic/foodcomp/index.html). The primary players in this group include the anthocyanins (eg, cyanidin, pelargonidin, petunidin), the flavonols (quercetin, kaempferol), flavones (luteolin, apigenin), flavanones (myricetin, naringin, hesperetin, naringenin), flavan-3-ols (catechin, epicatechin, gallocatechin), and, although sometimes classified separately, the isoflavones (genistein, daidzein). Phytochemicals in this class are frequently referred to as bioflavonoids due to their multifaceted roles in human health maintenance, and anthocyanins in food are typically ingested as components of complex mixtures of flavonoid components. Daily intake is estimated from 500 mg to 1 g, but can be several g/d if an individual is consuming flavonoid supplements (grape seed extract, ginkgo biloba, or pycnogenol; see, eg, [1]).

The colorful anthocyanins are the most recognized, visible members of the bioflavonoid phytochemicals. The free-radical scavenging and antioxidant capacities of anthocyanin pigments are the most highly publicized of the modus operandi used by these pigments to intervene with human therapeutic targets, but, in fact, research clearly suggests that other mechanisms of action are also responsible for observed health benefits [2, 3, 4, 5]. Anthocyanin isolates and anthocyanin-rich mixtures of bioflavonoids may provide protection from DNA cleavage, estrogenic activity (altering development of hormone-dependent disease symptoms), enzyme inhibition, boosting production of cytokines (thus regulating immune responses), anti-inflammatory activity, lipid peroxidation, decreasing capillary permeability and fragility, and membrane strengthening [6, 7, 8, 9, 10]. The chemical structure (position, number, and types of substitutions) of the individual anthocyanin molecule also has a bearing on the degree to which anthocyanins exert their bioactive properties [11, 12] and the structure/function relationships also influence the intracellular localization of the pigments [7]. The anthocyanin literature includes some controversy over the relative contributions of glycosylated anthocyanins versus aglycones in terms of bioavailability and bioactive potential [7, 13, 14, 15, 16]. Originally, it was assumed that only aglycones could enter the circulation circuit, however, absorption and metabolism of anthocyanin glycosides has now been demonstrated. The nature of the sugar conjugate and the aglycone are important determinants of anthocyanin absorption and excretion in both humans and rats [15].

The roles of anthocyanin pigments as medicinal agents have been well-accepted dogma in folk medicine throughout the world, and, in fact, these pigments are linked to an amazingly broad-based range of health benefits. For example, anthocyanins from *Hibiscus sp* have

historically been used in remedies for liver disfunction and hypertension; and bilberry (*Vaccinium*) anthocyanins have an anecdotal history of use for vision disorders, microbial infections, diarrhea, and diverse other health disorders [17, 18, 19]. But while the use of anthocyanins for therapeutic purposes has long been supported by both anecdotal and epidemiological evidence, it is only in recent years that some of the specific, measurable pharmacological properties of isolated anthocyanin pigments have been conclusively verified by rigorously controlled in vitro, in vivo, or clinical research trials [4]. In many other cases, the exact roles of the anthocyanins in human health maintenance versus other phytochemicals in a complex mixture from a fruit extract or whole food have not been completely sorted out. In fact, some reports suggest that anthocyanin activity is potentiated when delivered in mixtures [20, 21, 22].

For example, visual acuity can be markedly improved through administration of anthocyanin pigments to animal and human subjects, and the role of these pigments in enhancing night vision or overall vision has been particularly well documented [23]. Oral intake of anthocyanosides from black currants resulted in significantly improved night vision adaptation in human subjects [24], and similar benefits were gained after administration of anthocyanins from bilberries [25]. Three anthocyanins from black currant stimulated regeneration of rhodopsin (a G-protein-coupled receptor localized in the retina of the eye), and formation of a regeneration intermediate was accelerated by cyanidin 3-rutinoside [26]. These studies strongly suggest that enhancement of rhodopsin regeneration is at least one mechanism by which anthocyanins enhance visual acuity.

In both in vitro and in vivo research trials, anthocyanins have demonstrated marked ability to reduce cancer cell proliferation and to inhibit tumor formation [27, 28, 29, 30]. The capacity of anthocyanin pigments to interfere with the process of carcinogenesis seems to be linked to multiple potential mechanisms of action including inhibition of cyclooxygenase enzymes and potent antioxidant potential. Hou et al [20] revealed that anthocyanins inhibit tumorigenesis by blocking activation of a mitogen-activated protein kinase pathway. This report provided the first indication of a molecular basis for why anthocyanins demonstrate anticarcinogenic properties. In other research, fruit extracts with significant anthocyanin concentrations proved to be effective against various stages of carcinogenesis [18, 28, 31, 32], but the individual role of anthocyanins versus other components was not determined, in part because the anthocyanins were too easily degraded during bioassays if separated from stabilizing cofactors such as other phenolic constituents [33].

The role of anthocyanins in cardiovascular disease protection is strongly linked to oxidative stress protection. Since endothelial dysfunction is involved in initiation and development of vascular disease, four anthocyanins isolated from elderberries were incorporated into the plasma lemma and cytosol of endothelial cells to directly examine the protective roles [34]. These tests demonstrated not only that anthocyanins could be directly incorporated into endothelial cells, but that significant oxidative stress protection was the result. Delphinidin, but not malvidin or cyanidin, provided endothelium-dependent vasorelaxation in the rat aorta, providing a pharmacological benefit comparable to red wine polyphenolics [35]. In a rat model, little influence of feeding purified anthocyanins (cyanidin 3-*O*-glucoside) or anthocyanin-rich extracts from elderberry or blackcurrant could be detected on cholesterol levels or fatty acid patterns in liver, but the pigments were capable of sparing vitamin E [36]. Crude anthocyanin extracts from bilberry have been administered both orally and via injection to reduce capillary permeability [13]. In other research related to cardiovascular impairment, the roles of anthocyanin pigments versus other flavonoids delivered in the phytochemical extract have not been completely sorted out. Protection from heart attacks through administration of grape juice or wine was strongly tied to the ability of the anthocyanin-rich products to reduce inflammation and enhance capillary strength and permeability, and to inhibit platelet formation and enhance nitric oxide (NO) release [37]. Similarly, delivery of a black currant concentrate with intense anthocyanin content caused endothelium-dependent vasorelaxation in rat aorta rings in vitro [38]. The mechanism of vasorelaxation was attributed to increased levels of NO production, but the active compounds in the concentrate were not isolated. When rats were pretreated to create increased susceptibility to oxidative damage, then fed anthocyanin-rich extracts, significant reduction in indices of lipid peroxidation and DNA damage resulted [9]. Ingestion of these extracts, which contained mixtures of delphinidin, cyanidin, petunidin, peonidin, and malvidin in the 3-glucopyranoside forms, also increased plasma antioxidant capacity.

Tsuda et al [4] recently provided evidence that anthocyanins extracted from purple corn, when provided to mice in tandem with a high-fat diet, effectively inhibited both body weight and adipose tissue increases. Typical symptoms of hyperglycemia, hyperinsulinemia, and hyperleptinemia provoked by a high-fat diet did not occur when mice also ingested isolated anthocyanins. The experiments suggest that anthocyanins, as a functional food component, can aid in the prevention of obesity and diabetes.

Anthocyanins have been credited with capacity to modulate cognitive and motor function, to enhance memory, and to have a role in preventing age-related declines in neural function. Cho et al [39] reported that administration of isolated semipurified anthocyanins from purple sweet potato enhanced cognitive performance as assessed by passive avoidance tests in ethanol-treated mice, and also effectively inhibited lipid peroxidation in rat brain tissues. By administering blueberry extracts with significant anthocyanin content (but not purified pigments), it was noted that the blueberry-supplemented

diets led to effective reversal of age-related deficits in various neural and behavioral parameters (memory and motor functions) [40]. Further investigations by this laboratory team demonstrated that anthocyanins (in particular, cyanidin-3-sambubioside-5-glucoside and cyanidin-3, 5-diglucoside) were highly bioavailable in endothelial cells, which was linked to their roles in prevention of atherosclerosis and neurodegenerative disorders [34, 41].

Anthocyanins exerted multiple protective effects against pleurisy in a rat model and were capable of attenuating inflammation. Anthocyanin treatment also downregulated expression of enzymes involved in inflammation in the lung [10]. The antimicrobial activity of anthocyanins in general has been well established, including significant inhibition of aflatoxin biosynthesis [42]. The experimental evidence demonstrating anthocyanin benefits for diabetes and pancreatic disorders has also accumulated in recent years, and again the efficacy is attributed to the multiple, simultaneous biological effects these pigments cause in the body, including prevention of generation of free radicals, decreased lipid peroxidation, reduced pancreatic swelling, and decreased blood sugar concentrations in urine and blood serum [43, 44].

THE ANTHOCYANIN ENIGMA

An enigma is defined as anything that perplexes because it is inexplicable, hidden, or obscure; something that serves as a puzzle to be solved. The whole realm of anthocyanin consumption and human health fits into this definition, because several aspects of anthocyanin's pharmacological roles have remained elusive to the scientist. In most of the interventions of anthocyanins in human health, details on the mechanisms of action for bioactivity, uptake, absorption, bioavailability, whole body distribution, and tissue localization are still not fully elucidated.

There are at least four primary obstacles that have impeded the formulation, by medical professionals, of robust dietary or prescriptive guidelines on consumption of anthocyanins.

Probably the most complicated piece of the puzzle is that, in terms of biological activity in the human body, an anthocyanin pigment is (almost) never acting independently. Typically, anthocyanins and other flavonoid components, or anthocyanins and other nonflavonoid phytochemicals, are interacting together in order to provide full potency. Interactions between phytochemicals within a plant are a key evolutionary strategy for the host plant. There are over 4000 flavonoids described, with multiple substitution patterns and often large complex structures in the mixtures. Bioflavonoids like anthocyanins occur in mixtures within edible foods and are ingested in mixtures. Any plant containing anthocyanins includes a complex phytochemical cocktail. The anthocyanins and related flavonoids are secondary products typically produced by plants as defensive, protective, or attractive agents, and it makes good evolutionary sense for the plant to use a variety of strategies and multiple fronts of attack to accomplish these functions, rather than single compounds to which a pathogen or predator could become resistant. This same multiplicity in bioactive phytochemical synthesis is also a bonus for animals and humans who ingest the plant material donors, and benefit from the interaction of the flavonoids with therapeutic targets. When the interactions between co-occurring phytochemicals are positive (eg, additive effects or synergies), they are called potentiating interactions. In other cases, components in the donor plant can actually inhibit the bioactivity of the flavonoid compound (eg, pectin interference with antioxidant capacity in in vitro assays), and in other cases, concomitant compounds which are not themselves bioactive may work together with a bioflavonoid to enhance bioavailability or absorption. Synergy among flavonoids including anthocyanins has been cited as responsible for antiplatelet activity of red wine and grape juice, with strong interactions between components of grape skin and grape seed required to potentiate antiplatelet activity in human and animal systems [45]. Co-occurring flavonoids working synergistically to antagonize hydrogen peroxide formation are most effective in depressing platelet function [46]. Traditional bioprospecting approaches, which search for single purified plant-derived compounds as a means of drug discovery, will not capture the full potency of a plant extract when multiple potentiating interactions are responsible for bioactivity.

Another common well-recognized handicap to scientists exploring the bioactive properties of the flavonoids, and the second part of the anthocyanin puzzle, is the fact that these phytochemicals can be of an evanescent nature [33]. The susceptibility of anthocyanins to oxidation and degradation is one of the concerns of food processors who wish to maximize the shelf life of products enhanced with natural pigment colors. In particular, many of the classic phytochemical methods (including column chromatography), used to extract from plant tissues and fractionate components out of a crude extract, can degrade anthocyanins and/or inactivate them during purification steps. As a result, research that aims to identify bioactive entities and gauge potencies of extracts can easily fail to assess the actual sources of biological activity in situ.

Strict attention to the ephemeral nature of some flavonoid constituents in berries (especially during extraction/fractionation sequences) led to the adaptation of a vacuum chromatography technique in our laboratory, which was designed to (as much as feasible) preserve the integrity of the compounds and keep natural mixtures intact until final separation for purposes of identification [32, 47]. Using whole individually-quick-frozen berries as a starting point, fruits are extracted in a Waring blender with 70% aqueous acetone ($\sim$2L solvent kg^{-1} fruit) then filtered through cheesecloth. Acetone is removed from the filtrate under vacuum in a 40°C water bath, and water is then removed by lyophilization, resulting in a dark purple powder. A portion of the crude dry extract is then

redissolved in water and poured over a Toyopearl resin polymer column for vacuum chromatography. Vacuum chromatography on Toyopearl with a series of solvents (water, 50% MeOH, 100% MeOH, 100% acetone, and 50% acetone) elutes 5 TP fractions which are then concentrated under vacuum, and water is removed by lyophilization. Sugars are very quickly and efficiently removed in the first fraction, which greatly simplifies the handling and analysis of remaining fractions.

Once bioactive fractions are identified, a second, third, and subsequent rounds of separation are accomplished on silica gel, also by vacuum, sometimes open column gravity chromatography. At each step of the procedure, isolated mixtures are compared using silica gel thin layer chromatography and 2 spray reagents (vanillin-HCl and dichromate reagent) in order to gauge the composition and number of components in each fraction. In general, this fractionation strategy has permitted rapid separation of relatively large volumes of extract, with less exposure to damaging and expensive solvents, less exposure to column support materials and air, minimal losses, and reliable separation of flavonoids. In tandem with all of the fractionations is a consistent sequence of bioassays (for multiple stages of carcinogenesis) because the fractionation scheme is bioactivity-guided. As fractions become more highly purified, analysis with HPLC, HPLC-MS, and NMR can be used to conclusively determine the origins of the bioactivity.

A third piece of the puzzle is the inducible nature of many of the bioactive flavonoids including anthocyanins. As is true of a plethora of secondary plant products, the initial production and accumulation of phytochemicals is triggered by physical or chemical microenvironmental triggers, usually a stress factor. The genes responsible for flavonoid synthesis are highly inducible. As such, a researcher intent on maximizing production of anthocyanin pigments must recognize the induction factors and deliberately elicit production of bioactive flavonoids by providing these stimuli to the plant material of interest. Elicitation mimics stresses that provoke secondary product formation in nature, and activates otherwise dormant biochemical pathways. This triggering of productivity can, of course, be very difficult to accomplish in a field setting, but can be accomplished reliably in controlled growth facilities.

The final puzzle piece in the "anthocyanin enigma" is the inability of the scientist or medicinal practitioner to track metabolic progress of anthocyanins after ingestion, due to the plethora of metabolic breakdown products that are rapidly produced in situ. There is substantial current interest in the quest to follow the transport of bioflavonoids through the body, to determine absorption and bioavailability, and to see where breakdown products accumulate and for how long. However, since these phytochemicals are highly metabolized after consumption of anthocyanin-rich foods or supplements, metabolic tracking has not been possible. Despite active research and increasing interest in the realm of natural products and health maintenance, there is a paucity of information on the absorption, biodistribution, and metabolism of anthocyanins and interacting flavonoids. Various plant secondary products have been implicated in the promotion of good health or the prevention of disease in humans, but little is known about the way they are absorbed in the gut, or in which tissues they are deposited throughout the body. While these issues could be studied if the phytochemicals were isotopically labeled, generating labeled molecules often is problematic because many compounds of interest can be synthesized only *in planta* at present.

IN VITRO ANTHOCYANIN PRODUCTION SYSTEMS

The development and optimization of plant cell culture systems which reliably and predictably synthesize anthocyanins in a controlled environment has provided a unique and useful model for in-depth research on anthocyanins, and has helped at least in part to circumvent the obstacles presented in all four cases of the "anthocyanin enigma" as described above. Callus and cell suspension cultures from a wide and diverse range of plant genera have been cultivated to produce anthocyanin pigments in vitro [48, 49, 50, 51, 52, 53, 54, 55, 56]. In most of these past reports, the overall goal of the plant cell culture production system was to explore an alternative resource for natural plant pigments, for possible use as food colorants. More recently, some anthocyanin-producing plant species have been intensively cultured in vitro in order to harvest the bioactive pigments and related phytochemicals as medicinally-active compounds [47, 57, 58, 59]. By controlling both the physical and the chemical microenvironment of the plant cell cultures, anthocyanin production could in many cases be boosted to higher concentrations than available in the parent plant in vivo. Some of the most intensively-researched cell culture production systems used selections from the genus *Vitis* (grape), where scaled-up bioreactor-based systems approached semicommercial productivity [60, 61]. The cell culture systems can be quite stable, and many have been selected for high anthocyanin yield and lack of dependence on irradiance. Anthocyanin profiles from cell cultures do not necessarily mirror the profiles from the parent plant, and isolation of pigments from the simplified cell culture tissues is substantially more streamlined than from complex fruit or vegetative tissues [47, 53, 58]. This simplicity can be a particular advantage for investigation of the health properties of bioflavonoids including anthocyanins.

In most cases, the systems begin with vegetative plant materials (leaves, petioles, stems) and not fruit tissues. Explants from in vivo plants are surface-disinfested and introduced into cell culture to produce rapidly proliferating callus, then cell suspension cultures, which are eventually induced to produce flavonoids (usually with a trigger such as light, elevated carbohydrate, changed nitrogen profile,

or elicitation with a fungal extract or other chemical elicitor).

Because cell culture anthocyanin production systems are comprised of simple tissues which can be engineered to reliably and predictably accumulate pigment, these systems circumvent many of the obstacles in the anthocyanin enigma. Interactions between potentiating phytochemicals are still in force in cell culture systems, but because the tissues are much simpler to extract, the nature of phytochemical interactions is much easier to sort out and to quantitatively test. Cell cultures permit rapid and efficient isolation without many of the interfering compounds (pectins, excess polysaccharides, enzymes) that can complicate extraction or bioassay from fruits [18]. Aqueous extracts of an anthocyanin-producing sweet potato line exhibited higher potency (antiproliferative and antimutagenic potential) than extract from field-grown crops [58]. Similarly, when antioxidant capacity of cell cultures and various fruit extracts were compared side by side in a galvinoxyl free radical assay, the potency of the cell culture extract was substantially higher than that of all fruit extracts, and only grape seed proanthocyanidins exhibited higher activity [47]. Because these other substances are not present, the flavonoids are much easier to isolate without the degradation that can occur rapidly when isolating slowly from complex, recalcitrant fruit or vegetative tissues.

While the flavonoid content of a fruit may comprise only 1% or less of the total substance, a cell culture can be crafted to accumulate much higher concentrations of flavonoids, in the range of 20%–30% by volume. Many flavonoids, in particular, high-molecular weight proanthocyanidin oligomers or complex anthocyanin isomers, are either not available commercially or prohibitively expensive. By producing these phytochemicals in volume in cell cultures, a source of ready standards is available for testing unknowns [32].

Cell cultures are a superb model system for testing the effects of elicitation on the inducible bioflavonoid genes, which is a means of resolving yet another aspect of the anthocyanin enigma. In fact, elicitation of cell cultures (using chemical or environmental triggers to production) is a recognized and efficient means of maximizing anthocyanin pigment towards commercialization of product recovery [55, 56, 62, 63], and since the in vitro production environment is rigorously controlled, investigators have the opportunity to test multiple elicitation triggers without interference from uncontrolled environmental conditions in field settings.

Perhaps the most significant advantage to investigation using in vitro anthocyanin-accumulating cell cultures is that the cultures can serve as a vehicle for delivery of isotopic labels (13C or 14C) to the metabolizing cells while the pigments are being biosynthesized [59, 64, 65]. By using a radioisotope-labeled source of compounds, an administered phytochemical can be included in a defined diet and can be discerned from preexisting, endogenous sources of the same phytochemical or breakdown product. These large molecules must be synthesized *in planta*. In this emerging research area, radiolabel or isotopic label has been introduced to metabolizing cell cultures using a carbohydrate source (sucrose or glucose) or a precursor which is much further along the metabolic pathway to anthocyanin synthesis, such as phenylalanine. Levels of incorporation range between 15% and 30%, and levels achieved now allow organ and neuronal localization of the ^{14}C-labeled compounds and monitoring using autoradiography and scintillation counting. Accelerator mass spectrometry (AMS) technology will even permit monitoring of small levels in human systems. With these innovations, it is clear that the effective use of cell-culture-produced anthocyanins can now elucidate previously hidden roles of anthocyanin pigments in human health and metabolism.

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REVIEW ARTICLE

Nature's Swiss Army Knife: The Diverse Protective Roles of Anthocyanins in Leaves

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Anthocyanins, the pigments responsible for spectacular displays of vermilion in the leaves of deciduous trees, have long been considered an extravagant waste of a plant's resources. Contemporary research, in contrast, has begun to show that the pigments can significantly influence the way a leaf responds to environmental stress. Anthocyanins have been implicated in tolerance to stressors as diverse as drought, UV-B, and heavy metals, as well as resistance to herbivores and pathogens. By absorbing high-energy quanta, anthocyanic cell vacuoles both protect chloroplasts from the photoinhibitory and photooxidative effects of strong light, and prevent the catabolism of photolabile defence compounds. Anthocyanins also mitigate photooxidative injury in leaves by efficiently scavenging free radicals and reactive oxygen species. Far from being a useless by-product of the flavonoid pathway, these red pigments may in some instances be critical for plant survival.

INTRODUCTION

It is no coincidence that the leaves of most higher plants are green. Chlorophyll and the associated accessory pigments allow plants to maximise use of the visible light spectrum for photosynthesis [1]. By establishing internal gradients in light capture, the green pigments enable plants to respond rapidly to changes in the spectral environment, as well as to exploit niche habitats. Equally, they provide some protection against photoinhibition and photooxidation, the damaging effects of excess quanta. Green leaves are engineered to optimise productivity.

Given the obvious benefits to their being green, why then do many plant species produce red-pigmented leaves at one or more stages in their life cycles? Red-leafed flora are common throughout all orders of the plant kingdom, from the basal liverworts to the most advanced angiosperms [2]. They occur in habitats as diverse as the Antarctic shoreline and the tropical rainforests, are as abundant in arid deserts as in freshwater lakes, and seem equally at home in the light-starved forest understorey as in the sun-drenched canopy. In many red-leafed species the manufacture of red pigments is transient, often associated with a discrete developmental stage such as in the growth flushes of tropical trees [3, 4, 5], or in the senescing autumn foliage of deciduous trees [6, 7, 8, 9]. In certain other species, however, red pigments can persist throughout the leaf's entire life span [10], or else they are induced and retained only after the plant has experienced stress [11]. Functional implications of these red pigments in plants have been the focus of a significant research output over the past decade.

For most vascular plants, red colouration in leaves is achieved by anthocyanins, predominantly cyanidin-3-*O*-glucoside, as a solution located in the vacuole of the plant cell. Other pigments—notably the betalains, certain carotenoids, thiarubrine A, some terpenoids, and the 3-deoxyanthocyanins—also impart reddish colours in various species; these pigments have been less well studied than the anthocyanins, but at least some of them have comparable functions in leaves [12, 13, 14, 15, 16].

The synthesis and vacuolar sequestration of anthocyanin molecules represent a considerable metabolic investment for plant cells. First, there are metabolic costs associated with enzyme production and activity; at least seven enzymes are involved in the biosynthesis of cyanidin from its precursors, 4-coumaroyl-CoA and malonyl-CoA [17]. Then there is a cost associated with the conjugation of each cyanidin molecule to a monosaccharide molecule. Finally, there are costs associated with the transport of cyanidin-3-*O*-glucoside into the cell vacuole via a tonoplast Mg-ATP-requiring glutathione carrier [18]. This investment suggests that the accumulation of anthocyanins in leaf cells is unlikely to be an "extravagancy without a vital function" [19]. Neither is it likely that these red pigments are simply the default product of a saturated flavonoid biosynthetic pathway, since the timing of anthocyanin production is usually tightly controlled and often occurs in tissues remote from those associated with other flavonoids [10]. On the contrary, a wealth of recent evidence, both empirical and theoretical, ascribes a remarkable diversity of functions to anthocyanins in leaves, many of them associated with stress responses and some potentially critical to a plant's survival. Anthocyanins are

arguably the most versatile of all pigments, their multifarious roles in plant stress responses stemming as much from the physicochemical property of light absorption as from their unique combination of biochemical reactivities. Recent advances in our understanding of these various functions are the subject of this review.

CONSEQUENCES OF BEING RED

Anthocyanins in vivo absorb the green and yellow wavebands of light, commonly between 500 and 600 nm [20, 21, 22, 23, 24, 25]. Foliage appears red because of the subtraction of yellow-green light from the spectrum of light reflected from the leaf's surface. Interestingly, the amount of red light that is reflected from red leaves often only poorly correlates to anthocyanin content [20]; leaf morphology and the amount and distribution of chlorophyll are apparently the stronger determinants of red reflectance. The property of anthocyanins to absorb light provides a mechanism for several important functions in leaves.

Herbivory

The red colours of anthocyanic leaves have been proposed both to attract and to repel various animal species. Burns and Dalen [26] postulated that red-orange autumn foliage of Canadian shrub species would accentuate the conspicuousness of black-coloured fruits to birds. Experimental manipulations of fruit and background foliage colours confirmed that the black-red contrast was indeed an effective enhancer of fruit-removal rates by avian dispersers. Certain insects, on the other hand, seem to preferentially avoid eating red-pigmented leaves. California maple aphids, for example, readily colonise yellow-orange leaves of Japanese maples, yet they largely ignore red-leafed individuals [27]. Similarly, leaf-cutting ants from the tropical forests of Panama browse significantly less on red leaves than on green leaves [28]. To these and other insects the anthocyanins may serve as aposematic signals of defensive commitment against herbivory [29]. Alternatively, the red pigments may simply render the leaves unpalatable. Leaves that are rich in chlorophyll as well as anthocyanin tend to be brown or even black, mimicking dead foliage or else serving to camouflage leaves against the exposed soil and litter of forest floors [30, 31, 32, 33]. Even brilliant red or scarlet leaves can appear dark to nonmammalian folivores, which lack red light receptors [5, 34]. The gains to be had from herbivore deterrence would offset metabolic costs to the plant associated with anthocyanin biosynthesis.

Protection of photolabile defence compounds

By intercepting the high-energy quanta, anthocyanic cell vacuoles can prevent important photolabile molecules from degradation by green light. An elegant example of this was described recently for the silver beachweed (*Ambrosia chamissonis*), a composite that grows at exposed, sunny locations along the California coast. The plant holds large amounts of thiarubrine A, a potent defence compound that is toxic to insects, bacteria, and fungi [35]. Thiarubrine A is photolabile; even short exposures to visible light and/or ultraviolet radiation render it inactive [36, 37]. However, the tissues in *A chamissonis* that contain thiarubrine A are shielded from light by a sheath of cells containing a mix of two anthocyanins, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-(6'-*O*-malonylglucoside). The anthocyanins absorb quanta that would otherwise lead to the destruction of thiarubrine A, and thereby contribute significantly to the defensive armoury of the plant.

Protection of photosynthetic apparatus

When leaves receive more light energy than can be used in photochemistry, they show a characteristic decline in the quantum efficiency of photosynthesis, termed photoinhibition [38]. Under severe conditions the chloroplasts generate reactive oxygen species, which have the potential to destroy thylakoid membranes, damage DNA, and denature proteins associated with photosynthetic electron transport. Anthocyanins have been shown in many plant species to reduce both the frequency and severity of photoinhibition, as well as to expedite photosynthetic recovery [23, 39, 40, 41, 42, 43, 44]. In red-osier dogwood (*Cornus stolonifera*), for example, a 30-minute exposure to strong white light reduced the quantum efficiency of photosynthesis by 60% in red leaves, but by almost 100% in acyanic leaves [23]. When the plants were returned to darkness, the red leaves recovered to their maximum potential after only 80 minutes, yet their acyanic counterparts had not achieved the pretreatment state even after six hours.

Anthocyanins protect leaves from the stress of photoinhibitory light fluxes by absorbing the excess photons that would otherwise be intercepted by chlorophyll *b*. Although red leaves absorb more green light in total, their photosynthetic tissues actually receive fewer quanta than do those of acyanic leaves because the energy absorbed by the cell vacuole cannot be transferred to the chloroplasts [45]. As a result, under light-limiting environments the photosynthetic efficiencies of red leaves are often slightly lower than those for acyanic leaves [4, 22, 45, 46, 47, 48, 49]. Under strong light, however, the anthocyanins serve as a useful optical filter, diverting excess high-energy quanta away from an already saturated photosynthetic electron transport chain. Chloroplasts irradiated with light that has first passed through a red filter have been shown to generate fewer superoxide radicals, thereby reducing the propensity for structural damage to the photosystems [25]. The anthocyanins are therefore clearly a useful supplement to other nonphotochemical quenching mechanisms such as the xanthophyll cycle pigments. Recent studies involving mutants of *Arabidopsis thaliana* indicate that whereas xanthophylls have a greater role in the protection of plants from short-term

light stress, the anthocyanins can be the more effective photoprotectants over the long term [50].

The photoprotection hypothesis potentially explains why the leaves of many deciduous trees turn red in the autumn. As leaves senesce, nitrogen associated with their chloroplasts is resorbed into the branches. Anthocyanins would protect the degrading chlorophyll from damaging light levels, thereby restricting the formation of reactive oxygen that could jeopardize the resorptive process [2, 8, 9, 23, 51, 52]. Consistent with this hypothesis, nitrogen resorption has recently been shown to be more efficient in wild-type than in anthocyanin-deficient mutants of three woody species [53].

Protection from ultraviolet radiation

Interest in the flavonoid family has increased in recent years following the observation that these compounds act as sunscreens against potentially damaging UV-B radiation. Foliar anthocyanins have generally been included with other flavonoids in this UV-B protective role. Consistent with this hypothesis, the anthocyanins, particularly when acylated, absorb strongly in the UV region [54, 55], are induced or upregulated in plant tissues in response to UV irradiation [56, 57, 58, 59, 60], and mitigate DNA damage in UV-B-irradiated cell cultures [61, 62, 63]. Furthermore, certain anthocyanin-deficient mutants of *Arabidopsis* are hypersensitive to UV-B [64], and red-leafed *Coleus* varieties retain higher photosynthetic efficiencies after UV irradiation than do green-leafed varieties [49].

Notwithstanding this body of evidence, there is now a growing conviction that foliar anthocyanins cannot be primarily concerned with UV protection. Unlike the colourless flavonoids, the anthocyanins are usually located in the internal mesophyll tissue rather than in the epidermis, the optimal site for UV interception [33, 65]. Moreover, UV vulnerability often correlates only poorly to anthocyanin content. For example, an *Arabidopsis* mutant with enhanced sensitivity to UV radiation was found deficient in certain flavonoids, yet it held normal amounts of anthocyanin [66]. Similarly, the responses of *Brassica rapa* mutants to supplementary UV-B treatment were for the most part independent of anthocyanin levels in the leaves [67]. Indeed, red-leafed plants of petunia (*Impatiens*) and rice have all been observed to perform significantly worse than their green-leafed counterparts under UV-enriched environments [68, 69, 70]. Hada et al. [71] noted that DNA damage after prolonged UV treatment was substantially greater in purple-leafed rice than in a near-isogenic green line. To repair UV-damaged DNA, plants employ photolyase, an enzyme that uses blue/UV-A light to re-monomerise the pyrimidine dimers. The anthocyanins in purple rice prevented the photoactivation of photolyase by absorbing some of the blue/UV-A light incident on the leaves. Thus, any short-term gain from the absorption of UV-B by anthocyanins would be offset by their property to absorb visible light and thereby limit the rate of DNA repair.

FREE RADICAL SCAVENGING

Anthocyanins diminish the oxidative load in a leaf simply by filtering out yellow-green light, since the majority of reactive oxygen in plant cells is derived from the excitation of chlorophyll. Anthocyanins are, in addition, excellent scavengers of free radicals. Purified solutions scavenge almost all species of reactive oxygen and nitrogen with an efficiency up to four times greater than those of ascorbate and α-tocopherol [72, 73, 74]. Recent experimental evidence indicates that this antioxidant potential is indeed utilised by plant cells. In *Arabidopsis*, for example, strong light and low temperatures caused more lipid peroxidation in anthocyanin-deficient mutants than in wild-type plants [50]. Similarly, upon gamma irradiation, only those *Arabidopsis* plants that contained both anthocyanin and ascorbic acid were able to grow and flower normally [75].

Microscopic examinations of wounded leaf peels have shown that red-pigmented cells eliminate H_2O_2 significantly faster than do green cells [76]. It is not clear, however, whether scavenging occurs predominantly by the red tautomers of anthocyanin found inside the cell vacuole, or else by the colourless tautomers in the cytosol. Both forms have impressive antioxidant potentials [77, 78, 79]. In a model in vitro system, the colourless tautomers of cyanidin 3-(6-malonyl)glucoside were found capable of scavenging up to 17% of the superoxide radicals generated by irradiated chloroplasts [25]. Given their proximity to the organelle sources of reactive oxygen, it may be that the cytosolic anthocyanins, rather than those in the cell vacuole, provide the greater contribution to antioxidant defence.

The degree to which anthocyanins contribute to the arsenal of low-molecular-weight antioxidants (LMWA) varies among species. In the young, red leaves of *Elatostema rugosum*, an understorey herb from New Zealand, anthocyanins are the predominant phenolic component of the LMWA pool [78]. In contrast, the red- and green-leafed morphs of the canopy tree *Quintinia serrata* both hold hydroxycinnamic acids as their most concentrated LMWA [79]. Similar differences have been reported across ecotypes of wild-type *Arabidopsis* [75]. Thus it would seem that anthocyanin biosynthesis can enhance but is not usually a prerequisite for protection from oxidative stress.

AMELIORATION OF STRESS RESPONSES

The induction of foliar anthocyanins has been implicated in the acquisition of tolerance to many different kinds of environmental stressors [11, 80, 81]. Anthocyanins, for example, are associated with enhanced resistance to the effects of chilling and freezing [82, 83, 84, 85, 86], to heavy metal contamination [87, 88, 89, 90], to desiccation [91, 92, 93], and to wounding [76, 94, 95]. It is not clear at this stage whether the apparent ameliorative properties stem from one or more types of mechanism. Chalker-Scott [11, 80] provided a compelling case for

a generalised role of anthocyanins as osmoregulators in plant cells, since most types of suboptimal environments induce water stress, either directly or indirectly. Others have argued that the photoprotective [96] or the antioxidant [97] properties of anthocyanins are paramount. Regardless of their mechanism, it is clear that anthocyanins offer multifaceted, versatile, and effective protection to plants under stress. They are the Swiss army knife of the plant kingdom.

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REVIEW ARTICLE

Molecular Mechanisms Behind the Chemopreventive Effects of Anthocyanidins

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Anthocyanins are polyphenolic ring-based flavonoids, and are widespread in fruits and vegetables of red-blue color. Epidemiological investigations and animal experiments have indicated that anthocyanins may contribute to cancer chemoprevention. The studies on the mechanism have been done recently at molecular level. This review summarizes current molecular bases for anthocyanidins on several key steps involved in cancer chemoprevention: (i) inhibition of anthocyanidins in cell transformation through targeting mitogen-activated protein kinase (MAPK) pathway and activator protein 1 (AP-1) factor; (ii) suppression of anthocyanidins in inflammation and carcinogenesis through targeting nuclear factor *kappa* B (NF-κB) pathway and *cyclooxygenase* 2 (*COX*-2) gene; (iii) apoptotic induction of cancer cells by anthocyanidins through reactive oxygen species (ROS) / c-Jun NH_2-terminal kinase (JNK)-mediated caspase activation. These data provide a first molecular view of anthocyanidins contributing to cancer chemoprevention.

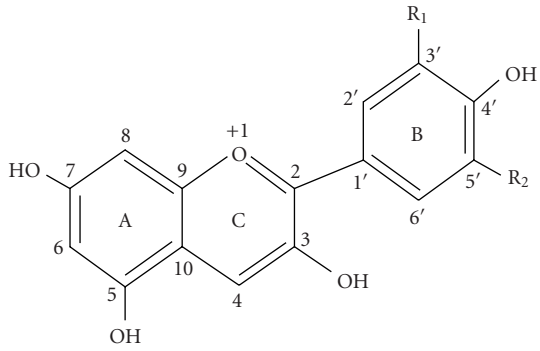
INTRODUCTION

Anthocyanins are naturally occurring polyphenolic compounds that give the intense color to many fruits and vegetables such as berries, red grapes, purple sweet potato, and red cabbages [1, 2]. In contrast to the other flavonoids, anthocyanins carry a positive charge in the central ring structure and are thus cations. In plants, they are present exclusively as glycosidic compounds. The number and nature of the different attached sugar moieties are responsible for the high number of anthocyanins, more than 500 compounds [3]. The aglycone (named anthocyanidin) is a diphenylpropane-based polyphenolic ring structure, and is limited to a few structure variants including delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Figure 1), that represent the aglycones of most anthocyanins in plants.

Based on food composition data, we consume considerable amounts of anthocyanins from crops, fruits, and vegetable-based diets [4] although the range is from several milligrams to several hundred milligrams, depending on the nutrition customs. An enhanced intake of anthocyanin is now increasing because extracts with higher anthocyanin contents from bilberry or elderberry are commercially available. Epidemiological investigations have indicated that moderate consumption of anthocyanins through the intake of products such as red wine [5] or bilberry extract [6] is associated with a lower risk of coronary heart disease. Recent studies indicated that anthocyanins have strong free radical scavenging and antioxidant activities [7, 8, 9, 10, 11, 12, 13], and show inhibitory effects on the growth of some cancer cells [14, 15, 16, 17, 18]. Animal experiments showed that oral intake of anthocyanins from purple sweet potato (*Ipomoea batatas* L.) and red cabbage (*Brassica oleracea* L.) suppressed rat colon carcinogenesis induced by 1, 2-dimethylhydrazine and 2-amino-1-methyl-6-phenylimidazo- [4,5-b] pyridine [19]. In addition, anthocyanins can be directly absorbed and distributed to the blood in human and rats after consumption of dietary anthocyanins [18, 19, 20, 21, 22, 23, 24]. These facts suggest that anthocyanins may play a role in cancer chemoprevention. Thus, mechanisms behind chemopreventive effects of anthocyanins need to be considered at molecular level.

ANTICARCINOGENESIS THROUGH TARGETING MAPK PATHWAY AND AP-1 FACTOR

AP-1 is a transcription factor and has been shown to play a critical role in promoting carcinogenesis [25, 26]. In mouse epidermal cell line JB6 (+), tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), epidermal growth factor (EGF), and tumor necrosis factor (TNF) alpha can induce AP-1 activity and neoplastic transformation by activating MAPK including extracellular signal-regulated kinase (ERK), JNK, or p38 kinase



Delphinidin, $R_1 = R_2 = OH$ Pelargonidin, $R_1 = R_2 = H$
Cyanidin, $R_1 = OH, R_2 = H$ Peonidin, $R_1 = OCH_3, R_2 = H$
Malvidin, $R_1 = R_2 = OCH_3$ Petunidin, $R_1 = OCH_3, R_2 = OH$

Figure 1. Basic chemical structures of the major anthocyanidins.

[27, 28]. Delphinidin, cyanidin, and petunidin have been shown to inhibit TPA-induced AP-1 transcriptional activity and cell transformation in JB6 cells [29]. Structure-activity studies indicated that the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins seems essential for the inhibitory action because pelargonidin, peonidin, and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effects in both AP-1 activity and cell transformation. The results from signal transduction analysis indicated that delphinidin blocked ERK phosphorylation at early times and JNK phosphorylation at later times, but not p38 phosphorylation at any time [29]. Moreover, delphinidin blocked the phosphorylation of MAPK/ERK kinase (MEK, an ERK kinase), SAPK/ERK kinase (SEK, a JNK kinase), and c-Jun (a phosphorylation target of ERK and JNK). The data suggest that the inhibition of TPA-induced AP-1 activity and cell transformation by delphinidin involves the blockage of ERK and JNK signaling cascades (Figure 2). Furthermore, a greater inhibition was observed in combinations of superoxide dismutase (SOD) with anthocyanidins that have the ortho-dihydroxyphenyl structure on the B-ring. Multiplicative model analysis suggested that this greater inhibition between SOD and delphinidin is synergistic, not additive [29]. Thus, the inhibitory effects of anthocyanidins on AP-1 activation and cell transformation would be due in part to their potent scavenging activity for superoxide radicals and in part to MAPK blockage. SOD has been shown to selectively inhibit the TPA-induced activation of protein kinase Epsilon and to prevent subsequent activation of JNK2 in response to TPA, thereby delaying AP-1 activation and inhibiting mouse skin tumor promotion [30]. Thus, the signaling pathways blocked by delphinidin or SOD may differ in part although both are considered to be important in the cancer prevention activity of anthocyanidins.

ANTI-INFLAMMATION THROUGH TARGETING NF-κB PATHWAY AND *COX-2* GENE

Cyclooxygenase (COX) is the rate-limiting enzyme for synthesis of dienoic eicosanoids such as prostaglandin (PG) E2. COX exists in three isoforms [31, 32]. COX-1 is expressed constitutively in many types of cells and is responsible for the production of PGs under physiological conditions. COX-3 is a COX-1 variant and is mainly expressed in cerebral cortex. Analgesic/antipyretic drugs such as acetaminophen, phenacetin, antipyrine, and dipyrone can selectively inhibit this enzyme. Thus, inhibition of COX-3 could represent a primary central mechanism by which these drugs decrease pain and possibly fever [31]. COX-2 is induced by proinflammatory stimuli, including mitogens, cytokines, and bacterial lipopolysaccharide (LPS) in cells in vitro and in inflamed sites in vivo [33]. Data indicate that COX-2 is involved in many inflammatory processes and induced in various carcinomas, suggesting that COX-2 plays a key role in tumorigenesis [34]. Interestingly, some antioxidants with chemopreventive effects inhibit the expression of COX-2 by interfering with the signaling mechanisms that regulate the *COX-2* gene [35]. Wang et al [36] found that anthocyanins and their aglycone, cyanidin, from tart cherries could inhibit the activities of COX-1 and COX-2. Seeram et al [37] found that cyanidin showed superior inhibition of the cyclooxygenase activity in vitro. In our laboratory, we used mouse macrophage cell line RAW264 to demonstrate the molecular mechanism of anthocyanins in the inhibition of the *COX-2* gene. We found that anthocyanin extracts from bilberry or purified delphinidin inhibited LPS-induced COX-2 expression at protein and transcriptional levels. Studies on signal pathway indicated that delphinidin at least blocked LPS-induced IκB degradation and then suppressed NF-κB activation and *COX-2* gene expression (Hou et al, unpublished data). These data demonstrated that the blockage of NF-κB signaling pathway is involved in the inhibition of *COX-2* gene expression by anthocyanins (Figure 2).

ANTITUMOR PROGRESSION THROUGH OXIDATIVE/JNK-MEDIATED APOPTOSIS

Apoptosis has been reported to play an important role in elimination of seriously damaged cells or tumor cells by chemopreventive agents [38, 39]. The cells that have undergone apoptosis have typically shown chromatin condensation and DNA fragmentation [40]. They are rapidly recognized by macrophages before cell lysis, and then can be removed without inducing inflammation [38, 41]. Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs of which human promyelocytic leukemia cell line (HL-60) provides a valid model for testing antileukemic or general antitumoral compounds [42]. Delphinidin, cyanidin, and petunidin induced apoptosis of HL-60 cells detected by morphological changes and by DNA fragmentation, whereas pelargonidin, peonidin,

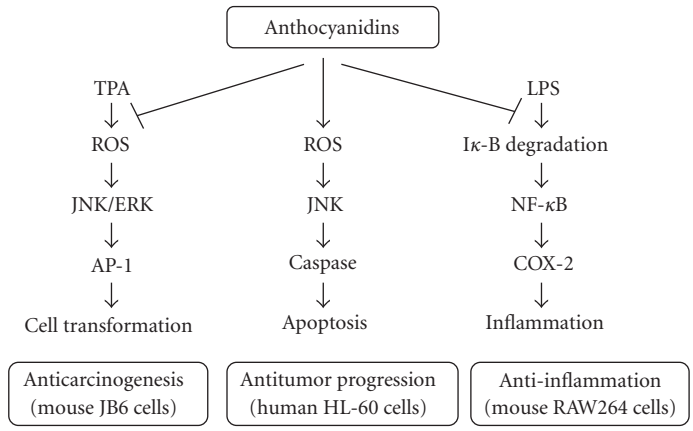


FIGURE 2. A schematic molecular view of cancer chemoprevention by anthocyanidins. Anthocyanidins may contribute to cancer chemoprevention through targeting three different signal transduction pathways and downstream genes. Abbreviations: AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

and malvidin showed no induction of apoptosis [43]. The anthocyanidin glycosides (anthocyanins) extracted from bilberry such as delphinidin glycosides and cyanidin glycosides also induced the apoptosis in HL-60 cells [44]. Structure-activity studies indicated that the potency of apoptosis induction of anthocyanidins is associated with the number of hydroxyl groups at the B-ring, and the ortho-dihydroxyphenyl structure at the B-ring appears essential for apoptosis actions [43]. It is noteworthy that anthocyanidins increased the levels of hydrogen peroxide in HL-60 cells with a structure-activity relationship that depends on the number of hydroxyl groups at the B-ring [43] and appears in the order of delphinidin > cyanidin, petunidin > pelargonidin, peonidin, and malvidin.

The mechanistic analysis indicates that the apoptosis induction by delphinidin may involve an oxidation/JNK-mediated caspase pathway. Delphinidin treatment increased the levels of intracellular ROS, which may be a sensor to activate JNK. Concomitant with the apoptosis, JNK pathway activation such as JNK phosphorylation, *c-jun* gene expression, and caspase-3 activation was observed in delphinidin-treated cells [43]. Antioxidants such as *N*-acetyl-*L*-cysteine (NAC) and catalase effectively blocked delphinidin-induced JNK phosphorylation, caspase 3 activation, and DNA fragmentation [43]. Thus, delphinidin may trigger an apoptotic death program in HL-60 cells through an oxidative stress-mediated JNK signaling cascades (Figure 2). It is interesting that anthocyanidins produced ROS, showing pro-oxidant activities, to induce apoptosis in HL-60 cells, contrary to the antioxidant activities of anthocyanidins in the inhibition of TPA-induced cell transformation in mouse skin JB6 cells [29].

It should be noted that anthocyanidins are the aglycons of the naturally occurring anthocyanins. Most of the molecular results on biological activities of anthocyanins were from anthocyanidins due to the fact that anthocyanidins are easier to be prepared than anthocyanins. Thus, it is not yet known whether the naturally occurring anthocyanins will also activate these molecular pathways. Accumulated results on structure-activity studies have shown that the biological activities of anthocyanins appear to increase with a decreasing number of sugar units and/or with an increasing number of hydroxyl groups at their aglycons [7]. For example, both antioxidant activity and cyclooxygenase inhibitory activity of cyanidin glycosides increased with a decreasing number of sugar units. Cyanidin-rutinose showed better activity than cyanidin-glucosylrutinose, and cyanidin aglycone showed the best activity at much lower concentrations [37]. In our laboratory, we found that the aglycons with ortho-dihydroxyphenyl structure at the B-ring, such as delphinidin and cyanidin [29, 43] and their glycosides (Hou et al, unpublished data), possessed the activities of anticarcinogenesis, anti-inflammation, and apoptosis induction. The ortho-dihydroxyphenyl structure on the B-ring appears essential for these actions, and the activities of aglycons such as delphinidin and cyanidin are stronger than that of their glycosides.

CONCLUSION

The molecular mechanisms of anticarcinogenesis, anti-inflammation, and apoptosis induction of malignant cells by anthocyanidins have been demonstrated at molecular level. These data provide a first molecular view of the chemopreventive effects of anthocyanidins. Based on many genes that may be associated with cancer chemoprevention, a genome-wide gene analysis by using microarray technology will be required to get the whole view of molecular mechanisms of cancer chemoprevention by anthocyanidins.

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CORRESPONDENCE ARTICLE

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-inflammtory, 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 5000*g* 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 5000*g* 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 (°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 reticule 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 (°Brix)	Titratable 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

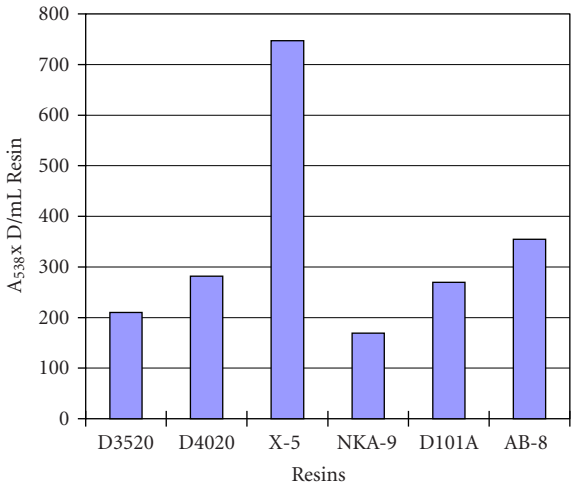


Figure 1. The mulberry pigment adsorbent capabilities of different resins (D = dilution factor).

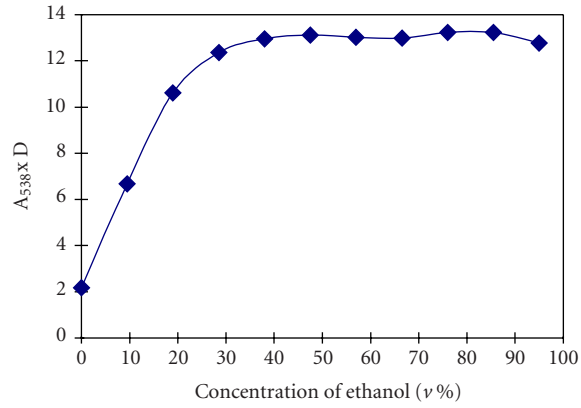


Figure 2. The elution efficacy of different concentrations of acidified ethanol (D = dilution factor).

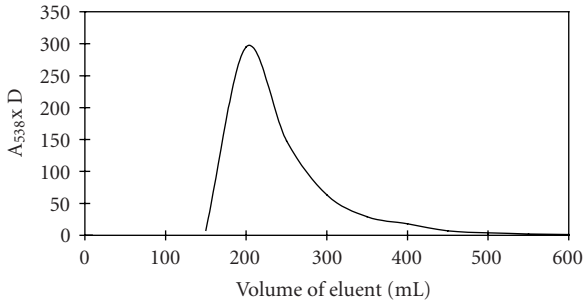


Figure 3. The elution of anthocyanins with acidified 80% (v/v) ethanol applied to mulberry pigment adsorbed onto resin (D = dilution factor).

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

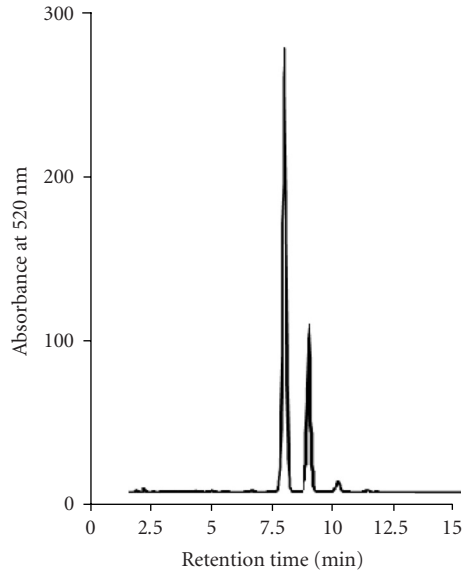
Crude mulberry juice is rich in anthocyanins and, after concentration, can be directly used as a natural pigment. However, the concentration of anthocyanins in such juice is low, with a color value usually less than 4.0. That is due to the presence of nonpigment components such as mono-, di-, and polysaccharides, 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.

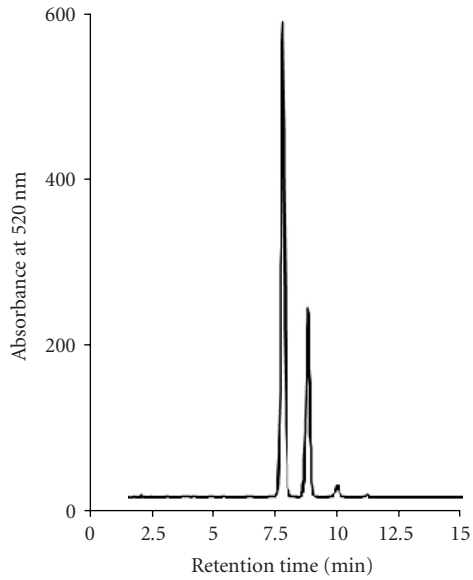
When tested by HPLC under the same conditions (520 nm), the HPLC profiles of the purified and crude pigment extracts were identical, consisting of four peaks (Figure 4). This suggests that pigment purification using the resins did not change the composition of the anthocyanin mixture.

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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CORRESPONDENCE ARTICLE

The Effect of Two Methods of Pomegranate (*Punica granatum* L) Juice Extraction on Quality During Storage at 4°C

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The effect of two extraction methods of pomegranate juice on its quality and stability was evaluated. The first method consisted of separation of the seeds from fruits and centrifugation. The second method consisted of squeezing fruit halves with an electric lemon squeezer. During a period of 72 hours of cold storage at 4°C, the juices were evaluated for the presence of sugars, organic acids, and anthocyanins. Delphinidin 3-glucoside was identified to be the major anthocyanin present at the level of 45–69 mg/L. Among the organic acids, oxalic and tartaric acids dominated. The major sugars detected in pomegranate juice were glucose and sucrose. No significant differences in the content of sugars, organic acids, or anthocyanins in juices obtained through application of the two different extraction methods were detected, with the exception of the drastic decrease of cyanidin 3, 5-diglucoside level in juice obtained by seed centrifugation. The pH did not show differences between treatments. Titrable acidity and the level of sugars expressed as °Brix decreased after 32 and 15 hours after extraction, respectively, when juice was obtained by centrifuging the seeds.

INTRODUCTION

Phenolic compounds are important components of many fruits, vegetables, and beverages contributing to their colour and sensory properties. Epidemiological studies have demonstrated that the composition of phenol-rich food retards the progression of arteriosclerosis and reduces the incidence of heart diseases by preventing the oxidative stress, that is, lipid peroxidation in arterial macrophages and in lipoproteins [1, 2]. More recently, some authors reported that anthocyanins decreased cadmium accumulation in liver and kidney, the concentration of bilirubin and urea in blood serum, and aspartate aminotransferase and alanine aminotransferase activities [3].

Pomegranate juice is an important source of phenolic compounds, with anthocyanins being one of the most important, especially the 3-glucosides and 3, 5-diglucosides of delphinidin, cyanidin, and pelargonidin [4]. These components along with gallagyl-type tannins, ellagic acid derivatives, and other hydrolysable tannins could contribute in some way to the antioxidant activity of pomegranate juice [2].

"Assaria" pomegranate is a Portuguese variety cultivated in the southern region of the country. Its edible seeds are a favourite snack due to sweet taste and tenderness, and its fruits are mainly used for direct consumption. However, they could also be used for production of fruit juices or production of processed products such as jams, jellies, syrups, or carbonated beverages [5]. At present the damaged fruits, with cracks, cuts, or bruises in the husk, are discarded. Their application for the production of processed food products could improve the economic yield of this crop.

The composition of pomegranate juice depends on cultivar type, environmental and postharvest factors, and storage and processing factors [2, 6, 7, 8, 9, 10].

Although Assaria pomegranate is the main Portuguese variety, the composition of its juice is not yet well studied. The objective of our research is to evaluate the composition of the Assaria pomegranate juices obtained using two different extraction methods and their effect on the juice quality during storage over 72 hours at 4°C.

MATERIALS AND METHODS

Fruits and treatments

Sweet pomegranates (*Punica granatum* cv Assaria) were harvested in a commercial orchard in eastern Algarve. Fruits were transported on the same day to the laboratory at the University of Algarve. The damaged fruits were removed and the healthy fruits of uniform size and appearance were washed and randomly distributed into groups of 10 fruits for juice extraction.

To obtain juice, two extraction methods were applied. The first method consisted of manually peeling the fruits, separating the seeds, and extracting the juice by a Phillips Electric juice centrifuge. In the second method, fruits were cut in two halves and the juice was immediately extracted using a Phillips Electric lemon squeezer. Each extraction was replicated 4 times.

The obtained juices were immediately stored at 4°C in the dark. Samples were collected at 0, 5, 15, 32, 48, and 72 hours after extraction. At each sampling point, the juices were analysed for °Brix (which is a percentage by weight of sugar in a solution at room temperature), pH, titrable acidity, anthocyanins, sugars, and organic acids. The changes in colour were monitored according to the Munsell Colour Chart [11].

Standards and reagents

Delphinidin 3, 5-diglucoside (Dp3, 5), delphinidin 3-glucoside (Dp3), cyanidin 3, 5-diglucoside (Cy3, 5), cyanidin 3-glucoside (Cy3), pelargonidin 3, 5-diglucoside (Pg3, 5), and pelargonidin 3-glucoside (Pg3) standards were purchased from Apin Chemicals Ltd, UK. Methanol (HPLC gradient grade) was purchased from Sigma-Aldrich Quimica, SA (Spain). Formic, oxalic, tartaric, pyruvic, malic, ascorbic, maleic, citric, fumaric, and sulphuric acids glucose, and fructose were purchased from Riedel-de-Haën (Germany). The ultrapure water was purified with the MilliQ system, from Millipore, USA.

Titrable acidity, pH, and °Brix

Titrable acidity was calculated as percentage of citric acid by titrating 10 mL of the pomegranate juice with a solution of NaOH (0.1 N) till pH 8.1. The pH was measured by a pH meter (Crison micropH 2001—Crison Instruments, SA (Spain)). The level of sugars was measured as °Brix by a digital refractometer, model PRI-Atago Co LTD (Japan).

Anthocyanins

The juice sample (1 mL) was centrifuged for 2 minutes at 10 000 rpm and filtered through a 0.45 μm filter (Millipore). The identification of anthocyanins was performed by HPLC with a System Gold Programmable Detector Module 166-UV-Vis (Beckman Coulter, USA), using a LiChroCART 100 RP-18 column (25 cm×0.4 cm i.d.; 5 μm particle size; Merck (Germany)). The mobile phase was 5% formic acid (A) and methanol (B) in a linear gradient from 15% to 35% B at 15 minutes, followed by isocratic run until 20 minutes. The flow rate was 1 mL/min. Chromatograms were recorded at absorbance of 510 nm. The different anthocyanins were identified by comparison of their retention times with those of pure standards. The concentrations of anthocyanins were calculated from standard curves of Dp3, 5, Dp3, Cy3, 5, Cy3, Pg3, 5, and Pg3, at four concentrations (0.01, 0.02, 0.04, 0.08 mg/L). Injection volume was 20 μL using an injector with a 20 μL loop (Rheodyne, USA).

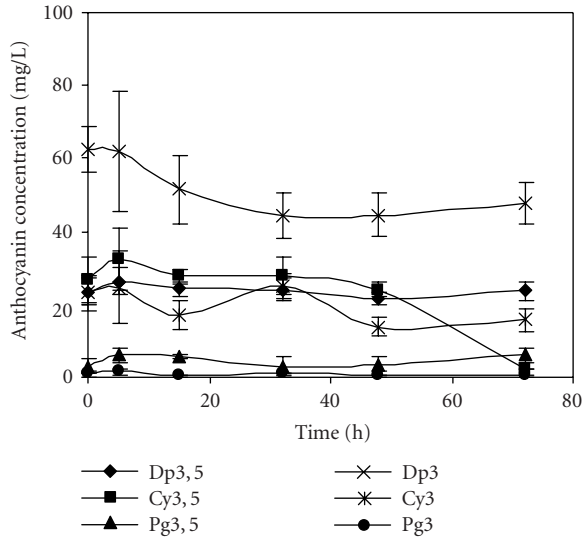

Figure 1. Evolution of concentration of anthocyanins present in the pomegranate juice obtained by seed centrifugation and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

Sugars and organic acids

To determine the content of sugars and organic acids in juice, samples (1 mL) were centrifuged for 20 minutes at 13 000 rpm and filtered through a 0.45 μm filter (Millipore). The composition of sugars and acids was detected with an HPLC (Beckman) equipped with a Jasco (Japan) refractive index (RI) 1530 detector. The column Polyspher OA HY (30 cm × 0.65 cm i.d.; 9 μm particle size) from Merck was used at 35°C. The mobile phase consisted of 0.0025 N H_2SO_4 applied at a flow rate of 0.4 mL/min. The injection volume was 20 μL using an injector with a 20 μL loop (Rheodyne). The different sugars and organic acids were identified by comparison of their retention times with those of pure standards. The concentrations of these compounds were calculated from standard curves of the respective sugars and organic acids.

RESULTS AND DISCUSSION

The levels of major anthocyanins detected in pomegranate juices obtained through two different extraction methods, centrifugation of seeds or squeezing of fruit halves with an electric lemon squeezer, and stored at 4°C over 72 hours are presented in Figures 1 and 2. No significant differences in the composition of anthocyanins were detected among the treatments. In both cases, the main anthocyanin was Dp3, followed by Dp3, 5, Cy3, 5, and Cy3. Pg3, 5 and Pg3 were present in the lowest amounts. The anthocyanins detected in our analysis of the Assaria pomegranate juices were as identified in other cultivars [12]; however, concentrations of the individual pigments differed.

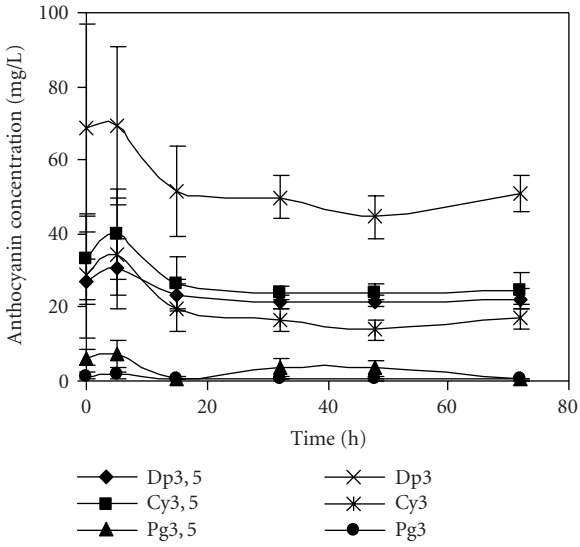

Figure 2. Evolution of concentration of anthocyanins present in the pomegranate juice obtained by squeezing of fruit halves with an electric lemon squeezer and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

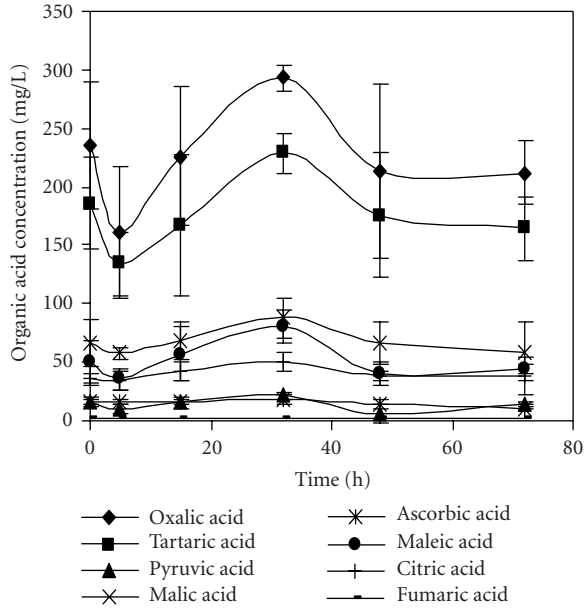

Figure 3. Evolution of concentration of organic acids present in the pomegranate juice obtained by seed centrifugation and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

Colour is one of the most important parameters when making a sensorial evaluation of food quality. No significant differences were observed between the colours of juices obtained through various extraction methods. At the extraction time, the Assaria juice colour was noted as 53A, according to the Munsell Colour Chart. The juice colour did not change during experimental time.

The bright colour of pomegranate fruit and juice is due to anthocyanins, so their stability through juice processing is of major importance. The anthocyanin content in both extraction methods was similar as was the respective evolution profile over time. During the first 5 hours of storage at 4°C, a slight increase (0.3%–4%) in the amounts of each anthocyanin was registered, followed by a decrease in the next ten hours and then stabilisation occurred. Additionally, the fruit juice obtained by centrifugation of seeds showed a pronounced decrease of Cy3, 5, mainly after 48 hours of storage (Figure 1). It was reported previously that in POM Wonderful pomegranate juices the diglucoside anthocyanins were more stable than the monoglucosides [8], therefore the high decrease of Cy3, 5 observed in our experiment was unexpected.

The main organic acids present in the pomegranate juices were oxalic and tartaric acids, either in juices obtained by seed centrifugation or by squeezing of fruit halves, respectively (Figures 3 and 4). These results were quite different from those reported by others [6, 13] for 11 Spanish pomegranate cultivars in which citric acid was the main organic acid, sometimes followed closely by malic acid. Oxalic and tartaric acids were present only in small amounts. In contrast, a great variation in the organic acid composition among the Turkish pomegranate cultivars has been reported [14]. Citric and malic acids were predominant in the majority of varieties, but in some cultivars large amounts of oxalic and tartaric acids were detected. In those varieties, only one had oxalic acid as the major organic acid.

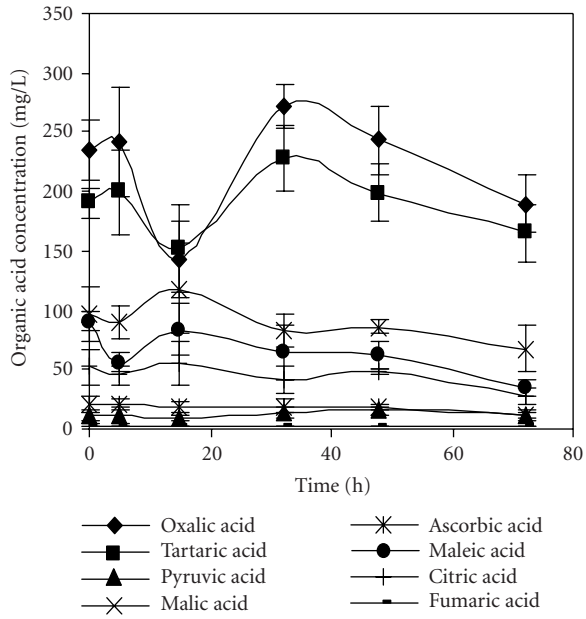

Figure 4. Evolution of concentration of organic acids present in the pomegranate juice obtained by squeezing of fruit halves with an electric lemon squeezer and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

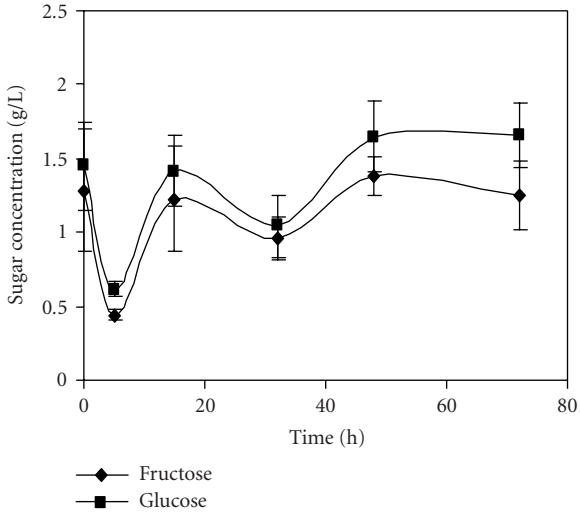

Figure 5. Evolution of sugar concentration in the pomegranate juice obtained by seed centrifugation and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

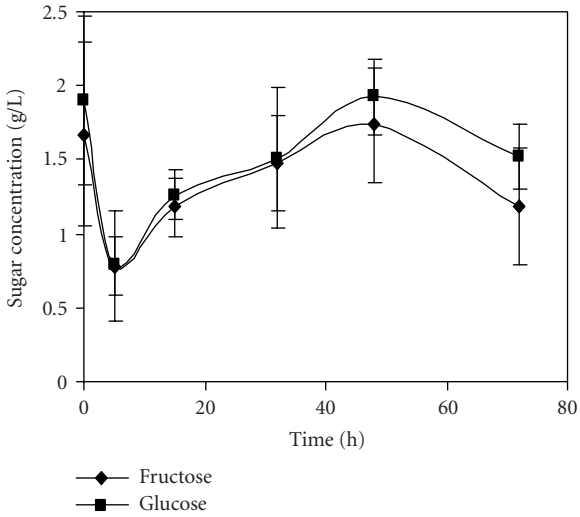

Figure 6. Evolution of sugar concentration in the pomegranate juice obtained by the squeezing of fruit halves with an electric lemon squeezer and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

The evolution profiles of organic acids over time in juices obtained through application of both methods were quite similar, with only a few exceptions. A decrease of each organic acid during the first 5–15 hours of cold storage was observed, followed by an increase reaching the maximal values after 32 hours of storage. At this point, the levels of oxalic acid were of 292.9 and 271.1 mg/L and those of tartaric acids were of 228.9 and 228.0 mg/L in juices obtained by seed centrifugation and squeezing of fruit halves, respectively. The exceptions reported above were malic, maleic, and citric acids in the samples obtained by squeezing the fruits, and ascorbic acid in those obtained through application of both methods (Figure 4). The concentrations of malic and citric acids reached the maximum after 15 hours of storage. The highest level of maleic acid was observed just after juice extraction. The ascorbic acid content remained quite stable over the whole storage period independently of the method application.

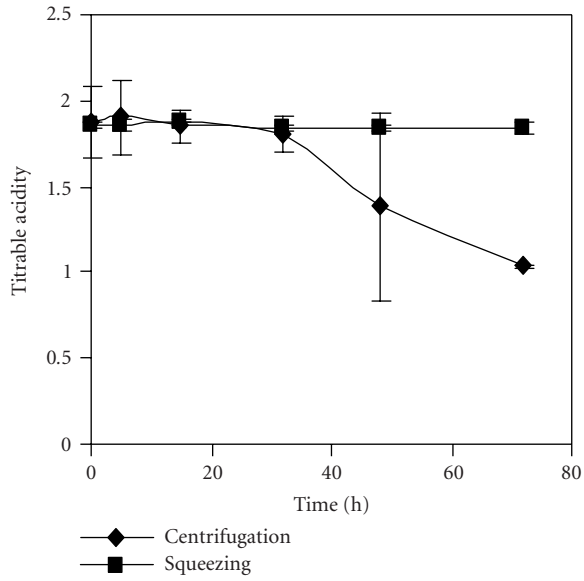

Figure 7. Evolution of titrable acidity as percentage of citric acid in the pomegranate juices obtained by centrifugation of seeds and by the squeezing of fruit halves with an electric lemon squeezer and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

The main carbohydrates detected in the pomegranate juices were glucose and fructose (Figures 5 and 6). This supports previously reported results for other pomegranate cultivars [6]. Only traces of sucrose were found and therefore sucrose was not considered in the present work. The amounts of glucose and fructose were quite similar in juices obtained through application of both methods. A pronounced decrease of total carbohydrate content of about 50% was observed in juices independently of the extraction method after 5 hours of storage at 4°C. It can be assumed that the decrease of sugar and organic acids content during the first 15 hours of storage occurs due to *de novo* synthesis of anthocyanins, whose level increased exactly at the same time in juices obtained through both methods.

Titrable acidity showed a clear decrease after 32 hours of storage when juice was obtained by centrifuging the seeds, that was less marked in the juice extracted by squeezing fruits (Figure 7). The level of sugars measured as °Brix changed over time but the main feature was the sharp decrease after 15 hours until the end of the experiment in the seed centrifugation procedure (Figure 8). This behaviour may be related to the different chemical composition of juices due to the presence of tannins as a result

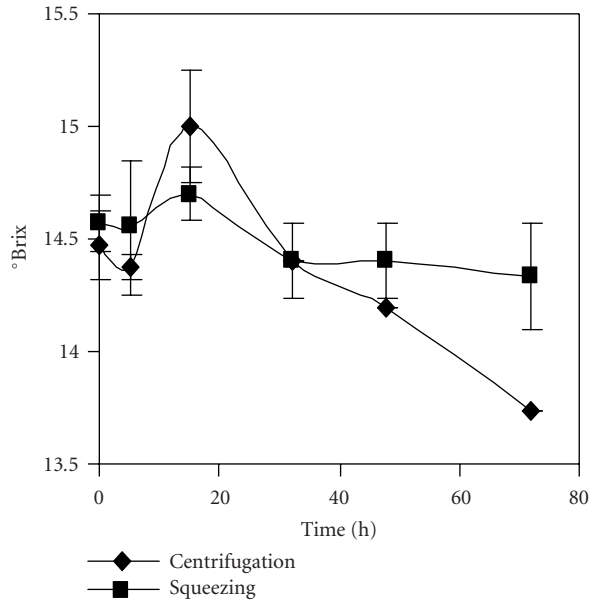

Figure 8. Evolution of °Brix of pomegranate juices obtained by centrifugation of seeds and by the squeezing of fruit halves with an electric lemon squeezer and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

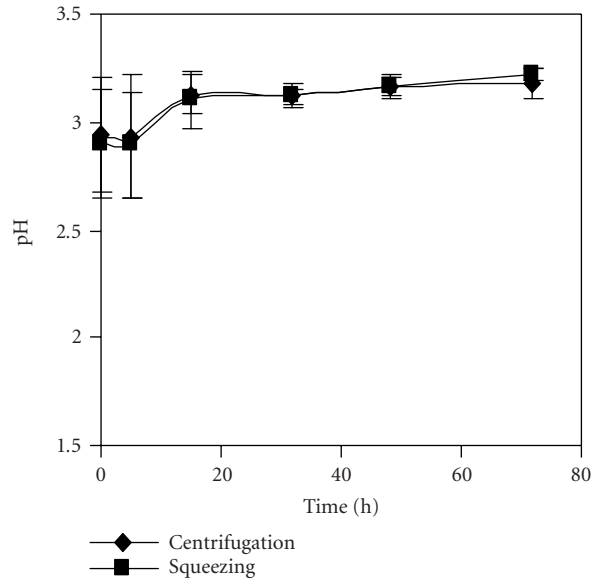

Figure 9. Evolution of pH of pomegranate juices obtained by centrifugation of seeds and by the squeezing of fruit halves with an electric lemon squeezer and stored for 72 hours at 4°C. Bars represent standard deviation of 4 replications.

of rind cell damage during fruit squeezing. The presence of tannins is the main problem when juices are extracted from whole fruits. As a result, a bitter taste develops that must be corrected by industrial processing [15]. In our experiment, according to panelist evaluation of the fresh juices, the juice obtained by fruit squeezing showed a bitter taste in comparison to the sweet taste of the juice obtained by centrifuging seeds.

The pH presented a slight increase over time, more pronounced from 5 to 15 hours after extraction (Figure 9). There were no differences in pH related with the method used for juice extraction. This could partially explain the relative stability of anthocyanins found in the juices obtained by both extraction methods.

Both methods used for pomegranate juice extraction did not affect the evaluated characteristics of juice quality; namely, the anthocyanins content, the juice colour, the organic acids and sugars composition, as well as the pH values. Squeezing unpeeled fruit halves is the most economical and easy method to use. The juice obtained by that method was more stable over time as indicated by the titrable acidity and °Brix determinations. The main disadvantage of the squeezing method is the production of juice with bitter taste if additional treatments are applied. Besides, the bitterness could be overcome because the actual trend in juice production is the blending of several fruit juices. Additionally, the use of Assaria pomegranate in fruit juice mixtures will be beneficial for human health in reducing the risk from oxalic acid consumption [16].

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CORRESPONDENCE ARTICLE

Anthocyanin Concentration of "Assaria" Pomegranate Fruits During Different Cold Storage Conditions

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The concentration of anthocyanins in fruits of "Assaria" pomegranate, a sweet Portuguese cultivar typically grown in Algarve (south Portugal), was monitored during storage under different conditions. The fruits were exposed to cold storage (5°C) after the following treatments: spraying with wax; spraying with 1.5% $CaCl_2$; spraying with wax and 1.5% $CaCl_2$; covering boxes with 25 μc thickness low-density polyethylene film. Untreated fruits were used as a control. The anthocyanin levels were quantified by either comparison with an external standard of cyanidin 3-rutinoside (based on the peak area) or individual calculation from the peak areas based on standard curves of each anthocyanin type. The storage time as well as the fruit treatment prior to storage influenced total anthocyanin content. The highest levels were observed at the end of the first month of storage, except for the fruits treated with $CaCl_2$, where the maximal values were achieved at the end of the second month. The anthocyanin quantification method influenced the final result. When total anthocyanin was calculated as a sum of individual pigments quantified based on standard curves of each anthocyanin type, lower values were obtained.

INTRODUCTION

Pomegranate has been considered a fruit tree species of minor importance, but the increased consumer demand for exotic fruits as well as for high nutritional quality foods opens new perspectives for consumption of that traditional species. Pomegranate seeds, the edible portion of the fruit, are rich in sugars, vitamins, polysaccharides, polyphenols, and minerals [1]. They have low oil content and are rich in polyunsaturated (n-3) fatty acids. The pomegranate seed extract possesses high antioxidant activity [2]. The antioxidant capacity of commercial pomegranate juice is three times higher than those of red wine and green tea [3]. The antioxidant qualities of pomegranate juice make it appealing for the production of health supplements and nutraceuticals [2].

The antioxidant activity was suggested to be related, in part, to the three major anthocyanidins found in pomegranate seed extract [4]. Therefore evaluation of the anthocyanin content in food products is very important. At present, various methods for anthocyanin quantification are used [5]. In pomegranate juice, anthocyanin content is determined usually according to the method developed by Gil et al [6]. Application of various methods may influence the final result. Besides the quantification method, the anthocyanin content also depends on factors like species, varieties, maturity index, seasonal conditions, processing type, or storage conditions of the food products [3, 5, 7]. Application of various anthocyanin extraction methods also influences the evaluation of antioxidant activities of extracts [3].

In the present paper, two quantification methods of anthocyanins as well as the influence of storage conditions on anthocyanin content in "Assaria" pomegranate juice were evaluated.

MATERIALS AND METHODS

Fruits and treatments

Sweet pomegranates (*Punica granatum* cultivator "Assaria") were harvested in an orchard in eastern Algarve (Portugal). Fruits were transported, on the same day, to the laboratory at the University of Algarve. After selection (diseased, bruised, and injured fruits were rejected), healthy fruits of uniform size and appearance were randomly distributed into alveolated boxes and stored in 4 modalities of fruit covering. The fruits were subjected to several treatments; treatment 1: control (no covering); treatment 2: wax coating by spraying fruits with Brillaqua wax emulsion (polyethylene wax (3.8%) (w/w), shellac (1.5%) (w/w), and wood resin (10%) (w/w), from Brillaqua, Valencia, Spain); treatment 3: covering boxes with a 25 μc low-density polyethylene film; treatment 4: 1.5% $CaCl_2$ fruit spraying; treatment 5: 1.5% $CaCl_2$ and Brillaqua wax emulsion (combination of treatments 2 and 4). The fruits were stored at 5°C.

At harvest and monthly, for 4 months, 10 fruits of each replication were removed and the concentration of anthocyanins was measured. For each sampling point, there were 4 replications.

Anthocyanins quantification

Pomegranates were manually peeled and the seeds liquefied by hand. The tegmina were discarded. The juice sample (1 mL) was centrifuged (2 minutes at 10 000 rpm) and filtered through a 0.45 μm filter.

The identification of anthocyanins was performed by HPLC with a detector UV-Vis Beckman 166 (USA), using a Li-Chrochart 100 RP-18 column (25 cm $\times$ 0.4 cm inner diameter; 5 μm particle size, Merck (USA)). The mobile phase was 5% formic acid (A) and methanol (B) in a linear gradient starting with 15% B to reach 35% B in 15 minutes, then isocratic until 20 minutes, at a flow rate of 1 mL/min. Chromatograms were recorded at the absorbance of 510 nm. Injection volume was 20 μL using an injector with a 20 μL loop (Rheodyne, Calif, USA).

Anthocyanins were identified by comparison of their retention times with those of pure standards.

To quantify total concentration of anthocyanins, two methods were used. Method 1: the concentration of anthocyanins was calculated from their peak areas in the chromatograms and compared with an external standard of cyanidin-3-rutinoside as previously reported [6]. Method 2: anthocyanins were identified and quantified individually based on standard curves of each anthocyanin type: delphinidin 3,5-diglucoside (Dp3,5), delphinidin 3-glucoside (Dp3), cyanidin 3,5-diglucoside (Cy3,5), cyanidin 3-glucoside (Cy3), pelargonidin 3,5-diglucoside (Pg3,5), and pelargonidin 3-glucoside (Pg3), at four concentrations (0.01, 0.02, 0.04, and 0.08 mg/L). Total amount of anthocyanin in the samples was calculated as the sum of the mean of individual pigments.

RESULTS

The total anthocyanin concentration in the "Assaria" pomegranate juice determined by two different quantitative methods, method 1 and method 2, monitored over 4 months of storage, is presented in Figures 1 and 2. Application of two quantification methods resulted in obtaining different results: the concentration of anthocyanins was always 2-fold higher when cyanidin 3-rutinoside, as external standard, was used. Both methods showed That during the first month of storage, an increase of the total anthocyanin level occurred in all treatments, reaching a maximal value at the end of the first month of storage with the exception of fruits treated with $CaCl_2$. The highest concentration was found in the fruits treated with wax, independent of the quantification method used (439.0 mg/L and 210.9 mg/L for methods 1 and 2, respectively). In the fruits treated with $CaCl_2$, the maximal concentration of anthocyanins was obtained only after two months of storage (307.2 and 155.4 mg/L, for methods 1 and 2, respectively). At the end of the first month of storage, these fruits showed the lowest levels of anthocyanins (236.5 and 113.3 mg/L, respectively).

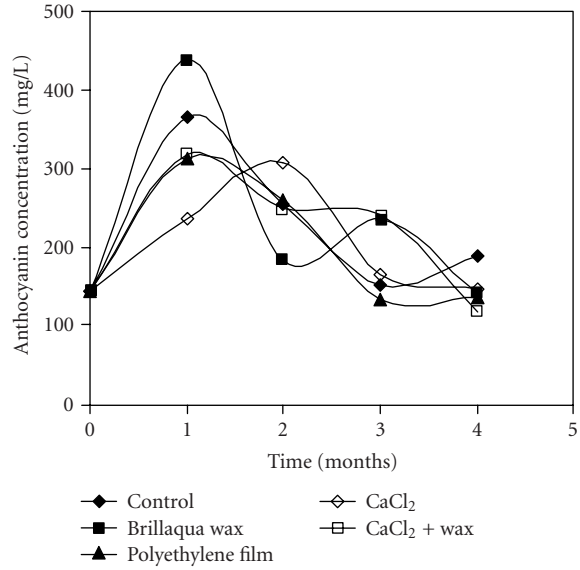

Figure 1. Evolution of total anthocyanin concentration in juice of the "Assaria" pomegranate fruits, during storage at 5°C, quantified by comparison with an external standard of cyanidin 3-rutinoside (Apin Chemicals, UK). Total amount of anthocyanins in the samples was calculated as the sum of the mean of individual pigments.

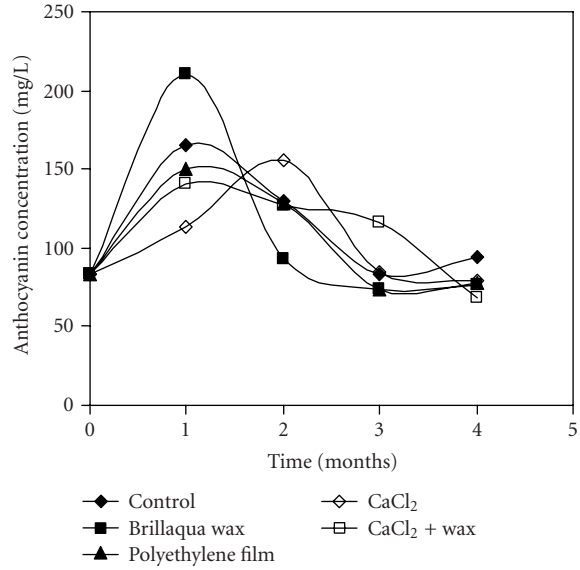

Figure 2. Evolution of total anthocyanin concentration in juice of the "Assaria" pomegranate fruits, during storage at 5°C, calculated from standard curves of Dp3,5; Dp3; Cy3,5; Cy3, Pg3,5, and Pg3. Total amount of anthocyanins in the samples was calculated as the sum of the mean of individual pigments.

During the second, third, and fourth months of storage in all treatments, with the exception of $CaCl_2$ treatment, significant decrease in anthocyanin levels was observed. The same tendency was observed with the $CaCl_2$ treatment in the third and fourth months of storage. Nevertheless, the anthocyanin amount still remained above the initial values in all treatments. The greatest decrease was observed in the wax treatment.

Six anthocyanins were detected in the "Assaria" pomegranate juice: delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), pelargonidin 3-glucoside (Pg3), delphinidin 3,5-diglucoside (Dp3,5), cyanidin 3,5-diglucoside (Cy3,5), and pelargonidin 3,5-diglucoside (Pg3,5). Their relative amounts were different among treatments.

Figures 3a and 3b represent the time course of concentration of each anthocyanin in the control fruits, using the quantitative methods 1 and 2, respectively. Application of both methods showed that the levels of Pg3 and Pg3,5 were very low in comparison to the remaining anthocyanins. The quantification method influenced identification of the major anthocyanins. Thus, when the levels of anthocyanins were calculated based on cyanidin-3-rutinoside as an external standard, Dp3,5 was the major anthocyanin present in samples (Figure 3a). When the quantification was obtained from standard curves of each pigment, Dp3 was the dominating anthocyanin (Figure 3b). In both methods, the same evolution profile of anthocyanins was observed: higher accumulation of anthocyanins during the first storage month followed by their decrease in the following months.

In order to make the results easier to interpret, hereafter the quantification of anthocyanins is made comparing the peak areas of each anthocyanin with those obtained from calibration curves of each standard stock solution (method 2). This methodology is the most adequate because the standards' composition was close to that of the samples, as required in analytical chemistry.

As mentioned earlier, the fruits treated with wax showed the greatest accumulation of anthocyanins after one month of storage (Figure 4). Among the anthocyanins monitored, Dp3 was the major pigment. The maximal value reached after the first month of storage was 81.7 mg/L, and was superior to the control with 66.5 mg/L (Figure 3b).

The polyethylene film treatment also induced a great accumulation of Dp3 (58.0 mg/L) during the first month of storage (Figure 5). Contrary to the significant changes in the levels of Dp3,5, Cy3,5, and Cy3, the variations of the Cy3,5 level over time were lower.

The fruits treated with $CaCl_2$ showed the lowest amounts of anthocyanins (Figure 6). As mentioned earlier, the highest concentration was detected at the end of the second month of storage. Similarly to the previous treatments, the major anthocyanin was Dp3. The highest concentration of this pigment, observed at the end of the second month of storage, was 50.5 mg/L.

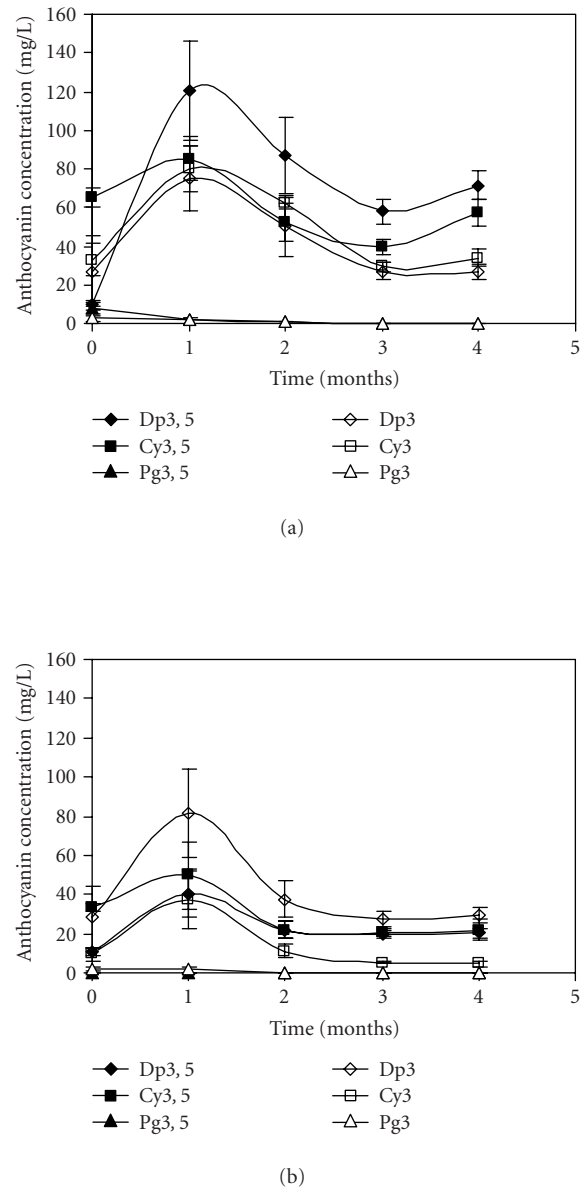

Figure 3. Evolution of delphinidin 3,5-diglucoside (Dp3,5), cyanidin 3,5-diglucoside (Cy3,5), pelargonidin 3,5-diglucoside (Pg3,5), delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) concentration in "Assaria" pomegranate juice from control fruits, during storage at 5°C. (a) Individual anthocyanins were quantified by comparison with an external standard of cyanidin 3-rutinoside (Apin Chemicals). (b) The concentrations of anthocyanins were calculated from standard curves of Dp3,5; Dp3; Cy3,5; Cy3, Pg3,5, and Pg3. Bars represent standard deviations of four replicates.

Contrary to the treatments described above in which Dp3 was the major pigment after one month of storage, two main pigments were detected in the fruits treated with $CaCl_2$ plus wax: Cy3,5 (46.7 mg/L) and Dp3 (42.8 mg/L) (Figure 7).

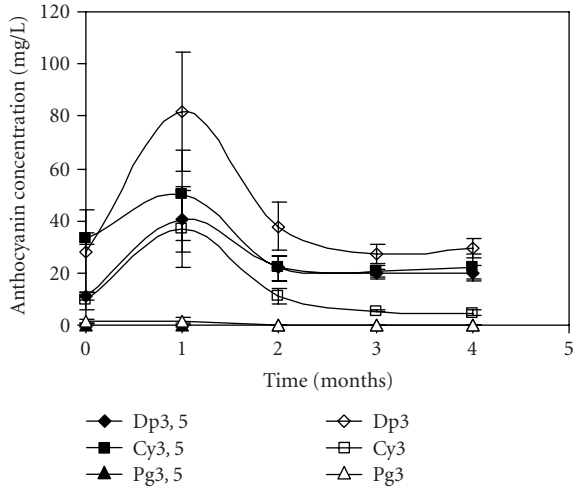

Figure 4. Evolution of delphinidin 3, 5-diglucoside (Dp3, 5), cyanidin 3, 5-diglucoside (Cy3, 5), pelargonidin 3, 5-diglucoside (Pg3, 5), delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) concentration in "Assaria" pomegranate juice from fruits treated with Brillaqua wax, during storage at 5°C. The concentrations of anthocyanins were calculated from standard curves of Dp3, 5; Dp3; Cy3, 5; Cy3, Pg3, 5, and Pg3. Bars represent standard deviations of four replicates.

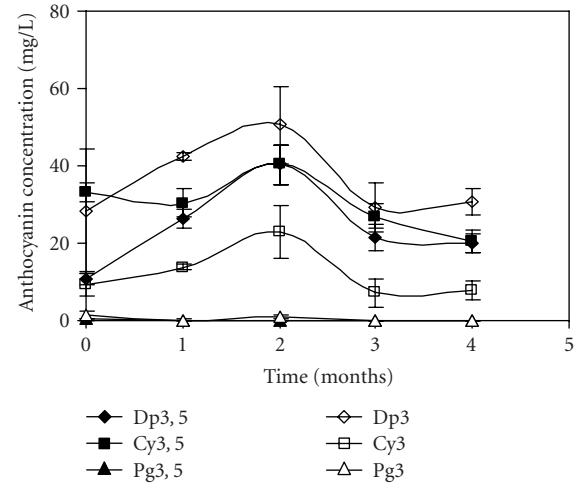

Figure 6. Evolution of delphinidin 3, 5-diglucoside (Dp3, 5), cyanidin 3, 5-diglucoside (Cy3, 5), pelargonidin 3, 5-diglucoside (Pg3, 5), delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) concentration in "Assaria" pomegranate juice from fruits treated with 1.5% $CaCl_2$, during storage at 5°C. The concentrations of anthocyanins were calculated from standard curves of Dp3, 5; Dp3; Cy3, 5; Cy3, Pg3, 5, and Pg3. Bars represent standard deviations of four replicates.

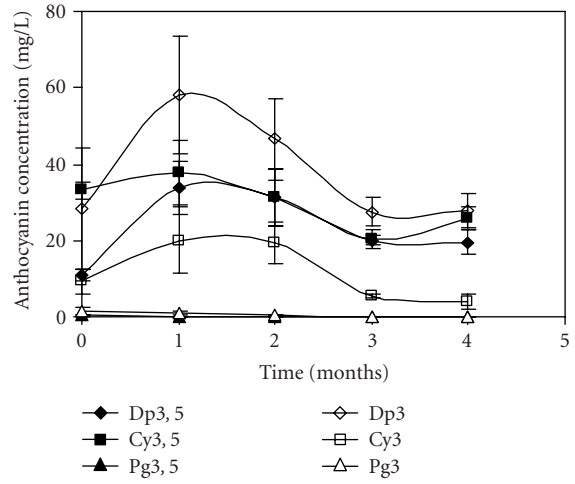

Figure 5. Evolution of delphinidin 3, 5-diglucoside (Dp3, 5), cyanidin 3, 5-diglucoside (Cy3, 5), pelargonidin 3, 5-diglucoside (Pg3, 5), delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) concentration in "Assaria" pomegranate juice from fruits covered with polyethylene film, during storage at 5°C. The concentrations of anthocyanins were calculated from standard curves of Dp3, 5; Dp3; Cy3, 5; Cy3, Pg3, 5, and Pg3. Bars represent standard deviations of four replicates.

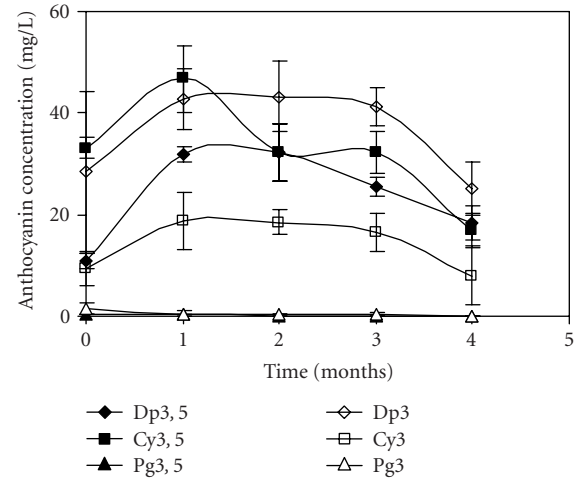

Figure 7. Evolution of delphinidin 3, 5-diglucoside (Dp3, 5), cyanidin 3, 5-diglucoside (Cy3, 5), pelargonidin 3, 5-diglucoside (Pg3, 5), delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) concentration in "Assaria" pomegranate juice from fruits treated with 1.5% $CaCl_2$ plus Brillaqua wax, during storage at 5°C. The concentrations of anthocyanins were calculated from standard curves of Dp3, 5; Dp3; Cy3, 5; Cy3, Pg3, 5, and Pg3. Bars represent standard deviations of four replicates.

DISCUSSION

The anthocyanin profiles of some food products derived from red fruits are used to verify the authenticity and control the quality of these products [5]. Nevertheless, a great number of the laboratories that perform such analyses use their own methods. This fact renders the comparison and validation of these methods difficult. The most advisable method for quantitative chromatographic analysis involves the preparation of a series

of standard solutions that approximate the composition of the unknown sample. This does not happen when cyanidin-3-rutinoside is used in the method that is frequently used by some authors for anthocyanin quantification in pomegranate juices [6]. In the present paper, it was considered that applications of the standard solutions of each anthocyanin were more appropriate as the samples than the standard solution of cyanidin-3-rutinoside. Therefore, the results were obtained comparing the peak areas with those obtained from the standard solutions of each anthocyanin, maintaining the same assay conditions.

The anthocyanins present in pomegranate seeds of cultivator "Assaria" were as those previously isolated and identified for cultivator "Mollar" [6]. However, their amounts were different. A great variation of the amounts of Dp3, 5, Dp3, and Cy3 was registered over time, regardless of whether the levels of Cy3, 5, were more stable. The variation was more evident between the harvesting time and the first month of storage, when a great increase of anthocyanin level was monitored, followed by decrease until the end of storage. Previously, it was reported that Dp3, 5 and Dp3 were the best substrates to undergo enzymatic oxidation for pomegranate cultivar "Mollar" [8]. This could partly explain the important decrease that was registered in the amounts of these anthocyanins in the "Assaria" pomegranate juice.

The increase in the total amount of anthocyanins during the first month of storage may be due to the continued biosynthesis of phenolic compounds after harvest, related to the ripening processes. The increase of anthocyanin concentration after harvest was reported previously in pomegranates [6, 7] and that was correlated with the activity of the enzymes of the anthocyanin biosynthetic pathway: phenylalanine ammonia lyase (PAL) and UDP-glucose: flavonoid-3-O-glucosyltransferase (GT). Nevertheless, in juice of pomegranates stored in different atmospheres, Holcroft et al [7] observed that the increase in the total amount of anthocyanins was correlated with PAL activity but not with GT activity.

The pattern of anthocyanin content variation was distinct among the treatments during storage period. The greatest value was observed in wax treatment at the end of the first month but the fruits of that treatment presented the lowest content a month later. In contrast, the fruits treated with $CaCl_2$ that presented the greatest value in the second month had the lowest content in the first month. Therefore, using the appropriate storage treatment, it is possible to have fruits with high anthocyanin content until two months after harvest.

The effect of the different treatments could be related to changes in the fruit internal atmosphere. Holcroft et al [7] showed that in juice of pomegranates stored in air enriched with CO_2, the anthocyanin concentration increased in time in both air-stored fruits and fruits stored in 10 kPa CO_2, but remained stable for four weeks and subsequently decreased in fruits stored in 20 kPa CO_2. In our work, the treatments used affected distinct biochemical mechanisms that could modify anthocyanin stability in different ways, making the treatment effect difficult to interpret.

Besides the storage treatments being very important for maintaining the external appearance, delaying senescence, and controlling decay of pomegranates, they can also contribute to increasing anthocyanin amounts during the first month of storage.

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CORRESPONDENCE ARTICLE

Urinary Excretion of Cyanidin Glucosides and Glucuronides in Healthy Humans After Elderberry Juice Ingestion

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In a pilot study with 6 females and 1 male, the metabolism of various cyanidin glucosides after oral administration of elderberry juice was investigated. The anthocyanin metabolites were detected in urinary excretion. After ingestion of a bolus quantity of 3.57 g total anthocyanins in a 150 mL elderberry juice concentrate, 0.053% of the administered dose was excreted in urine as glucosidically bound cyanidins within the first 5 hours. Only 0.003% of the ingested anthocyanin glucosides was excreted as cyanidin glucuronide, suggesting that this conversion step might be of minor importance in urinary excretion.

INTRODUCTION

Anthocyanins, as ubiquitous constituents of berries and coloured vegetables, are widespread in the plant dominion. Much attention has been paid in recent years to their radicals scavenging activity in vitro and in vivo and possible health benefits. The main anthocyanins being analysed in elderberries are various glucosylated cyanidins (Figure 1). Besides glucose, sambubiose is the typical sugar conjugate in elderberries [1, 2]. Rate and extent of absorption, metabolisation, and excretion of elderberry anthocyanins are to date not fully understood. First of all, controversially discussed hitherto is whether also conjugated glucuronides of cyanidin are formed and excreted in vivo.

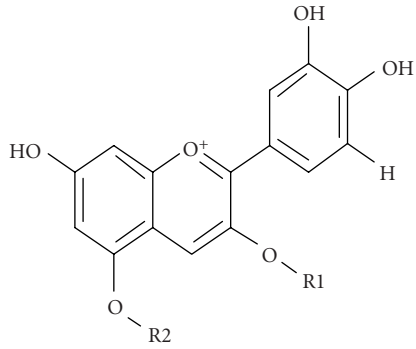
STUDY DESIGN

The study protocol was approved by the Ethics Committee of the Giessen University, Germany. After an overnight fasting, seven healthy nonsmoking volunteers (6 female and 1 male with a mean BMI of 21.5) consumed a bolus quantity of 150 mL of concentrated elderberry juice (containing 3.57 g of total anthocyanins) together with rolls and cheese. The elderberry juice concentrate was obtained from Wild (Heidelberg, Germany). Urine samples were collected initially and in hourly intervals over a period of 5 hours. The concentration of the excreted cyanidin conjugates was determined by HPLC analysis.

SAMPLE EXTRACTION AND HPLC ANALYSIS

All chemicals were purchased from Merck (Darmstadt, Germany). HPLC-DAD analyses of urinary samples (0–5 hours after ingestion) were performed before and after hydrolysis of glucuronide conjugates with β-glucuronidase [3, 4, 5, 6]. The anthocyanins were extracted by using a solid phase extraction cartridge (Sep-PakVac 12, Waters, Milford, Mass). The cartridge was first washed with 5 mL methanol and equilibrated with the same volume of 5% aqueous formic acid. Seven mL urine samples diluted with 2 mL formic acid were applied to the equilibrated cartridge. After washing with 5 mL of 5% formic acid, the anthocyanins were eluted with 5% formic acid in 5 mL methanol. The eluate was evaporated to dryness in a vacuum rotary evaporator at 30°C and the extract redissolved with 200 μL mobile phase before HPLC analysis. The chromatographic conditions adopted from Netzel et al [3] comprised separation on a Prontosil Eurobond RP-18 (5 μm, 250 × 4 mm ID, Bischoff, Leonberg, Germany), protected by a guard column (LiChrospher 100 RP-18, 4×4 mm, Merck, Germany), and isocratic elution with water/formic acid/acetonitril (v/v/v) = 81/10/9 (flow rate 0.5 mL/min) by using a high-precision pump (model L-6200, Merck-Hitachi, Darmstadt, Germany). The cyanidin glucosides were detected at 520 nm with the aid of a UV-VIS detector (L-4200, Merck-Hitachi).

Cyanidin glucosides excreted were identified by spiking blank urine samples with authentic compounds and comparing the retention time in the HPLC analysis and



Anthocyanins	R1	R2
Cyanidin 3-sam-5-gluc	Sambubiose	Glucose
Cyanidin 3, 5-digluc	Glucose	Glucose
Cyanidin 3-sam	Sambubiose	H
Cyanidin 3-gluc	Glucose	H

Figure 1. Chemical structures of elderberry anthocyanins.

Table 1. Urinary excretion of anthocyanins as unchanged glucosides and glucuronides after elderberry juice consumption (means ± SD).

Anthocyanins	Doses	Total excretion (glycosides + glucuronides)		Glucuronide excretion	
	(mg/subject)	(mg/5 h)	(%)[2]	(mg/5 h)	(%)[2]
cya-3, 5-digluc[1]	215.0	0.313 ± 0.227	0.145 ± 0.105	0.032 ± 0.021	0.015 ± 0.010
cya-3-sam	2245.8	0.962 ± 0.521	0.043 ± 0.023	0.045 ± 0.024	0.002 ± 0.001
cya-3-gluc	1108.2	0.601 ± 0.321	0.054 ± 0.029	0.038 ± 0.025	0.003 ± 0.002
sum	3569.0	1.876 ± 1.063	0.053 ± 0.030	0.116 ± 0.049	0.003 ± 0.001

[1]Amounts of cya-3-sam-5-gluc and cya-3, 5-digluc were calculated as cya-3, 5-digluc.

[2]Calculated as the ratio of amounts excreted (within 5 h) to amounts/doses ingested.

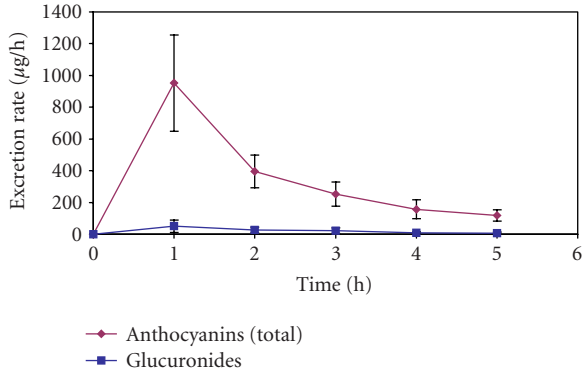


Figure 2. Time-course plots (mean ± SD) of total anthocyanins (glucosides + glucuronides) and anthocyanin glucuronides in human urine following ingestion of 150 mL of elderberry juice.

UV-visible spectra. Standard curves were prepared for quantification prior to the preparation procedure. The detection limit (S/N ≥ 10) was between 0.13 ng and 0.60 ng/injection volume (100 μL). Aliquots of each urine sample were acidified with formic acid (2 mL/0.2 mL) and stored frozen at −80°C until analysis.

RESULTS AND DISCUSSION

The analysed anthocyanin conjugates in urine are shown in Table 1. The urinary excretion of total anthocyanins (unchanged cyanidin glucosides and their glucuronide conjugates) within 5 hours was 0.053 ± 0.030% of the administered dose. Only 6.2 ± 2.2% of the excreted amount consisted of glucuronides. Based on this recovery, the percentage of glucuronides in urinary excretion was 0.003±0.001% (calculated as the ratio of anthocyanin glucuronides excreted to anthocyanin glucosides ingested). The excretion pattern of total anthocyanins in Figure 2 demonstrates that a maximal excretion rate is reached 1 hour after intake, followed by a rapid drop to initial values about 5 hours after intake, resembling a first-order excretion kinetics.

The metabolic conversion of anthocyanins within the organism remains to be elucidated. In principle, glucuronidation or conjugation with sulphuric acid is a common final metabolic step to facilitate urinary excretion. Various metabolic conversion products of anthocyanins were found by several authors. Tsuda et al [7] found no cyanidin glucuronides in livers and kidneys of rats after oral administration of cyanidin 3-glycoside, but cyanidin was converted to peonidin and protocatechuic acid.

A different situation seems to exist to date in man. In the urinary excretion of elderly women, minor amounts of glucuronides of peonidin and cyanidin 3-glucoside could be deteced in half of the volunteers besides unchanged glucosides after elderberry consumption, which does correspond to our results [2, 5]. Only glucosides of elderberry anthocyanins, however, could be found in plasma and urine of volunteers by other authors [8, 9].

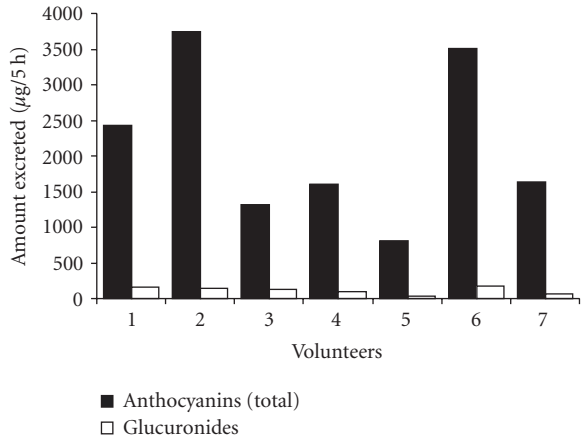


FIGURE 3. Individual amounts excreted of total anthocyanins (glucosides + glucuronides) and anthocyanin glucuronides within 5 hours following ingestion of 150 mL of elderberry juice.

To entirely estimate the extent of glucuronidation of anthocyanins as metabolic fate, it has to be considered that besides urinary excretion, biliary secretion may also serve as a possible way of elimination, particularly known for glucuronides. Newer studies, however, revealed that after intake of elderberry extract, the identical pattern of glucosylated cyanidins could be detected in plasma as in urine [10]. Thus, the results of the quantification of excretion products in the present study demonstrate that, at least in the given dose range, glucuronidation of cyanidin obviously represents a negligible conversion step in the metabolism of cyanidin ingested from elderberries. The proportion of glucuronide conjugates seems to represent a rather constant but very small proportion despite interindividual differences of total anthocyanin excretion (Figure 3). This may be in contrast to other fruits such as strawberry anthocyanins, being predominantly excreted in urine as glucuronides besides small amounts of sulfoconjugates [11]. It remains to show the extent to which the administered dose level does determine the site of metabolism. There exists some body of evidence that large doses of polyphenols are primarily metabolised in the liver, whereas small doses may be metabolised by the intestinal mucosa, with the liver playing a secondary role to further modify the polyphenol conjugates [12].

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