JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Detection of Anthocyanins/Anthocyanidins in Animal Tissues

Farrukh Aqil,[†,‡] Manicka V. Vadhanam,[†] Jeyaprakash Jeyabalan,[†] Jian Cai,[‡] Inder P. Singh,[§] and Ramesh C. Gupta*[,†,#]

[†]James Graham Brown Cancer Center, [‡]Department of Medicine, and [#]Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky 40202, United States

[§]Department of Natural Products, National Institute of Pharmaceutical Education and Research , S. A. S. Nagar, Punjab, India

Supporting Information

ABSTRACT: Dietary polyphenols may contribute to the prevention of several degenerative diseases, including cancer. Anthocyanins have been shown to possess potential anticancer activity. The aim of this study was to determine anthocyanin bioavailability in lung tissue of mice fed a blueberry diet (5% w/w) for 10 days or a bolus dose (10 mg/mouse; po) of a native mixture of bilberry anthocyanidins. All five anthocyanidins present in the blueberry were detected in the lung tissue using improved methods. The effect of various solvents on the stability of anthocyanins and their recovery from the biomatrix was analyzed. Detection of anthocyanins and their metabolites was performed by UPLC and LC-MS. Although anthocyanins were not detected, cyanidin was detected by UPLC-PDA and other anthocyanidins were detected by LC-MS, following conversion to anthocyanidins and selective extraction in isoamyl alcohol. The results show that anthocyanins can be detected in lung tissue of blueberry-fed mice and thus are bioavailable beyond the gastrointestinal tract.

KEYWORDS: *anthocyanins, anthocyanidins, bioavailability, lung tissue, blueberry diet*

INTRODUCTION

Berries are gaining increased attention lately for their chemopreventive and therapeutic potential against several cancers.[1,2] Blueberry contains an abundance and distinct spectrum of anthocyanins, namely, the glycosides of cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pe), and malvidin (Mv). A compelling body of literature suggests berry phytochemicals, including anthocyanins, have multifunctional chemopreventive and therapeutic effects, including anti-inflammatory,[3] radiation protection,[4] and antioxidant.[5] Anthocyanins, which comprise the largest group of water-soluble pigments, are widely distributed in dark-colored fruits, vegetables, grains, and flowers and are responsible for their red, purple, and blue hues. Anthocyanins from different plant sources, including blueberry, have been shown to possess potential anticancer activities.[6−10]

Several studies have shown that anthocyanins can inhibit cellular growth, induce apoptosis, and kill cancer cells in vitro.[3] Animal studies, although limited, have also demonstrated the chemopreventive potential of berries and their bioactive constituents such as anthocyanins and ellagitannins. The protective effects of these bioactives could be related to their potent antioxidant activity, as demonstrated in various in vitro and in vivo studies,[5,11,12] among other effects. In our previous studies, we demonstrated the chemopreventive potential of blueberry against breast cancer using the ACI rat model[8,13] and the therapeutic potential of blueberry anthocyanidins against lung cancer using the nude mouse xenograft model.[3] In the latter study, we also demonstrated that a mixture of anthocyanidins exhibited synergistic therapeutic activity compared with individual entities, both in vitro and in vivo.[3] The synergistic effects in this study presumably resulted from the effect of the anthocyanidins on some distinct and overlapping protein targets associated with cell proliferation, apoptosis, inflammation, invasion, and metastasis.[3]

Despite reports of berry anthocyanins' protection against cancers and other diseases,[6−10] a significant gap exists between what was shown in many in vitro studies and what can be achieved in vivo. Several studies were conducted to evaluate the bioavailability/pharmacokinetics in blood and tissue using high doses of individual anthocyanins.[14] Extraction of anthocyanins or their metabolites from blood generally relies upon solid phases such as Water's Oasis HLB or C18 Sep-Pak cartridges. However, tissue bioavailability data are scarce because there are few standardized methods for extractions from tissue. Moreover, the stability of anthocyanins and anthocyanidins during the workup has always been a concern.

Given these scenarios and multiple biological effects, anthocyanin/anthocyanidin bioavailability in non-gastrointestinal (GI) tissues is considered an important issue and needs to be demonstrated. Studies on the bioavailability of anthocyanins from a single berry can provide direct and valuable information about their absorption. Our unpublished data show 30−35% reduction of cigarette smoke-induced lung tumor in A/J mice by 2.5% w/w dietary berries. We have also demonstrated that anthocyanidins delivered intraperitoneally have antitumor activity against lung cancer xenograft.[3] Hence, this study was designed to evaluate and compare stability parameters of anthocyanidins and develop a sensitive method to assess the

Special Issue: 2013 Berry Health Benefits Symposium

Received: October 13, 2013
Revised: March 15, 2014
Accepted: March 21, 2014
Published: March 21, 2014

bioavailability of anthocyanins/anthocyanidins and their metabolites in lung tissues.

MATERIALS AND METHODS

Chemicals. HPLC grade water, acetonitrile, methanol, and other HPLC solvents, hydrochloric acid (HCl), formic acid, acetic acid, and trifluoroacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Authentic anthocyanin standards were obtained from Chromadex (Irvine, CA, USA). All other chemicals used in the study were of analytical grade. Freeze-dried highbush blueberry powder (50:50 blend of Tifblue and Rubel) was received from the U.S. Highbush Blueberry Council (Folsom, CA, USA). The native mixture of anthocyanidins (~95% pure) was isolated in our laboratory from 36% anthocyanin-enriched bilberry extract (Indena, Seattle, WA, USA), which contains five major anthocyanidins, delphinidin, cyanidin, malvidin, peonidin, and petunidin in the ratio of 33:28:16:16:7, as described in our previous study.[3]

Extraction and Isolation of Purified Anthocyanins/Anthocyanidins from Bilberry. Extraction, enrichment, and hydrolysis of the bilberry were carried out using essentially the same method as described previously.[15] Briefly, the enriched bilberry extract powder was extracted with 75% aqueous ethanol containing 0.1% HCl and enriched by loading the concentrated extracts on an XAD-761/Diaion HP-20 (1:1) column. The polyphenols, including anthocyanins, were eluted with methanol. Pooled elutes were concentrated and hydrolyzed with 2 N HCl (~5 volumes). Hydrolysates were purified using C18 Sep-Pak cartridges (Waters, Milford, MA, USA). Anthocyanidins and other polyphenols were eluted with acidified (0.01% HCl) methanol. The enriched extracts were dried under reduced pressure using a Savant Speed-Vac (Thermo Scientific, USA) and stored at −20 °C until use. The enriched extracts were dissolved in acidified water, and anthocyanidins were selectively extracted in isoamyl alcohol and dried under vacuum.[16] The extracted anthocyanidins were further purified by loading on the C18 cartridges per the manufacturer's guidelines.

Diet. AIN-93 M diet supplemented with blueberry powder at 5% (w/w) was prepared in pellet form by Harlan-Teklad (Madison, WI, USA) and stored at 4 °C in the dark in vacuum-sealed bags until use.

Animal Study. Animal experiments were performed in agreement with an approved protocol from the Institutional Animal Care and Use Committee of the University of Louisville. Female athymic nude mice (5−6 weeks old) were purchased from Harlan Sprague−Dawley, Inc. (Indianapolis, IN, USA). Two animal studies were conducted to test different routes of administration. In study 1, after a week of acclimatization, animals were randomized into two groups ($n = 4$) and provided either AIN-93 M diet or diet supplemented with 5% blueberry powder (w/w). Animals received diet and water ad libitum. The diet was changed every other day, and the food intake was monitored. No difference was found in food consumption in control and experimental groups. The animals had free access to food and water until euthanasia. After 10 days of treatment, animals were euthanized by CO_2 asphyxiation, and lung was collected, snap frozen, and stored at −80 °C until use. Blood was collected by cardiac puncture; plasma was separated and stored at −80 °C.

In study 2, animals were treated with a bolus dose of the native mixture of anthocyanidins isolated from bilberry (10 mg/mouse) by gavage in 10% dimethyl sulfoxide. Two hours after the treatment, animals were euthanized; lung tissue was collected, snap frozen, and stored at −80 °C until use. Blood was collected by cardiac puncture; plasma was separated and stored at −80 °C.

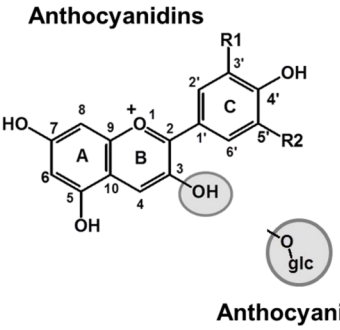
Effect of Acids/Solvents on Anthocyanidin Stability. To test the stability of anthocyanidins in various solvents, cyanidin chloride (100 μg/mL) was dissolved in (i) acetonitrile, (ii) methanol, and (iii) methanol acidified with 0.1% HCl. The solutions were immediately analyzed by ultraperformance liquid chromatography (UPLC). Samples maintained at room temperature were analyzed at 1 h intervals for 5 h. The concentration was plotted against percent of cyanidin chloride at initial time point. Because methanol provided complete stability as described under Results, all of the analyses from tissue were done by using methanol containing 0.1% HCl.

Recovery of Anthocyanins from Biological Matrix. Anthocyanins in biological samples (see below) were analyzed at anthocyanidin level. Conversion of anthocyanins to anthocyanidins in the presence of biological matrix was optimized as follows: To 500 μL of plasma was added 100 μL of bilberry extract (1 mg/mL), the mixture was acidified with 0.1% formic acid, acetic acid, trifluoroacetic acid, phosphoric acid, or hydrochloric acid. Samples were incubated for 15 min at 37 °C and then extracted with 5 volumes of acetonitrile. The sample was centrifuged at 10000*g*, and the supernatant was collected and evaporated to dryness under vacuum (Savant Speed-Vac). The dried extracts were dissolved in acidified (0.1% HCl) methanol and analyzed by UPLC.

Extraction of Anthocyanidins from Tissues. Parameters such as the stability of anthocyanidins in different buffers and acid environment, extraction efficiency, and selectivity in different solvents, etc., were established before tissue extractions. First, we analyzed the recovery of anthocyanidins by spiking lung tissues collected from untreated rats from another study. Methods described previously[14] were used to detect berry anthocyanins/anthocyanidins,[14] except for the following modifications: extraction of anthocyanins in acidified (0.1% HCl) acetonitrile, evaporation of solvent, and reconstitution in 50% methanol containing 2 N HCl followed by acid hydrolysis and selective extraction of anthocyanidins in isoamyl alcohol without any use of solid−liquid chromatography. Furthermore, UPLC separation method was developed by identifying solvents to separate reference anthocyanidins and protocatechuic acid (PCA), a bioactive metabolite of Cy,[17] and spiking the tissue homogenate with Dp, Cy, and PCA.[14] Briefly, after the optimization, lung tissue from two mice was pooled (two pools per group) in both animal studies and homogenized in 400 μL of 1.15% KCl; anthocyanins were extracted in acetonitrile containing 0.1% HCl as described above. The supernatant was evaporated and reconstituted in 50% methanol containing 2 N HCl. The extract was then hydrolyzed (100 °C/1 h) to convert anthocyanins to anthocyanidins, and the latter were selectively extracted in isoamyl alcohol.[16] Finally, the samples were dried under reduced pressure (Savant Speed-Vac) and reconstituted in 40 μL of acidified (0.1% HCl) methanol just before injection, and 10 μL was analyzed by UPLC. The limits of detection for the various anthocyanidins (Cy, Dp, Pt, Pe, and Mv) (0.3−0.75 ng) and PCA (0.2 ng) were established.

UPLC Analysis. Anthocyanins and anthocyanidins were analyzed on a Shimadzu UPLC system composed of two LC-20AD-XR pumps, an SIL-20A-XR autosampler, and an SPD-M20A photodiode array detector (PDA) controlled by Class VP software (ver 7.4, SP3) attached to a Shim-pack XR-ODS-II column (3.0 × 150 mm; 2.2 μm). A linear gradient of 3.5% phosphoric acid (solvent A) and acetonitrile (solvent B) with a flow rate of 0.75 mL/min was used. In the gradient, solvent B was initially 15% for 2 min and increased to 20% by 3 min. Solvent B was further increased to 60% from 3 to 10 min, held for 1 min, and returned to 15% by 12 min.

LC-MS Analysis. Reversed-phase chromatography of anthocyanidins was performed on a Thermo Scientific (San Jose, CA, USA) Accela LC system. The mobile phases consisted of buffer A, water/formic acid (100:0.1, v/v), and buffer B, acetonitrile/formic acid (100:0.1, v/v). Five microliters of sample was injected onto a Hypersil GOLD C_{18} column (50 × 2.1 mm, 1.9 μm, 175 Å) from Thermo Scientific. A step gradient at a flow rate of 100 μL/min was used to elute the compounds. The gradient started at 5% buffer B and increased to 40% buffer B in 10 min, then increased to 90% buffer B in 3 min, and was maintained at 90% buffer B for 7 min. Elute from the LC was directed to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). The compounds were ionized by electrospray ionization and detected by Orbitrap at 30000 mass resolution (full scan, m/z 220−1000) or by multiple reaction monitoring (MRM). The spray voltage was 4.0 kV, and the capillary temperature was 250 °C. The sheath, auxiliary, and sweep gas flows were set to 15, 5, and 0, respectively. In MRM, molecular ions of anthocyanidins were selected with 3.0 m/z isolation window and fragmented by collision-induced



	R_1	R_2
Delphinidin	-OH	-OH
Cyanidin	-OH	-H
Peonidin	$-OCH_3$	-H
Petunidin	-OH	$-OCH_3$
Malvidin	$-OCH_3$	$-OCH_3$

Figure 1. Blueberry fruits and chemical structure of anthocyanins and anthocyanidins found in blueberry/bilberry. Shaded portion shows the difference in anthocyanidins and anthocyanins.

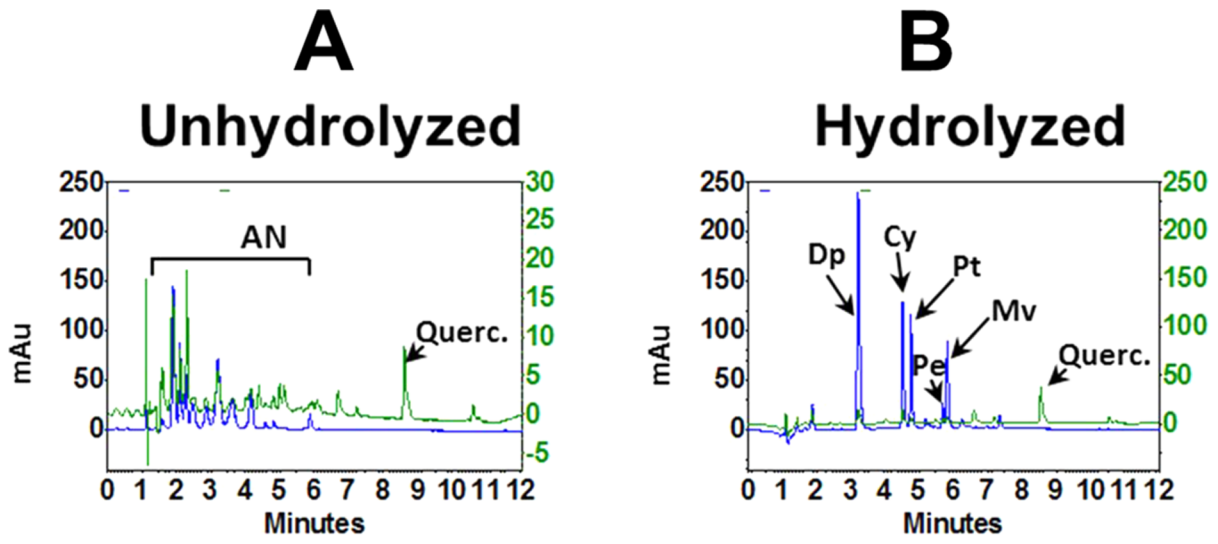


Figure 2. UPLC profiles of unhydrolyzed and hydrolyzed extract of blueberry in anthocyanins (A) and anthocyanidins (B). AN, anthocyanins; Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pe, peonidin; Mv, malvidin; Querc., quercetin.

dissociation (CID). For CID, collision energy, activation Q, and activation time (mS) were 35, 0.25, and 30, respectively. Full scan MS/MS spectra of the compounds (m/z 100−400) were acquired by Orbitrap at 7500 mass resolution. The transitions used for anthocyanidin detection are shown in panel C of Figure 4. The limits of detection by LC-MS for Dp and Cy were >0.5 and 0.25 ng, respectively, whereas those for Pt, Pe, and Mv were 2.5 pg.

RESULTS

Isolation of Blueberry Phytochemicals and Their Characterization. Blueberry extract was applied onto the XAD-761/HP-20 column (1:1) for the enrichment of anthocyanins and other polyphenols, and the enriched extract was hydrolyzed to convert the glycones to aglycones (i.e., anthocyanins to anthocyanidins; Figure 1). The enriched anthocyanidins were extracted in isoamyl alcohol and further purified by C18 column chromatography. The final extract contained highly pure (>94%) anthocyanidins (Figure 2). When analyzed by UPLC, the purified extract showed five anthocyanidins in the following descending order: Dp (33%), Cy (28%), Pt (16%), Ma (16%), and Pe (7%); a small amount of quercetin was also found in the sample (Figure 2).

Effect of Solvent on Stability of Anthocyanins. The cyanidin was highly unstable in acetonitrile. The rate of degradation was slow initially, but nearly 60% was degraded in 5 h (Supporting Information Figure S1). The stability was better in methanol, where there was only a slight decline and 80% of the compound was found intact. Interestingly, acidified methanol provided complete stability to cyanidin, and no degradation or loss was observed even after 5 h at room temperature. Hence, all of the analyses from tissues were done by using methanol containing 0.1% HCl.

Recovery of Anthocyanins and Anthocyanidins from Biomatrix. The recovery of anthocyanins and anthocyanidins was variable depending on the type of acid used. Formic acid and acetic acid did not provide any recovery of anthocyanins/anthocyanidins from PBS, whereas trifluoroacetic acid, phosphoric acid, and hydrochloric acid gave good recoveries. When these three acids were tested for recovery of anthocyanins/anthocyanidins from plasma, the recovery was in the following order: hydrochloric acid > phosphoric acid > trifluoroacetic acid (Table 1). However, hydrochloric acid extraction also resulted in conversion of some anthocyanins to anthocyanidins. Nearly 55−95% of anthocyanins and 63−100% of anthocyanidins were recovered from plasma (Table 1). Quantification of Pe and Mv was skewed due to the lack of separation of these two peaks in the UPLC conditions used. For measurement from biological matrices from animal experiments, the hydrochloric acid treatment was extended in a boiling water bath for 60 min to convert all anthocyanins to anthocyanidins and then quantified by UPLC-PDA.

Extractions and Detection of Anthocyanidins in Vivo. Blueberry has been reported to contain a variety of

dx.doi.org/10.1021/jf500467b | J. Agric. Food Chem. 2014, 62, 3912−3918

Table 1. Effect of Different Acids on the Recovery of Anthocyanins/Anthocyanidins from Plasma Spiked with Reference Compounds

	% recovery from spiked plasma[a]		
antho	hydrochloric acid	phosphoric acid	TFA
Dp-glu	53.7	36.5	not analyzed
Cy-glu	63.6	42.5	
Pt-glu	67.5	54.6	
Pe-glu	116.1	61.6	
Mv-glu	93.3	83.8	
Dp	123.4	17.7	9.9
Cy	62.9	not detected	32.5
Pt	124.1	2	9.0
Pe	89.2	not detected	21.8
Mv	140.9	1.2	8.3

[a]TFA, trifluoroacetic acid. When plasma was acidified with acetic acid or formic acid, below 2% amounts were recovered. When acidified with HCl, some of the anthocyanins were converted to anthocyanidins. Due to interfering peak with Pe-glucoside and Mv, their recoveries were skewed.

anthocyanins. In this study, we demonstrated the presence of anthocyanins in the blueberry-fed rodent tissues following conversion to anthocyanidins. Sensitivity of detection of the individual reference anthocyanidins was determined by LC-MS and UPLC-PDA and ranged from 0.25 ng to 2.5 pg and from 0.3 to 0.75 ng, respectively. The detection limit for PCA, a metabolite of Cy, was 0.2 ng. Cy was readily detected by UPLC (Figure 3) in the samples extracted from mouse lung with a limit of detection of 0.3 ng, whereas the other anthocyanidins were undetectable due to higher detection limits. A small peak was detected at the retention time for Dp; however, it was not confirmed by MS. However, with LC-MS analysis by specific ion monitoring, Pe, Pt, and Mv were readily detectable below

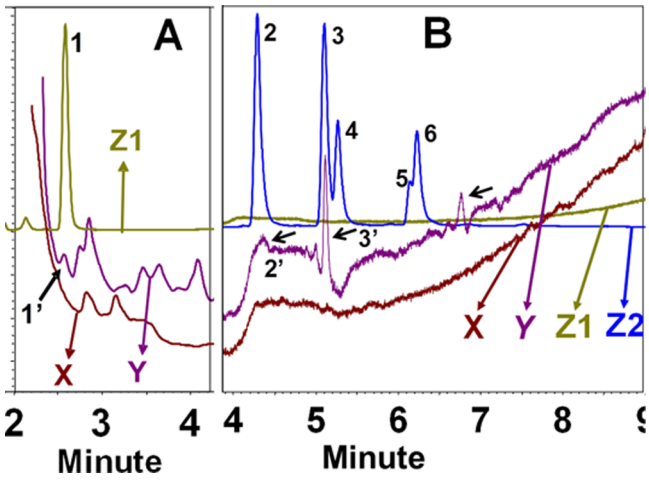


Figure 3. Detection of anthocyanidins at 520 nm (B) and 260 nm for protocatechuic acid (PCA) (A), a bioactive metabolite of cyanidin in lung tissue of rats given dietary blueberry (5% w/w) for 10 days by UPLC-PDA. Chromatograms shown are composites: X, lung tissue from rats on control diet; Y, lung tissue from rats on blueberry diet. Reference compounds: Z1, peak 1 (PCA); Z2, reference anthocyanidins. Peaks: 2, Dp; 3, Cy; 4, Pt; 5, Pe; 6, Mv. Solvent, gradient of 3.5% phosphoric acid in acetonitrile. Chromatograms are cropped for presentation purposes.

≤0.25 ng; Pe could be detected in the picogram range. No peak corresponding to anthocyanidins was detected in the lung of control animals by UPLC or LC-MS.

Several anthocyanidin peaks were found in the samples extracted from the lungs of mice fed blueberry diet by LTQ-MRM (low mass resolution). To confirm the presence of anthocyanidins in lung samples, an MRM with Orbitrap (high mass resolution) method was set up and used to reanalyze the extracted samples. Ion chromatograms of standards from Orbitrap MRM are shown in Figure 4, with a m/z window width of 0.06 that provides better selectivity. Dp and Cy were not detected in lung samples by Orbitrap MRM, but other anthocyanidins (Pe, Pt, and Mv) were detected by MRM (Figure 5) and further confirmed by MS/MS.

DISCUSSION

Several studies in recent years have focused attention on anthocyanin bioavailability both in humans and in experimental animals. Most of these studies reported that anthocyanins were poorly absorbed and were excreted unmetabolized.[18−20] Few studies have also reported that anthocyanins are bioavailable when delivered at very high doses; however, most of the bioavailability studies are focused on individual anthocyanidins, particularly Cy.[14,21−24]

The plausible reasons for lack of anthocyanins' detection include (i) instability of anthocyanins, (ii) metabolism by gut microflora, (iii) high rate of excretion, and (iv) unavailability of suitable analytical techniques. The aim of this work was to develop a method to assess the stability of anthocyanins/anthocyanidins, their selective extraction, and the impact on tissue bioavailability of anthocyanins.

There are several reports on the solubility and stability of anthocyanins in acidic environment.[25,26] Acidified methanol enhances the stability of anthocyanidins compared to acetonitrile. In methanol, the degradation of anthocyanins was very slow, with >80% of anthocyanins remaining intact after 5 h, whereas after acidification no degradation occurred. These results are consistent with published data demonstrating higher stability of anthocyanins in acidic pH.[25,26]

Several methods of anthocyanin extraction are available describing varying recovery of anthocyanins from a biological matrix.[27−30] These methods involve solid phase extraction using Water's Oasis HLB and Sep-Pak C18 cartridges. We standardized the extraction of anthocyanins from tissues of rats administered dietary blueberry by converting them to anthocyanidins, which reduces the number of compounds to five, and allowed us their detection due to their higher amounts. In complex matrices, where interference in peak separation was observed, use of isoamyl alcohol significantly improved the selectivity of anthocyanidins, albeit with a slight loss of recovery (6−7%, data not shown).

In this study, first, we determined stability and extractability of anthocyanidins isolated from bilberry and then determined their tissue bioavailability. Bilberry contains 15 anthocyanins containing galactose, glucose, and arabinose derivatives of Dp, Cy, Pt, Pe, and Mv. These anthocyanins were converted to five anthocyanidins upon acid hydrolysis. Glycones of the same anthocyanidins are also reported in blueberry.[13]

In the present work, we initially failed to detect anthocyanins in lung tissue from rats fed for 10 days with a 5% blueberry diet. The dietary route provides a slow ingestion of the berry phytochemicals. Under these conditions, the lung anthocyanin levels were presumably too low to be detected. Anthocyanins

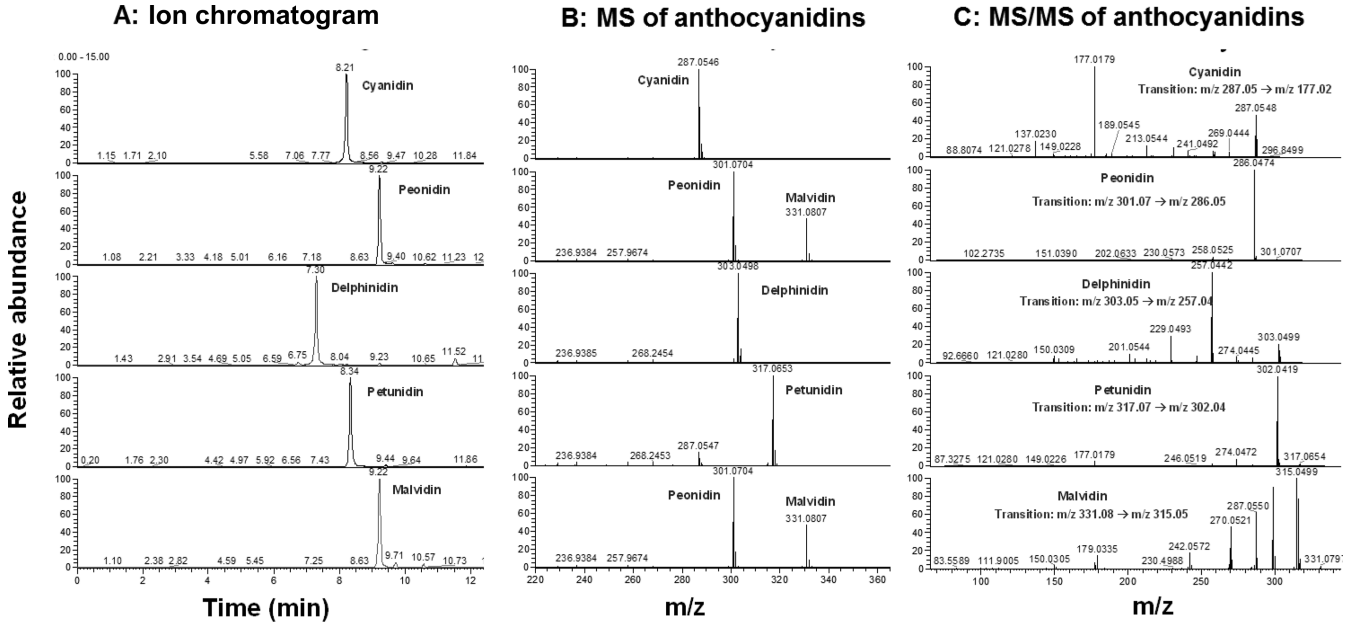


Figure 4. Multiple reaction monitoring (A) and the respective MS (B) and MS/MS (C) of indicated reference anthocyanidins. Peonidin and malvidin were eluted at the same retention time, and therefore they appear in the same spectrum. Anthocyanidins were separated with a Hypersil GOLD C18 column (50 mm × 2.1 mm) in a binary gradient of 1% formic acid and acetonitrile containing 1% formic acid as described under Materials and Methods.

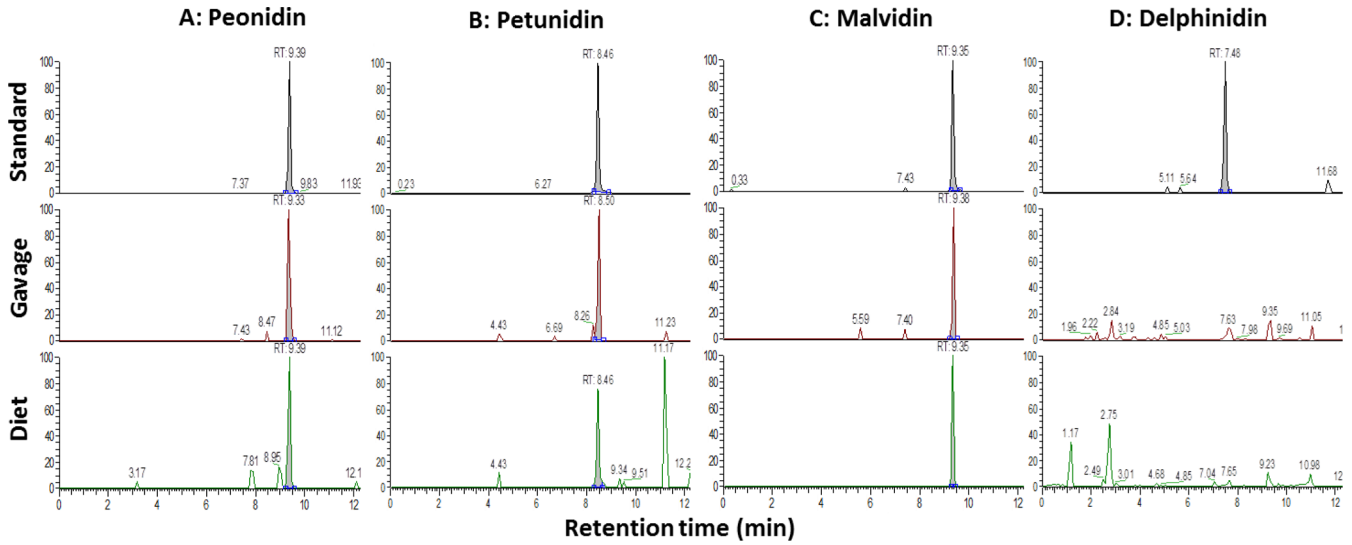


Figure 5. MRM detection of peonidin (A), petunidin (B), and malvidin (C) from the lung tissue of rats provided a native mixture of anthocyanidins isolated from bilberry by gavage (a bolus dose of 10 mg/mouse) or by diet supplemented with blueberry (5% w/w) for 10 days. Delphinidin (D) and cyanidin (not shown) could not be detected by LC-MS analysis. Chromatographic conditions are described under Materials and Methods.

were also not detected in the lung tissue following a bolus dose (10 mg/mouse) by gavage. On the other hand, when the tissue anthocyanins were converted to their respective aglycons by acid hydrolysis and selectively extracted in isoamyl alcohol, Dp and Cy were detected in the lung tissue, indicating that anthocyanins can reach and exert their effects beyond the GI tract. The possibility of bioavailable proanthocyanins being converted to anthocyanidins during acid hydrolysis cannot be ruled out. This presence of anthocyanins in lung tissue could be explained by their ability to permeate the gastrointestinal barrier.[31] These data demonstrate that the conversion of anthocyanins to anthocyanidins prior to analysis is an effective method for detecting anthocyanins in tissues. However, conversion of proanthocyanidins to anthocyanidins in vivo could also enhance the levels on anthocyanidins in animal tissue. PCA, which is produced by degradation of Cy,[32] was also detected in lung tissue, suggesting that lung PCA and anthocyanins along with some unknown metabolites are presumably bioactives responsible for the known anticarcinogenic potential of blueberry in the lung.[33]

A series of papers have shown the bioavailability of anthocyanins in brain tissue,[21,22] and a few other studies report the presence of anthocyanins in other organs including the lung following treatment of rodents with individual anthocyanin at high doses.[14,23,24] Our study is the first demonstration indicating the presence of anthocyanidins in lung tissue

dx.doi.org/10.1021/jf500467b | J. Agric. Food Chem. 2014, 62, 3912−3918

following low-dose dietary blueberry. The blueberry dose used in this study is the same or just 2-fold higher as used in our previous study in which it was found to inhibit estrogen-mediated mammary tumorigenesis.[8,13] The results from our other study also showed the efficacy of the native mixture of anthocyanidins from bilberry against lung cancer xenografts.[3]

In summary, we show for the first time that anthocyanins are bioavailable in the lung following a low dose of dietary blueberry powder. The detection has been possible by conversion of anthocyanins to their native anthocyanidins, followed by extraction in isoamyl alcohol. This technique can be utilized to demonstrate bioavailability of anthocyanins in different tissues and correlate their levels with disease inhibition.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(R.C.G.) Phone: (502) 852-3682. Fax: (502) 852-3842. E-mail: rcgupta@louisville.edu.

Funding

This work was supported by USPHS Grants CA-118114 and CA125152 and the Agnes Brown Duggan Endowment. R.C.G. holds the Agnes Brown Duggan Chair in Oncological Research.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Harris, G. K.; Gupta, A.; Nines, R. G.; Kresty, L. A.; Habib, S. G.; Frankel, W. L.; LaPerle, K.; Gallaher, D. D.; Schwartz, S. J.; Stoner, G. D. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat. *Nutr. Cancer.* **2001**, *40*, 125−133.

(2) Stoner, G. D.; Wang, L. S.; Seguin, C.; Rocha, C.; Stoner, K.; Chiu, S.; Kinghorn, A. D. Multiple berry types prevent *N*-nitrosomethylbenzylamine-induced esophageal cancer in rats. *Pharm. Res.* **2010**, *27*, 1138−1145.

(3) Kausar, H.; Jeyabalan, J.; Aqil, F.; Chabba, D.; Sidana, J.; Singh, I. P.; Gupta, R. C. Berry anthocyanidins synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells. *Cancer Lett.* **2012**, *325*, 54−62.

(4) Svobodova, A.; Zdarilova, A.; Vostalova, J. *Lonicera caerulea* and *Vaccinium myrtillus* fruit polyphenols protect HaCaT keratinocytes against UVB-induced phototoxic stress and DNA damage. *J. Dermatol. Sci.* **2009**, *56*, 196−204.

(5) Zafra-Stone, S.; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J. A.; Bagchi, D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **2007**, *51*, 675−683.

(6) Neto, C. C. Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. *Mol. Nutr. Food Res.* **2007**, *51*, 652−664.

(7) Aiyer, H. S.; Gupta, R. C. Berries and ellagic acid prevent estrogen-induced mammary tumorigenesis by modulating enzymes of estrogen metabolism. *Cancer Prev. Res. (Phila.)* **2010**, *3*, 727−737.

(8) Aiyer, H. S.; Srinivasan, C.; Gupta, R. C. Dietary berries and ellagic acid diminish estrogen-mediated mammary tumorigenesis in ACI rats. *Nutr. Cancer* **2008**, *60*, 227−234.

(9) Ravoori, S.; Vadhanam, M. V.; Sahoo, S.; Srinivasan, C.; Gupta, R. C. Mammary tumor induction in ACI rats exposed to low levels of 17β-estradiol. *Int. J. Oncol.* **2007**, *31*, 113−120.

(10) Vadhanam, M. V.; Ravoori, S.; Aqil, F.; Gupta, R. C. Chemoprevention of mammary carcinogenesis by sustained systemic delivery of ellagic acid. *Eur. J. Cancer Prev.* **2011**, *20*, 484−491.

(11) Wang, H.; Cao, G. H.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304−309.

(12) Matsumoto, H.; Nakamura, Y.; Hirayama, M.; Yoshiki, Y.; Okubo, K. Antioxidant activity of black currant anthocyanin aglycons and their glycosides measured by chemiluminescence in a neutral pH region and in human plasma. *J. Agric. Food Chem.* **2002**, *50*, 5034−5037.

(13) Ravoori, S.; Vadhanam, M. V.; Aqil, F.; Gupta, R. C. Inhibition of estrogen-mediated mammary tumorigenesis by blueberry and black raspberry. *J. Agric. Food Chem.* **2012**, *60*, 5547−5555.

(14) Marczylo, T. H.; Cooke, D.; Brown, K.; Steward, W. P.; Gescher, A. J. Pharmacokinetics and metabolism of the putative cancer chemopreventive agent cyanidin-3-glucoside in mice. *Cancer Chemother. Pharmacol.* **2009**, *64*, 1261−1268.

(15) Aqil, F.; Gupta, A.; Munagala, R.; Jeyabalan, J.; Kausar, H.; Sharma, R. J.; Singh, I. P.; Gupta, R. C. Antioxidant and antiproliferative activities of anthocyanin/ellagitannin-enriched extracts from *Syzygium cumini* L. (jamun, the Indian blackberry). *Nutr. Cancer* **2012**, *64*, 428−438.

(16) Toki, K.; Saito, N.; Irie, Y.; Tatsuzawa, F.; Shigihara, A.; Honda, T. 7-O-Methylated anthocyanidin glycosides from *Catharanthus roseus*. *Phytochemistry* **2008**, *69*, 1215−1219.

(17) Vitaglione, P.; Donnarumma, G.; Napolitano, A.; Galvano, F.; Gallo, A.; Scalfi, L.; Fogliano, V. Protocatechuic acid is the major human metabolite of cyanidin-glucosides. *J. Nutr.* **2007**, *137*, 2043−2048.

(18) McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* **2003**, *51*, 4539−4548.

(19) Netzel, M.; Strass, G.; Janssen, M.; Bitsch, I.; Bitsch, R. Bioactive anthocyanins detected in human urine after ingestion of blackcurrant juice. *J. Environ. Pathol. Toxicol. Oncol.* **2001**, *20*, 89−95.

(20) Frank, T.; Netzel, M.; Strass, G.; Bitsch, R.; Bitsch, I. Bioavailability of anthocyanidin-3-glucosides following consumption of red wine and red grape juice. *Can. J. Physiol. Pharmacol.* **2003**, *81*, 423−435.

(21) Milbury, P. E.; Kalt, W. Xenobiotic metabolism and berry flavonoid transport across the blood-brain barrier. *J. Agric. Food Chem.* **2010**, *58*, 3950−3956.

(22) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Gil-Izquierdo, A.; Lamaison, J. L.; Remesy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J. Agric. Food Chem.* **2005**, *53*, 3902−3908.

(23) Sakakibara, H.; Ogawa, T.; Koyanagi, A.; Kobayashi, S.; Goda, T.; Kumazawa, S.; Kobayashi, H.; Shimoi, K. Distribution and excretion of bilberry anthocyanins [corrected] in mice. *J. Agric. Food Chem.* **2009**, *57*, 7681−7686.

(24) Datla, K. P.; Christidou, M.; Widmer, W. W.; Rooprai, H. K.; Dexter, D. T. Tissue distribution and neuroprotective effects of citrus flavonoid tangeretin in a rat model of Parkinson's disease. *Neuroreport* **2001**, *12*, 3871−3875.

(25) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Manach, C.; Lamaison, J. L.; Remesy, C. Anthocyanins are efficiently absorbed from the small intestine in rats. *J. Nutr.* **2004**, *134*, 2275−2279.

(26) Li, J.; Li, X. D.; Zhang, Y.; Zheng, Z. D.; Qu, Z. Y.; Liu, M.; Zhu, S. H.; Liu, S.; Wang, M.; Qu, L. Identification and thermal stability of purple-fleshed sweet potato anthocyanins in aqueous solutions with various pH values and fruit juices. *Food Chem.* **2013**, *136*, 1429−1434.

(27) Ling, Y.; Ren, C.; Mallery, S. R.; Ugalde, C. M.; Pei, P.; Saradhi, U. V.; Stoner, G. D.; Chan, K. K.; Liu, Z. A rapid and sensitive LC-MS/MS method for quantification of four anthocyanins and its application in a clinical pharmacology study of a bioadhesive black raspberry gel. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2009**, *877*, 4027−4034.

(28) Wu, X.; Cao, G.; Prior, R. L. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* **2002**, *132*, 1865−1871.
(29) Yi, W.; Akoh, C. C.; Fischer, J.; Krewer, G. Absorption of anthocyanins from blueberry extracts by Caco-2 human intestinal cell monolayers. *J. Agric. Food Chem.* **2006**, *54*, 5651−5658.
(30) Milbury, P. E.; Vita, J. A.; Blumberg, J. B. Anthocyanins are bioavailable in humans following an acute dose of cranberry juice. *J. Nutr.* **2010**, *140*, 1099−1104.
(31) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Lamaison, J. L.; Remesy, C. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *J. Nutr.* **2003**, *133*, 4178−4182.
(32) Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin 3-*O*-*β*-D-glucoside in rats. *FEBS Lett.* **1999**, *449*, 179−182.
(33) Balansky, R.; Ganchev, G.; Iltcheva, M.; Kratchanova, M.; Denev, P.; Kratchanov, C.; Polasa, K.; D'Agostini, F.; Steele, V. E.; De Flora, S. Inhibition of lung tumor development by berry extracts in mice exposed to cigarette smoke. *Int. J. Cancer* **2012**, *131*, 1991−1997.