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Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Human Subjects

G. MAZZA,*,† COLIN D. KAY,‡ TONY COTTRELL,† AND BRUCE J. HOLUB‡

Food Research Program, Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, British Columbia, Canada, and Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

In recent years, numerous studies have shown that the polyphenolics present in fruit and vegetable products exhibit a wide range of biological effects. However, there is little reliable information on the absorption of glycosylated and acylated anthocyanins in humans. In the present study, the absorption of anthocyanins in humans was investigated after the consumption of a high-fat meal with a freeze-dried blueberry powder containing 25 individual anthocyanins including 6 acylated structures. Nineteen of the 25 anthocyanins present in the blueberries were detected in human blood serum. Furthermore, the appearance of total anthocyanins in the serum was directly correlated with an increase in serum antioxidant capacity ($ORAC_{acetone}$, $P < 0.01$). These results show that anthocyanins can be absorbed in their intact glycosylated and possibly acylated forms in human subjects and that consumption of blueberries, a food source with high in vitro antioxidant properties, is associated with a diet-induced increase in ex vivo serum antioxidant status.

KEYWORDS: Anthocyanins; delphinidin; cyanidin; petunidin; peonidin; malvidin; glucoside; galactoside; arabinoside; acetylated; postprandial oxidation; antioxidant activity; functional foods; blueberries; HPLC analysis

INTRODUCTION

Anthocyanins are flavonoids widely distributed in the human diet through fruit, vegetable, and grain products. They are best known for their ability to impart red, blue, and purple colors to the plants or plant products in which they occur. Less well-known are their role as free radical scavengers and their potentially significant interactions with biological systems, such as enzyme-inhibiting, antibacterial, and antioxidant effects. Their tendency to influence biological systems is at least in part due to their characteristic ability to form complexes with macromolecules, combined with a polyphenolic nature. The anthocyanins are distinguished from other flavonoids as a separate class by virtue of their ability to form flavylium (2-phenylbenzopyrylium) cations (*1*, *2*). Flavonoids such as quercetin occur in nearly all common fruits and vegetables, and the average daily intake of flavonoids, expressed as aglycons, is estimated at a few hundred milligrams per day (*3*). Several flavonoids have been shown to exhibit antioxidant, anti-inflammatory, anticarcinogenic, and estrogenic activities, to inhibit enzymes, and to reduce the risk of coronary heart disease (*3*−*6*).

Lowbush "wild" blueberries (*Vaccinium angustifolium)* are one of the highest sources of anthocyanins (*7*, *8*) and have exhibited one of the highest recorded in vitro antioxidant capacities of various fruits and vegetables tested (*9*, *10*). The lowbush blueberry as studied herein is of particular interest as it has a higher in vitro antioxidant capacity than the cultivated highbush blueberry (*Vaccinium corymbosum*) (*11*). Wild blueberries are relatively low in antioxidant vitamins and minerals (*12*); their in vitro antioxidant capacity has been attributed to their high concentration of phenolic compounds, particularly anthocyanins (*11*, *13*).

Information about the absorption, metabolism, and excretion of individual flavonoids in humans is scarce (*14*). Flavonoids are absorbed from the intestinal tracts of humans and animals and are excreted either unchanged or as flavonoid metabolites in the urine or feces. Most studies have dealt with the absorption of flavonoids after the oral administration of pharmacological doses of individual flavonoids rather than dietary (food-based) levels of the flavonoids. Furthermore, most experiments have been conducted using flavonoids in aglycon form rather than glycosylated flavonoids, which predominate in plants.

One of the first studies to provide evidence for the uptake of anthocyanins in humans in vivo was published by Paganga and Rice-Evans (*15*). Anthocyanins, rutin, and other quercetin glycosides were detected in human serum in the glycosylated form by HPLC analysis. The results reveal that phloretin and quercetin are absorbed from the diet as glycosides. Lapidot et al. (*16*) traced anthocyanins in human urine after the intake of 300 mL of red wine (containing ∼218 mg of anthocyanins). Totals of 1.5−5.1% of the anthocyanins were recovered in the

* Author to whom correspondence should be addressed [telephone (250) 494-6376; fax (250) 494-0755; e-mail mazzag@agr.gc.ca].
† Pacific Agri-Food Research Center Contribution 2169.
‡ University of Guelph.

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urine within 12 h after wine consumption. Two of the compounds were unchanged, whereas other compounds seemed to have undergone molecular modifications. The anthocyanin levels in the urine reached a peak within 6 h of consumption. More recently, Miyazawa et al. (*17*) reported the results of a human study involving seven male and five female adults. The anthocyanins were obtained from spray-dried extracts of elderberry (*Sambucus nigra*) and black currant (*Ribes nigrum*) juice concentrates. The subjects orally ingested 2.7 mg of cyanidin 3-glucosides and 0.25 mg of cyanidin 2,5-diglycosides/kg of body weight. In plasma, 30 min after intake the level of cyanidin 3-glucosides was 11 μg/L. At 60 min after intake, the plasma cyanidin 3-glucoside level was 13 μg/L. Only traces of cyanidin 2,5-diglycoside were found in plasma. Other studies on bioavailability of anthocyanins have been reported by Cao and Prior (*18*), Cao et al. (*19*), and Matsumoto et al. (*20*).

Recently, several authors have reported that anthocyanins show strong antioxidative activity (*21*−*26*). Tsuda et al. (*23*) investigated the reaction products of cyanidin 3-*O*-D-glucoside with 2,2′-azobis(2,4-dimethylvaleronitrile) and concluded that the antioxidative mechanism of cyanidin 3-*O*-D-glucoside may be different from that of α-tocopherol; cyanidin 3-*O*-D-glucoside would produce another radical scavenger, as it would break down the structure and scavenge the radicals. Narayan et al. (*26*) investigated the antioxidant effect of anthocyanins on enzymatic and nonenzymatic lipid peroxidation. Their results show that in vitro enzymatic and nonenzymatic polyunsaturated fatty acid peroxidation was significantly inhibited in a dose-dependent manner by purified anthocyanin pigment from carrot cell culture. The kinetics data show that the anthocyanin is a noncompetitive inhibitor of lipid peroxidation. Anthocyanins were found to be a potent antioxidant compared to classical antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoulene (BHT), and α-tocopherol (*26*, *27*). Glycosylation of a flavonoid reduces radical scavenger activity compared with the aglycon, as it reduces the ability of the flavonoid radical to delocalize electrons. In accordance with this, Fukumoto and Mazza (*27*) reported increased antioxidant activity with increase in the hydroxyl groups and deceased antioxidant activity with glycosylation of anthocyanidins. A full understanding of the correlation between antioxidant activity and chemical structure is not yet clear.

Diet and oxidative stress have been implicated in the development of cardiovascular diseases (*28*, *29*), diabetes (*30*, *31*), and cancer (*32*, *33*). Increasing the blood antioxidant status has been proposed as a preventative means to reduce the development of these diseases (*29*, *31*, *33*).

The purpose of the present in vivo study was to evaluate the absorption of anthocyanins and to examine the effect of consuming freeze-dried wild blueberries rich in anthocyanins and phenolic compounds on the serum antioxidant status as measured in healthy human subjects. Previous studies have demonstrated the in vitro antioxidant properties of blueberries to be higher than that of most fruits and vegetables (*13*, *34*, *35*).

MATERIALS AND METHODS

Subjects. Five male subjects (46.9 ± 1.9 years, BMI of 23.8 ± 0.8 kg/m^2) were recruited from the Guelph area (ON, Canada). Baseline (fasting) values are given in **Table 1**. The principal criteria for eligibility were (1) absence of clinical disease, (2) no history of renal or gastrointestinal disorders, (3) no alcoholism, and (4) no smoking. Subjects taking lipid-altering or blood pressure medications were excluded. Subjects also refrained from taking aspirin or anti-inflammatory medications prior to, or during, the study and discontinued all forms of antioxidant supplementation one month prior to the investigation. Furthermore, subjects were instructed to maintain a consistent diet throughout the study period. This study conformed with the ethical guidelines of the University of Guelph (ON, Canada) and was approved by the Human Subjects Committee. All subjects gave written consent in advance.

Table 1. Fasting Baseline Characteristics of Study Subjects before Initiation of Treatment[a]

	treatment	
measurement	control mean ± SD (n = 5)	blueberry mean ± SD (n = 5)
TEAC[b] (μmol of Trolox equiv/L)	1370 ± 40	1270 ± 60
$ORAC_{total}$ (μmol of Trolox equiv/L)	2479 ± 360	2497 ± 263
$ORAC_{PCA}$ (μmol of Trolox equiv/L)	658 ± 69	672 ± 56
$ORAC_{acetone}$ (μmol of Trolox equiv/L)	759 ± 145	735 ± 96
triacylglycerols (mmol/L)	1.33 ± 0.33	1.22 ± 0.40

[a] x ± SD (n = 5). Abbreviations: TEAC, Trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity. [b] Significant difference between treatment groups at baseline (t = 0) (paired t test): $P < 0.05$.

Table 2. Composition of Treatment Supplements (100 g)

	concentration	
component	blueberry	control
ORAC[a] (μmol TE/g)	147	5
total phenolics (g/100 g)	2.79	0.00
anthocyanins (g/100 g)	1.16	0.00
vitamin C (g/100 g)	0.01	0.00
carbohydrate[b] (digestible) (g/100 g)	76.4	76.4
total dietary fiber[b] (g/100 g)	17.8	0.00
protein[b] (g/100 g)	2.7	0.00
fat[b] (g/100 g)	2.4	0.00
ash[b] (mineral content) (g/100 g)	1.0	0.00
calories[b] (/100 g)	338	305
kJ[b] (energy) (/100 g)	1410	1272

[a] ORAC, oxygen radical absorbance capacity (TE/g, Trolox equivalents per gram), conducted by Brunswick Laboratories. [b] Chemical analyses of freeze-dried blueberry powder conducted by Maxxam Analytics Inc.

Supplementation. The freeze-dried wild blueberry powder used in this trial was produced at the University of Guelph (Department of Human Biology and Nutritional Sciences) in conjunction with the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA, Guelph, ON, Canada) (**Table 2**). The powder was produced from lowbush blueberries (*V. angustifolium*) obtained from the Sudbury area of northern Ontario. The wild blueberries were carefully hand picked and frozen at −30 °C. The berries were then freeze-dried (whole) and ground into a fine powder using a household food processor. The blueberry supplement (100 g) contained 1.20 g of total anthocyanins (42% of the total phenolics) and had an oxygen radical absorbance capacity (ORAC) value of 147 μmol of Trolox equiv/g (as determined by Brunswick Laboratories, Wareham, MA). The control supplement was matched for digestible carbohydrate and contained 76.4 g (305 calories) of glucose (Alantic Sugar Ltd., Toronto, ON, Canada) and 0.5 g of sugar-free Kool-Aid (Kraft Canada, Don Mills, ON, Canada) and had an ORAC value of 5 μmol of Trolox equiv/g (**Table 2**).

Experimental Design. This study followed a single-blind crossover design. Subjects were admitted to the Human Testing Laboratory (Department of Human Biology and Nutritional Sciences, University of Guelph) on the morning of the study dates in a fasted state (12−14 h, no alcohol for 24 h). Subjects were cannulated with an Intima 20 gage intravenous catheter (Becton Dickinson, Rutherford, NJ) in the brachial vein, and overnight fasting blood samples were drawn. Subjects received the control supplement (dissolved in 500 mL of water) with a high-fat meal (853 calories, 46.7 g of fat with 15.5 g of saturated fat, as determined by Maxxam Analytics Inc, Mississauga, ON, Canada) consisting of one Egg McMuffin, one Sausage McMuffin, and two hash

Table 3. Composition of High-Fat Meal

component	amount	% calories
carbohydrate[a] (digestible)	75.2 g	35.4
protein[a]	32.4 g	15.3
fat[a]	46.7 g	49.3
total dietary fiber[a]	4.5 g	
ash (mineral content)[a]	9.1 g	
vitamin C	6.0 mg	
vitamin E	2.1 mg	
calories[a]	853	
kJ (energy)[a]	3564	

[a] Chemical analyses of the high-fat meal conducted by Maxxam Analytics Inc.

brown patties (McDonald's Corp.) (**Table 3**). Time $t = 0$ was obtained upon initiation of the high-fat meal; subsequent blood samples were performed at 1, 2, 3, and 4 h. Procedures were repeated (on the same subjects) 7 days later using the same high-fat meal, with 100 g of the freeze-dried wild blueberry powder (dispersed in 500 mL of water) in place of the control supplement. All subjects consumed both the blueberry and control supplements and were fully compliant with the study regime. No adverse effects were reported.

Sampling Procedures. Twenty milliliters of blood (per time point) was drawn from a brachial vein catheter into an evacuated glass tube (Vacutainer, Becton Dickinson, Rutherford, NJ). Samples were allowed to clot at room temperature for 25 min. Samples were then immediately centrifuged (3000 rpm, 1000*g*) for 15 min (at 5−15 °C) to recover serum, which was extracted and aliquoted into 2 mL vials (Cryovial, Fisher Scientific Ltd., Nepean, ON, Canada) over an ice bath (3−4 °C) using disposable glass pipets. The serum was then snap-frozen in liquid nitrogen and stored at −80 °C.

Extraction of Anthocyanins from Serum. Anthocyanins in human serum were extracted by solid phase extraction using an SPE C18 cartridge (Supelclean ENVI-18, 6 mL tube, 0.5 g loading) (Sigma-Aldrich/Supelco, Bellefonte, PA). The cartridge was preconditioned using 7 mL of methanol containing 0.1% trifluoroacetic acid (TFA) and then equilibrated with 7 mL of 10 mM oxalic acid before use. Human serum (1.5 mL) was acidified with a 1/40 volume of 6 N HCl. The acidified serum was diluted with 1.5 mL of 10 mM oxalic acid. The mixture was then loaded on the cartridge. The cartridge was washed with 7 mL of 10 mM oxalic acid. Before anthocyanins were eluted, the remaining aqueous phase was drained off using a vacuum. Finally, anthocyanins were eluted with 7 mL of methanol containing 0.1% TFA. The eluate was carefully evaporated using Speed Vac Plus SC 110A and Universal vacuum system UVS 400 (Savant Instruments, Inc., Holbrook, NY) at room temperature and finally taken to dryness under a stream of nitrogen. The dry residue was redissolved in 200 μL of 4.5% formic acid in water and methanol in a ratio of 90:10 and filtered through a 13 mm 0.45 μm GHP Acrodisc filter (Pall-Gelman) into a 300 μL vial insert for HPLC.

HPLC Analysis. The HPLC system used was an Agilent 1100 series (Agilent Technologies Inc., Palo Alto, CA) with a photodiode array detector. Anthocyanins were separated on a Zorbax SB C18, 250 × 4.6 mm (5 μm) reverse phase column (Agilent Technologies) with an Inertsil 30 × 4.6 mm (5 μm) guard column (Phenomenex, Torrance, CA). The solvents used were 4.5% (v/v) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). The flow rate was 1 mL/min with a gradient profile of solvent A with the following proportions of solvent B: 0 min, 10%; 0−30 min, 10−25%; 30−50 min, 25−45%; 50−55 min, 45−100%; 55−60 min, 100%; 60−65 min, 100−10%. Total run time was 70 min. The column temperature was maintained at 35 °C, the injection volume was 50 μL, the detector signal was 525 nm (16 nm bandwidth), and the reference was 700 nm (100 nm bandwidth). Anthocyanins in the blueberry diet and in blood serum extracts were identified as described by Gao and Mazza (*8*, *36*) by reversed-phase high-performance liquid chromatography (RP-HPLC) and using retention times and UV−vis absorption spectra. The aliphatic acylating acids and the sugars were characterized by capillary gas−liquid chromatography (GLC) analysis of trimethylsilyl derivatives of sugars and aliphatic acids and methyl esters of aliphatic acids.

Serum Antioxidant Capacity. Serum antioxidant capacity was measured using the Trolox equivalent antioxidant capacity (TEAC) and ORAC ($ORAC_{total}$, $ORAC_{PCA}$, and $ORAC_{acetone}$ fractions) assays. TEAC was measured on an automated Hitachi 911 Biochemical Analyzer using the Randox-TEAC assay (Randox Laboratories, Mississauga, ON, Canada). Briefly, the TEAC assay is based on the inhibition by antioxidants to absorb free radicals. Plasma samples were added to a 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) ($ABTS^{\bullet-}$) radical solution. The change in radical concentration over a 10 min incubation period was monitored spectrophotometrically (600 nm) and compared to the decrease of a known standard (Trolox). Plasma ORAC was determined by Genox Laboratories (Baltimore, MD) using the ORAC assay as outlined by Cao et al. (*37*). Modifications to the ORAC assay allowed for the isolation of lipid- and water-soluble phases of the plasma sample. The $ORAC_{PCA}$ fraction (serum treated with perchloric acid) is a nonprotein fraction that preserves the water-soluble antioxidants within the sample. $ORAC_{acetone}$ (plasma treated with acetone) is a nonprotein fraction containing both water- and lipid-soluble antioxidants (*38*). Briefly, the ORAC assay was conducted using the automated COBAS FARA II spectrofluorometric analyzer (Roche Diagnostics, Basel, Switzerland) with fluorescent filters at an excitation wavelength of 546 nm and an emission wavelength of 565 nm. *R*-Phycoerythrin (*R*-PE) was used as the target molecule for free radical attack, with 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) as the peroxyl radical generator. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an aqueous-soluble vitamin E analogue, was used as a control standard. The fluorescence of *R*-PE was recorded every 55 s and expressed relative to the initial reading. Final results were calculated by using the differences of the areas under the *R*-PE decay curves between the blank and the sample. ORAC readings are expressed as micromoles of Trolox equivalents, where 1 Trolox equivalent equals the net protection area under the curve provided by 1 μmol of Trolox. Specifics of the ORAC and comparisons between the ORAC and TEAC assays have been outlined by Cao and Prior (*38*).

Statistical Analysis. Results are expressed as means ± SD. Baseline characteristics of the treatment groups were compared using independent paired *t* tests. The effects of diet, treatment, and postprandial times, as well as interactions between them, were determined by repeated measures analysis of variance (ANOVA) using the Statistical Analysis Systems statistical software package version 6.1 (SAS Institute, Cary, NC). Linear regression analysis was also conducted using SAS.

RESULTS AND DICUSSION

Anthocyanins in Human Serum. Figure 1 shows typical HPLC chromatograms of human serum extracts before (A) and 1 and 3 h (B and C) after consumption of 100 g of freeze-dried blueberries that contained 1.20 g of total anthocyanins. No anthocyanins were detected before consumption of the blueberry anthocyanins. Blood samples collected after consumption of the blueberries contained most of the 25 anthocyanins present in lowbush blueberries, and 11 anthocyanins, namely, delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-arabinoside, malvidin 3-galactoside, and malvidin 3-glucoside, could be analyzed quantitatively in blood serum. The contents of total and major anthocyanins in the serum extracts 1, 2, 3, and 4 h after consumption of the freeze-dried berries are presented in **Table 4**. The serum concentration of total anthocyanins, in the five subjects, expressed as cyanidin 3-glucoside, varied from 5.43 to 16.9 ng/mL. Maximum levels were observed 4 h after consumption of blueberries, and the anthocyanins present most abundantly were malvidin 3-galactoside, malvidin 3-glucoside, and delphinidin 3-glucoside, accounting for 27, 20, and 10% of the total, respectively.

Table 4 shows the time course of changes in the concentrations of total and 11 individual anthocyanins in human serum after the consumption of lowbush blueberries. The mean

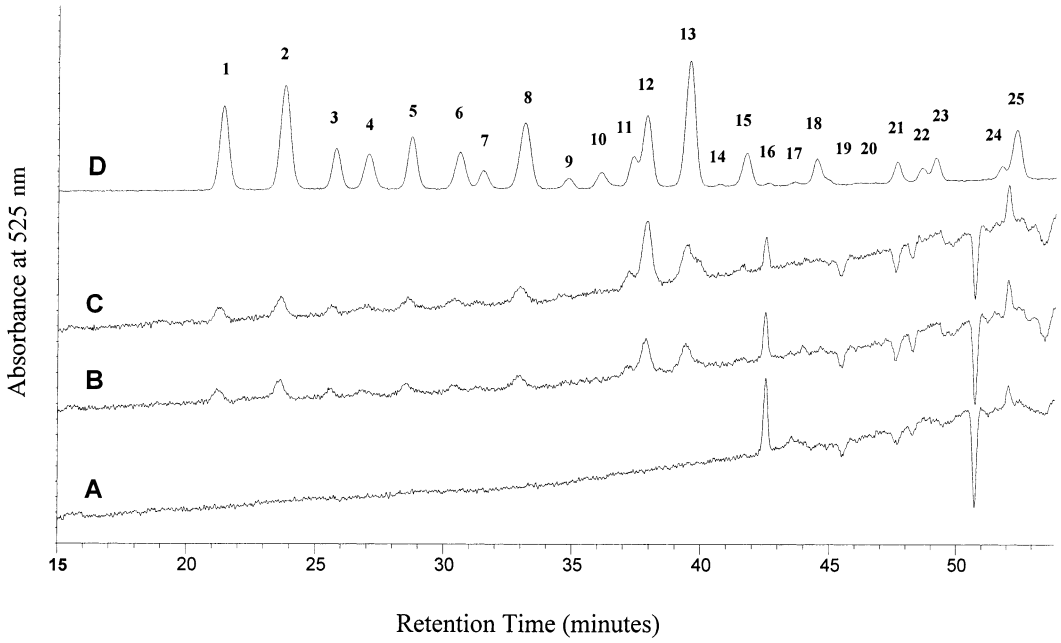

Figure 1. Typical chromatograms of blood serum extracts for a human subject before (A) and 1 (B) and 3 h (C) after diet ingestion and of blood serum spiked with blueberry diet extract (D). Peak identities: 1, delphinidin 3-galactoside; 2, delphinidin 3-glucoside; 3, cyanidin 3-galactoside; 4, delphinidin 3-arabinoside; 5, cyanidin 3-glucoside; 6, petunidin 3-galactoside; 7, cyanidin 3-arabinoside; 8, petunidin 3-glucoside; 9, peonidin 3-galactoside; 10, peonidin 3-arabinoside; 11, peonidin 3-glucoside; 12, malvidin 3-galactoside; 13, malvidin 3-glucoside; 14, delphinidin 3-acetylgalactoside; 15, malvidin 3-arabinoside; 16, cyanidin 3-acetylgalactoside; 17, cyanidin 3-acetylarabinoside; 18, delphinidin 3-acetylglucoside; 19, petunidin 3-acetylgalactoside; 20, peonidin 3-acetylgalactoside; 21, cyanidin 3-acetylglucoside; 22, malvidin 3-acetylgalactoside; 23, petunidin 3-acetylglucoside; 24, peonidin 3-acetylglucoside; 25, malvidin 3-acetylglucoside.

Table 4. Mean Concentration[a] and Standard Deviation of Anthocyanins in Blood Serum

	time after ingestion			
anthocyanin[b]	1 h	2 h	3 h	4 h
del-3-gal	0.49 ± 0.12	0.60 ± 0.09	0.68 ± 0.15	0.65 ± 0.12
del-3-glu	0.74 ± 0.21	0.98 ± 0.17	1.17 ± 0.41	1.25 ± 0.41
cy-3-gal	0.35 ± 0.00	0.39 ± 0.07	0.45 ± 0.13	0.39 ± 0.08
del-3-ara	0.38 ± 0.11	0.48 ± 0.22	0.64 ± 0.31	0.84 ± 0.25
cy-3-glu	0.52 ± 0.09	0.67 ± 0.16	0.91 ± 0.49	0.82 ± 0.43
pet-3-gal	0.31 ± 0.02	0.36 ± 0.08	0.42 ± 0.08	0.46 ± 0.09
cy-3-ara	0.27 ± 0.03	0.23 ± 0.03	0.29 ± 0.08	0.32 ± 0.06
pet-3-glu	0.67 ± 0.12	0.82 ± 0.12	0.98 ± 0.11	1.06 ± 0.19
pn-3-ara	0.44 ± 0.06	0.64 ± 0.13	0.83 ± 0.15	0.93 ± 0.19
mal-3-gal	1.59 ± 0.27	2.65 ± 0.73	3.30 ± 0.45	3.68 ± 0.73
mal-3-glu	1.09 ± 0.35	1.81 ± 0.51	2.44 ± 0.66	2.70 ± 0.59
total	6.63 ± 1.35	9.58 ± 2.05	12.10 ± 2.82	13.09 ± 2.74

[a] Serum concentration (ng/mL) expressed as cyanidin 3-glucoside chloride. All are means of five subjects, analyzed in duplicate. [b] Anthocyanin identities: del, delphinidin; cy, cyanidin; pet, petunidin; pn, peonidin; mal, malvidin; glu, glucoside; gal, galactoside; ara, arabinoside.

concentration of total anthocyanins increased from 6.6 ng/mL at 1.0 h postconsumption to 9.6, 12.1, and 13.1 ng/mL at 2, 3, and 4 h after intake, respectively. Considering that the average weight of the subjects used in this study was ~80 kg and that each subject consumed 1.20 g of anthocyanins, the average intake of anthocyanins was 15 mg/kg of body weight. Serum total anthocyanins (as cyandin 3-glucoside equivalents) in the subjects 3 h after consumption was 12.1 ± 2.8 μg/L (**Table 4**), or approximately 0.454 μg/kg of body weight (assuming 5 L of blood per person and 40−60% of the blood being serum). Thus, these results indicate that only 0.002−0.003% (or 20−30 ppm) of the ingested amount of anthocyanins was present in the human serum 3 h after ingestion. This level of absorption appears to be very low; but it is comparable to values recently reported by Matsumoto et al. (*20*). Similarly, the human serum anthocyanin concentrations from our study are comparable to the plasma total concentrations of anthocyanins reported by Miyazawa et al. (*17*) and Matsumoto et al. (*20*), although the time to reach maximum plasma concentration was longer than that reported by Miyazawa et al. (*17*) for cyanidin 3-glucosides and cyanidin 2,5-diglycosides and that reported by Matsumoto et al. (*20*) for black currant anthocyanins. This may be due to differences in the degree of processing of the anthocyanin-containing fruit product (freeze-dried blueberry powder versus spray-dried elderberry juice concentrate) and/or differences in the diets supplemented with anthocyanin-rich material. In this study, subjects (45−49 years) received the anthocyanin-rich blueberry powder with a high-fat meal; in the study reported by Miyazawa et al. (*17*) the subjects were 20−29 years old and received a typical Japanese diet. For other flavonoids, the concentration of quercetin glycosides from apples in human plasma has been reported to peak at 2.5 ± 0.72 h after oral intake, and peak levels for rutin (quercetin 3-rutinoside) were reached 9.3 ± 1.8 h after ingestion (*39*). Therefore, the absorption rate of anthocyanins and other flavonoids in humans may be affected by the chemical structure of the compound, as well as other factors including chemical interaction with other food and intestinal components, as well as structural and compositional characteristics of the source of the anthocyanin/flavonoid being studied.

HPLC chromatograms of the control serum samples spiked with the blueberry supplement extract (**Figure 1D**) revealed the presence of 25 anthocyanins, which were identified as described by Gao and Mazza (*8*, *36*). The results presented here show the presence in lowbush blueberries of the nonacylated and acetylated forms of galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin, and malvidin, and these results

Table 5. Recovery[a] of Anthocyanins from Cleanup Process

		peak areas		
anthocyanin[b]	RT[c]	diet extract	serum spike	% recovery
del-3-gal	21.40	21.82	17.07	78.2
del-3-glu	23.78	30.12	23.37	77.6
cy-3-gal	25.78	9.81	7.77	79.2
del-3-ara	27.05	11.05	7.93	71.7
cy-3-glu	28.73	13.17	10.54	80.1
pet-3-gal	30.61	10.37	8.34	80.4
cy-3-ara	31.52	5.10	3.91	76.7
pet-3-glu	33.14	20.27	16.38	80.8
pn-3-gal	34.85	2.39	1.98	83.1
pet-3-ara	36.13	4.90	3.70	75.5
pn-3-ara	37.37	6.67	5.36	80.3
mal-3-gal	37.89	18.33	15.10	82.4
mal-3-glu	39.56	32.89	26.70	81.4
mal-3-ara	41.75	8.13	6.29	77.4
del-3-glu-ac	44.49	7.47	4.92	65.9
cy-3-glu-ac	47.64	5.17	3.43	66.2
mal-3-gal-ac	48.64	3.37	2.35	69.9
pet-3-glu-ac	49.16	6.20	4.34	70.0
mal-3-glu-ac	52.37	14.47	12.21	84.4
mean				76.9

[a] Comparison of anthocyanin peak areas from diet extract before and after spiking into blood serum. [b] Anthocyanin identity as in **Table 4**; ac, acetylated. [c] Component retention time (minutes).

are in full agreement with our earlier findings (*8*). The major nonacylated anthocyanins were the 3-galactosides and 3-glucosides of delphinidin and malvidin (**Figure 1D**, peaks 1, 2, 12, and 13), and the major acetylated anthocyanins were the 3-acetylglucosides of delphinidin, cyanidin, petunidin, and malvidin (peaks 16, 21, 23, and 25). A comparison of the concentration of the major 19 anthocyanins present in the methanolic extract of the freeze-dried blueberry supplement and in control serum samples spiked with the blueberry supplement extract (**Table 5**) shows that the recovery of anthocyanins from the serum sample cleanup process averaged 76.9%. Lower recovery values were obtained for acylated anthocyanins (peaks 15−19, **Figure 1D**) than for nonacylated structures. These results indicate that during sample preparation some anthocyanins may be more or less effectively separated from the other blood serum components, although there is a possibility that some acetylated anthocyanins may be hydrolyzed to anthocyanin glucosides and acetic acid by hydrolase in the control blood serum samples. This, however, remains to be demonstrated, and the metabolism and functions of these circulating anthocyanins as reported here in human subjects require further studies.

Serum Antioxidant Status. The blueberry treatment appeared to prevent a mean decrease in serum antioxidant capacity as experienced in the control group following the consumption of a high-fat meal (as determined by TEAC analysis, treatment effect $P = 0.001$) (**Table 6**). The consumption of both of the treatment meals was associated with a significant time effect, which was a result of a progressive increase in serum $ORAC_{total}$ ($P < 0.001$) and $ORAC_{acetone}$ ($P < 0.001$), over time (**Table 6**). Furthermore, the blueberry treatment was associated with a significant increase in serum lipid-soluble antioxidant status ($ORAC_{acetone}$, $P = 0.04$) above the control at the 4 h time point (**Table 6**).

An increase from baseline in the serum antioxidant capacity using the ORAC assay ($ORAC_{total}$ and $ORAC_{acetone}$) following consumption of the high-fat meal occurred in the control group (as well as the blueberry treatment group) regardless of an essentially antioxidant-free control supplement. No such increase was found in the control group when using the TEAC assay.

Table 6. Percent Change in Serum Antioxidant Capacity and Triacylglycerol over Time

		treatment				
		control		blueberry		
measure	time	mean	SD	mean	SD	*P* value
TEAC[a,e]	0	0.0	0.0	0.0	0.0	
	1	–6.1	8.2	0.6	4.3	0.07
	2	–4.0	3.9	–1.1	6.8	0.55
	3	–3.5[d]	2.6	–2.7	5.4	0.84
	4	–4.3	4.0	–1.7	5.2	0.49
$ORAC_{total}$[b,f]	0	0.0	0.0	0.0	0.0	
	1	1.9	12.7	0.2	14.8	0.87
	2	15.2	17.6	11.3	7.7	0.68
	3	29.0	19.0	39.1[d]	10.0	0.16
	4	43.5[d]	14.1	50.5[d]	8.9	0.06
$ORAC_{PCA}$	0	0.0	0.0	0.0	0.0	
	1	0.2	3.8	6.5	10.7	0.24
	2	1.4	12.4	–1.3	8.5	0.79
	3	6.2	17.2	–4.1	4.4	0.23
	4	–1.5	7.1	1.9	11.2	0.36
$ORAC_{acetone}$[b,f]	0	0.0	0.0	0.0	0.0	
	1	2.1	17.9	0.6	12.8	0.88
	2	17.3	21.0	17.4	11.3	0.99
	3	40.3[d]	13.1	48.5[d]	11.4	0.10
	4	50.7[d]	14.2	58.9[d]	10.1	0.04[c]
triacylglycerol[b,f]	0	0.0	0.0	0.0	0.0	
	1	13.1[d]	2.5	4.4	5.7	0.46
	2	26.3[d]	15.8	21.6	11.7	0.63
	3	40.3[d]	13.2	40.5[d]	6.4	0.97
	4	50.6[d]	10.8	55.2[d]	5.3	0.44

[a] Treatment effect as determined by repeated measures ANOVA ($P < 0.05$). [b] Time effect as determined by repeated measures ANOVA ($P < 0.01$). [c] Significantly different from control group as determined by paired *t* test ($P < 0.05$). [d] Significantly different from baseline ($t = 0$) as determined by paired *t* test ($P < 0.05$). [e] Significant difference at baseline ($t = 0$) between treatment groups as determined by paired *t* test ($P = 0.05$). [f] Analyses sharing the same f-subscript are positively correlated ($P < 0.001$).

This dissimilarity in findings between the two assays is likely a result of mechanistic differences between the assays. The TEAC assay measures the inhibition of free radical action, which is similar to the basis of the ORAC assay; however, the TEAC assay measures the inhibition at a fixed time (10 min), whereas the ORAC assay measures the time it takes for this reaction to reach completion (≥60 min). Measuring the reaction to completion may allow the oxidation of fatty acids within absorbed triacylglycerol particles (TAG) (as a result of the high-fat meal) to retard the decay of the target molecule (*R*-PE) under these experimental conditions. Results of correlation analysis in fact reveal that the appearance of TAG in blood is correlated with increases in $ORAC_{total}$ and $ORAC_{acetone}$ fractions ($P < 0.01$). Although a correlation exists, further research is needed to determine the effect of TAG on the antioxidant assays used in this trial.

Correlation between Serum Anthocyanin Content and Postprandial Antioxidant Status. Figure 2 shows that there was a significant positive correlation between serum anthocyanin content and postprandial antioxidant status ($P < 0.01$). These results suggest that the compounds within the blueberry responsible for the increase in serum antioxidant status above the control group are most likely the anthocyanins. Anthocyanins comprise the highest concentration of all phenolic subgroups in the blueberry (*1*, *8*, *10*). Previous research has determined that the ORAC value of the blueberry correlates more strongly with anthocyanins than with total phenolics in the ripe berry

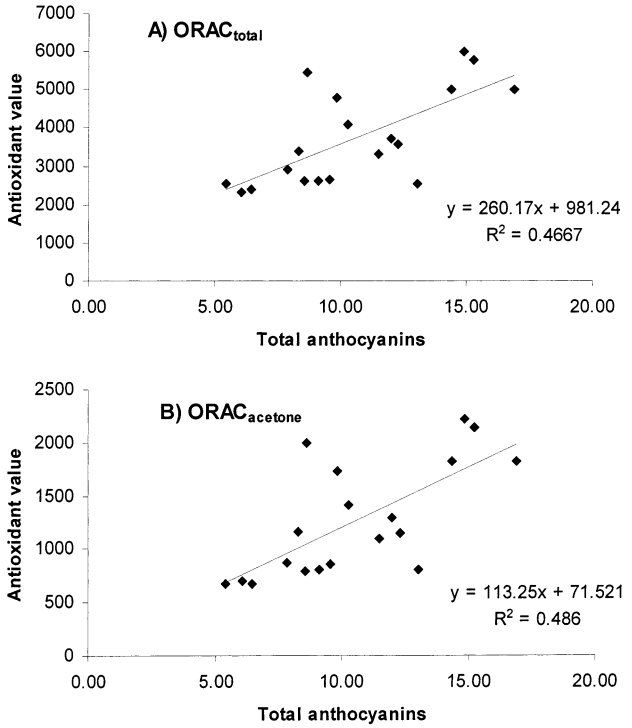

Figure 2. Correlation between serum antioxidant capacity and concentration of serum total anthocyanins. Antioxidant value expressed as micromoles of Trolox equivalents per liter, and anthocyanins expressed (nanograms per milliliter of serum) as cyanidin 3-glucoside chloride.

(*35*). Furthermore, the antioxidant properties of anthocyanins have been validated using other systems of oxidation such as their ability to prevent low-density lipoprotein (LDL) oxidation in vitro (*40*). Therefore, although we cannot exclude other phenolics within the blueberries as contributing to the observed effects in the blueberry treatment, studies have shown that the in vitro antioxidant properties of wild blueberries are mainly a result of their high concentration of anthocyanins and not their concentrations of antioxidant vitamins, minerals, or fibers (*11*−*13*).

Although we have demonstrated that supplementation with a freeze-dried wild blueberry powder was associated with an increased serum antioxidant capacity in the postprandial state, the magnitude of the observed response cannot be arbitrarily translated into a decreased risk of chronic degenerative disease. Studies have indicated an increased risk of chronic disorders in individuals with low levels of fasting antioxidants (*41*). However, at the present time there are insufficient data in the literature to determine the magnitude of chronic degenerative disease risk reduction with given increases in serum/plasma antioxidant status.

In conclusion, the results of this study show that anthocyanins can be absorbed in their intact glycosylated and possibly acylated forms in middle-aged men soon after the consumption of blueberries and that the presence of anthocyanins in the serum may be involved with a diet-induced increase in ex vivo serum antioxidant status.

LITERATURE CITED

(1) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press: Boca Raton, FL, 1993; Chapter 4, pp 85−130.

(2) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481−504.

(3) Hollman, P. C. H.; Katan, M. B. Dietary flavonoids: Intake, health effects and bioavailability. *Food Chem. Toxicol.* **1999**, *37*, 937−942.

(4) Peterson, J.; Dwyer, J. Flavonoids: Dietary occurrence and biochemical activity. *Nutr. Res.* **1998**, *18*, 1995−2018.

(5) Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401−436.

(6) Moncada, S.; Palmer, R. M.; Higgs, E. A. Nitic oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109−142.

(7) Francis, F. J. Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 273−314.

(8) Gao, L.; Mazza, G. Characterization of acylated anthocyanins in lowbush blueberries. *J. Liq. Chromatogr.* **1995**, *18*, 245−259.

(9) Wang H.; Cao, G.; Prior, R. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44*, 701−705.

(10) Camire, M. E. Bilberry and blueberries as functional foods and nutraceuticals. In *Herbs, Botanicals and Teas as Functional Foods and Nutraceuticals*; Mazza, G., Oomah, B. D., Eds.; CRC Press: Boca Raton, FL, 2000; pp 389−319.

(11) Kalt, W.; Forney, C. F.; Martin, A.; Prior, R. L. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* **1999**, *47*, 4638−4644.

(12) Bushway, R. B.; McGann, D. F.; Cook, W. P.; Bushway, A. A. Mineral and vitamin content of lowbush blueberries (*Vaccinium angustifolium*). *J. Food Sci.* **1983**, *48*, 1878−1880.

(13) Prior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwan, J.; O'Brein, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686−2693.

(14) Cook, N. C.; Samman, S. Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem.* **1996**, *7*, 66−76.

(15) Paganga, G.; Rice-Evans, C. A. The identification of flavonoids as glycosides in human plasma. *FEBS Lett.* **1997**, *401*, 78−82.

(16) Lapidot, T.; Harel, S.; Granit, R.; Kanner, J. Bioavailability of red wine anthocyanins as detected in human urine. *J. Agric. Food Chem.* **1998**, *46*, 4297−4302.

(17) Miyazawa, T.; Nakagawa, K.; Kudo, M.; Muraishi, K.; Someya, K. Direct intestinal absorption of red fruit anthocyanins, cyanindin-3-glucoside and cyanidin-3,5-diglucoside, into rat and humans. *J. Agric. Food Chem.* **1999**, *47*, 1083−1091.

(18) Cao, G.; Prior, R. L. Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clin. Chem.* **1999**, *45*, 574−576.

(19) Cao, G.; Muccitelli, H. U.; Sánchez-Moreno, C.; Prior, R. L. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. *Am. J. Clin. Nutr.* **2001**, *73*, 920−926.

(20) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Horayama, M.; Tsuda, T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J. Agric. Food Chem.* **2001**, *49*, 1546−1551.

(21) Serafini, M.; Maiani, G.; Ferro-Luzzi, A. Alcohol-free red wine enhances plasma antioxidant capacity in humans. *J. Nutr.* **1998**, *128*, 1003−1007.

(22) Cao, G.; Russell, R. M.; Lischner, N.; Prior, R. L. Serum antioxidant is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J. Nutr.* **1998**, *128*, 2383−2390.

(23) Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi, S.-W.; Kawakishi, S.; Osawa, T. Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-*β*-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407−2410.

(24) Mazza, G. Anthocyanins in edible plant: a qualitative and quanitiative assessment. In *Antioxidant Methodology*; Aruoma, O. I., Cuppett, S. L., Eds.; AOCS Press: Champaign, IL, 1997; pp 119−140.
(25) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304−309.
(26) Narayan, M. S.; Naidu, K. A.; Ravishankar, G. A.; Srinivas, L.; Venkataraman, L. V. Antioxidant effect of anthocyanin on enzymatic and non-enzymatic lipid peroxidation. *Prostaglandins Leukotrienes, Essent. Fatty Acids* **1999**, *60*, 1−14.
(27) Fukumoto, L. R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 3597−3604.
(28) Castelli, W. P. The new pathophysiology of coronary artery disease. *Am. J. Cardiol.* **1998**, *82*, 60T−65T.
(29) Kaplan, M.; Aviram, M. Oxidized low-density lipoprotein: Atherogenic and proinflammatory characteristics during macrophage foam cell formation. An inhibitory role for nutritional antioxidants and plasma paraoxonase. *Clin. Chem. Lab. Med.* **1999**, *37*, 777−787.
(30) Georgopoulos, A. Postprandial triglyceride metabolism in diabetes mellitus. *Clin. Cardiol.* **1999**, *22*, 28S−33S.
(31) Vendemiale, G.; Grattagliano, I.; Altomare, E. An update on the role of free radicals and antioxidant defense in human disease. *Int. J. Clin. Lab. Res.* **1999**, *29*, 49−55.
(32) Ames, B. N.; Gold, L. S.; Willett, W. C. The causes and prevention of cancer. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5258−5265.
(33) Willett, W. C. Diet and cancer: One view at the start of the millennium. *Cancer Epidemiol. Biomarkers Prev.* **2001**, *10*, 3−8.
(34) Satué-Gracia, T.; Heinonen, M.; Frankel, E. Anthocyanins as antioxidants on human low-density liposome systems. *J. Agric. Food Chem.* **1997**, *45*, 3362−3367.
(35) Wang, J.; Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 850−857.
(36) Gao, L.; Mazza, G. Rapid method for complete chemical characterization of simple and acylated anthocyanins by high-performance liquid chromatography and capillary gas−liquid chromatography. *J. Agric. Food Chem.* **1994**, *42*, 118−125.
(37) Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* **1993**, *14*, 303−311.
(38) Cao, G.; Prior, R. L. Comparison of different analytical methods for assessing total antioxidant capacity of human plasma. *Am. J. Clin. Nutr.* **1998**, *44*, 1309−1315.
(39) Hollman, P. C. H.; van Trijp, J. M. P.; Buysman, N. C. P.; Baag, M. S.; Mengelers, M. J. B.; de Vries, J. H. M.; Katan, M. B. Relative bioavailability of the antioxidant quercetin from various foods in man. *FEBS Lett.* **1997**, *418*, 152−156.
(40) Laplaud, P. M.; Lelubre, A.; Chapman, M. J. Antioxidant action of *Vaccinium myrtillus* extract on human low-density lipoproteins in vitro: initial observations. *Fundam. Clin. Pharmacol.* **1997**, *11*, 35−40.
(41) Lachance, P. A. Overview of key nutrients: Macronutrient aspects. *Nutr. Rev.* **1998**, *56*, S34−S39.

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