JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of Analyses by High-Performance Liquid Chromatography and Liquid Chromatography/Tandem Mass Spectrometry of Bilberry (*Vaccinium myrtilus*) Anthocyanins in Human Plasma and Urine

DARREN N. COOKE,† SARAH THOMASSET,† DAVID J. BOOCOCK,† MICHAEL SCHWARZ,‡ PETER WINTERHALTER,‡ WILLIAM P. STEWARD,† ANDREAS J. GESCHER,† AND TIMOTHY H. MARCZYLO*,†

Cancer Biomarkers and Prevention Group, Department of Cancer Studies and Molecular Medicine, University of Leicester, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, United Kingdom, and Institute of Food Chemistry, Technical University of Braunschweig, Braunschweig, Germany

Anthocyanins are potent antioxidants that may possess chronic disease preventive properties. Here, rapid, reliable, and reproducible solid-phase extraction, high-performance liquid chromatography (HPLC), and mass spectrometry techniques are described for the isolation, separation, and identification of anthocyanins in human plasma and urine. Recoveries of cyanidin-3-glucoside (C3G) were 91% from water, 71% from plasma, and 81% from urine. Intra- and interday variations for C3G extraction were 9 and 9.1% in plasma and 7.1 and 9.1% in urine and were less than 15% for all anthocyanins from a standardized bilberry extract (mirtoselect). Analysis of mirtoselect by HPLC with UV detection produced spectra with 15 peaks compatible with anthocyanin components found in mirtoselect within a total run time of 15 min. Chromatographic analysis of human urine obtained after an oral dose of mirtoselect yielded 19 anthocyanin peaks. Mass spectrometric analysis employing multiple reaction monitoring suggests the presence of unchanged anthocyanins and anthocyanidin glucuronide metabolites.

KEYWORDS: Anthocyanins; high-performance liquid chromatography; mass spectrometry; solid-phase extraction; *Vaccinium myrtilus*; bilberry

INTRODUCTION

Anthocyanins are a family of polyhydroxy and polymethoxy derivatives of 2-phenylbenzoyrylium (flavylium cation) found abundantly in nature. As glycosides or acylglycosides of anthocyanidins, their aglycons, anthocyanins are responsible for red and blue pigmentation in fruits, berries, and petals. Dietary anthocyanins are thought to decrease the risk of chronic degenerative disorders such as cardio- and cerebrovascular disease, atherosclerosis, cancer, diabetes, and failing vision (*1*−*6*). Such beneficial effects on health may be associated with the strong antioxidant characteristics of anthocyanins (*7*). The abundance of anthocyanins in the diet (180−215 mg/day; *8*) when compared with other flavonoids such as quercetin, luteolin, kaempferol, and apigenin (23 mg/day, *9*) warrants investigation into their role in human health. Anthocyanins have been shown to inhibit cancer cell growth in vitro and to decrease tumor burden in certain experimental models of carcinogenesis (for review, see ref *2*). Bilberry (*Vaccinium myrtilus*), a favorable source of dietary anthocyanins, contains 15 anthocyanins, glucosides, galactosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (for structures, see **Figure 1**).

In order to rationalize the potential effects of anthocyanins on human health, robust analytical techniques must be developed to allow detection and quantitation of parent species and metabolites in biomatrices. To our knowledge, there are five published analytical methods describing the analysis of anthocyanins in bilberry and other *Vaccinium spp* (*10*−*15*). In one of these methods, anthocyanins were deconjugated to anthocyanidins (*10*). There is only one report in which 25 anthocyanins found in low bush blueberry (*Vaccinium augustifolium*) were separated, albeit not all to baseline, employing a chromatographic run lasting 60 min (*11*). This run time is typical for most published methods. The noticeable exception is a capillary zone electrophoresis method that separated 12 anthocyanin peaks with a run time of only 10 min (*14*). Limited validation of some of these methods has been performed but not for anthocyanins from plasma and urine. Here, we describe a simple and fully validated solid-phase extraction (SPE)

* To whom correspondence should be addressed. Tel: +44 1162231859. Fax: +44 1162231855. E-mail: thm3@le.ac.uk.
† University of Leicester.
‡ Technical University of Braunschweig.

10.1021/jf061562q CCC: $33.50
Published on Web 08/23/2006

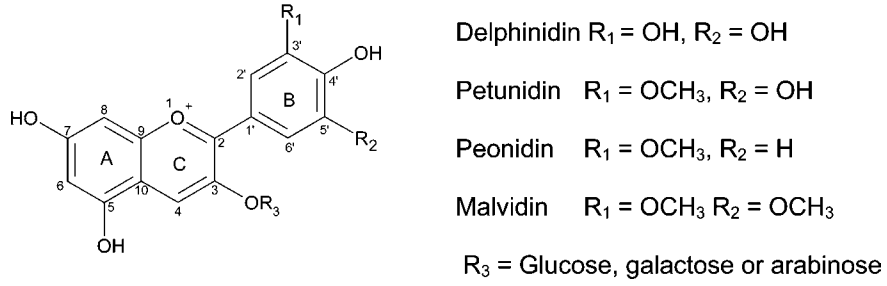

Figure 1. Structures of anthocyanins present in standardized bilberry extract (mirtoselect).

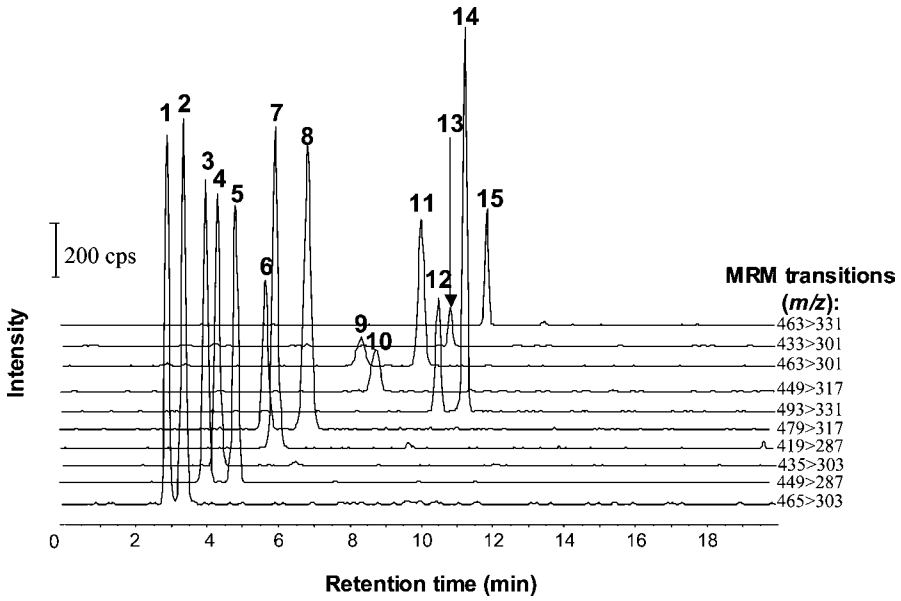

Figure 2. LC/MS/MS analysis of anthocyanins in mirtoselect. The following species were identified utilizing MRM transitions (values in *m*/*z*) obtained by loss of the sugar moiety: Peaks 1 and 2, delphinidin-3-galactoside/delphinidin-3-glucoside (465 > 303); peaks 3 and 5, cyanidin-3-galactoside/C3G (449 > 287); peak 4, delphinidin-3-arabinoside (435 > 303); peaks 6 and 8, petunidin-3-galactoside/petunidin-3-glucoside (479 > 317); peak 7, cyanidin-3-arabinoside (419 > 287); peaks 9 and 11, peonidin-3-galactoside/peonidin-3-glucoside (463 > 301); peak 10, petunidin-3-arabinoside (449 > 317); peaks 12 and 14, malvidin-3-galactoside/malvidin-3-glucoside (493 > 331); peak 13, peonidin-3-arabinoside (433 > 301); and peak 15, malvidin-3-arabinoside (463 > 331). Chromatograms are representative of five analyses.

technique for the isolation from plasma and urine of cyanidin-3-glucoside (C3G), the most abundant anthocyanin in diet, and of anthocyanins from a standardized bilberry extract and their metabolites. The described rapid high-performance liquid chromatography (HPLC) method is an improvement on previous methods in terms of speed and/or separation. Furthermore, an HPLC-electrospray ionization (ESI) tandem mass spectrometric (LC/MS/MS) method is described, which confirms the presence and identity of 15 bilberry anthocyanins and their metabolites in human urine.

MATERIALS AND METHODS

Extracts, Biomatrices, and Consumption of Bilberry Anthocyanins. C3G was purified from blackberries using a countercurrent chromatographic method as described previously (*16*). Mirtoselect, a bilberry extract suitable for human consumption and standardized for anthocyanin content, was provided by Indena S.p.A (Milan, Italy, see http://www.indena.it/pdf/mirtoselect.pdf). The extract comprises 36% anthocyanins consisting of 15 different components, i.e., glucosides, galactosides, and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (for structures, see **Figure 1**). Identities of these anthocyanin components, as provided by the manufacturer, were confirmed by LC/MS/MS analysis (see below) employing specific multiple reaction monitoring (MRM) transitions (**Figure 2**). Reagents used in the chemical analysis were purchased from Fisher Scientific UK (Loughborough, United Kingdom).

A healthy male volunteer provided human urine for method development purposes. Human plasma was obtained from the National Blood Transfusion Centre (Sheffield, United Kingdom). To assess the ability of these methods to analyze anthocyanin content in biomatrices, urine was also collected from a healthy male volunteer (37 years old, 105 kg) who had imbibed 450 mL of a 1% (w/v) solution of mirtoselect (containing 1.62 g of anthocyanins). Urine was collected before and 30, 60, 90, 120, 180, 360, and 420 min after administration.

Extraction of Anthocyanins from Biomatrices. The extraction method for urine or plasma was validated for extraction efficiency ($n = 20$), intraday ($n = 5$), and interday ($n = 3 \times 5$) variability. Urine and plasma were spiked with 5 ng/mL of C3G or 50 ng/mL mirtoselect, respectively. Aliquots of spiked plasma (1 mL) or urine (1 mL) were centrifuged (16000*g*, 5 min) to remove particulates. Oasis HLB SPE cartridges (1 mL; Waters, Elstree, United Kingdom) were conditioned sequentially with 1 mL of acetone:formic acid (9:1) and 1 mL of water:formic acid (9:1). Analytical samples (1 mL) were loaded and eluted at a flow rate of 1 mL/min. Anthocyanins were eluted sequentially with 0.2 and then 0.1 mL of acetone:formic acid (9:1). At all stages of the solid-phase extraction procedure, a vacuum was used to draw solvents through the cartridge until it was dry. Pooled eluants were

evaporated to dryness (stream of nitrogen). Residues were reconstituted in water:formic acid (9:1, 75 μL) and centrifuged (16000*g*, 10 min, 4 °C) prior to analysis.

Separation of Bilberry Anthocyanins by HPLC. Mirtoselect and human biomatrices were analyzed for anthocyanin content by HPLC with UV−visible detection (520 nm). The HPLC system (Varian Analytical Instruments, Oxford, United Kingdom) comprised a Varian 230 pump, Varian 410 autosampler, and Varian 325 UV−vis detector. Separation of anthocyanins was performed using an Xterra Phenyl column (Waters; 4.6 mm × 150 mm, 5 μm) with guard column (Waters, 4.6 mm × 10 mm, 5 μm) at 40 °C with a flow rate of 1.5 mL/min. The gradient elution system comprised two solvents: A, 9:1 water: formic acid, pH ≅ 1.6; and B, acetonitrile. The gradient employed was as follows: 99 to 97% A for 5 min, 97 to 90% A for 3 min, unchanged for 4 min, increase of A up to 70% within 1 min, and then maintained for 2 min.

Identification of Anthocyanins and Metabolites by LC/MS/MS. LC/MS/MS analysis was performed using an API2000 mass spectrometer (Applied Biosystems, Warrington, United Kingdom) with sample delivery via an 1100 series HPLC instrument (Agilent Technologies UK Ltd., South Queensferry, United Kingdom). The HPLC separation used was essentially as described above with the following modifications: Xterra phenyl column (2.1 mm × 150 mm, 3.5 μm) with guard (2.1 mm × 20 mm, 3.5 μm), solvent system A was held at 90% for an additional 5 min, and the flow rate was reduced to 0.31 mL/min to allow direct injection into the mass spectrometer without the need for eluant splitting. Mass spectrometric analyses were performed in positive ion mode under the following conditions: declustering potential, 55 V, focusing potential, 380 V; entrance potential, 10 V; collision energy, 50 V; collision energy exit potential, 16 V; ion spray voltage, 5000 V; and temperature, 450 °C. Anthocyanins were identified by MRM for fragments generated by the loss of the sugar moiety, glucuronides by monitoring of fragments that had lost glucuronic acid (176 amu), and anthocyanin glucuronides by combined loss of sugar and glucuronic acid. The presence of anthocyanidins was determined by specific MRM transitions as follows: delphinidin (303 > 229), cyanidin (287 > 137), petunidin (317 > 217), peonidin (301 > 201), and malvidin (331 > 242).

RESULTS AND DISCUSSION

Validation of Anthocyanin Extraction. Efficiency of extraction from human plasma for C3G was 71%, with intra- and interday variations of 9.0 and 9.1%, respectively. The efficiency of extraction from human urine was 81% with 7.1 and 9.1% for intra- and interday variations, respectively. Extraction efficiencies for bilberry anthocyanins averaged 91% and varied from 65% for peonidin-3-glucoside to 102% for malvidin-3-glycosides. These recoveries are in agreement with previously published data. Mazza et al. (*11*), using a SPE method, retrieved 70−84% of blueberry anthocyanins from human serum (1.5 mL). Although the method described here has no advantage over the earlier method (*11*) with respect to recovery, it is considerably more rapid, and as compared with evaporating 7 mL of methanol used to elute anthocyanins in the earlier paper, evaporation of 300 μL of acetone is quicker.

Separation of Anthocyanins in Bilberry Extract and Detection of Unchanged Anthocyanins and Their Metabolites in Human Urine. Analysis of an aqueous solution of mirtoselect using the HPLC method employed here was capable of resolving 15 anthocyanins within a run time of 15 min (**Figure 3A**). When the anthocyanins from mirtoselect were extracted by SPE and reconstituted for analysis by HPLC, the resultant chromatograms showed no discernible differences in terms of anthocyanin peaks between the original solution of authentic mirtoselect and the SPE eluant (**Figure 3B**). Hence, the SPE method did not introduce any significant bias in terms of extraction efficiency for any individual anthocyanins. The retention time of C3G (4.6 min) was characterized by a coefficient of variation of 1.23 (based on 10 injections; **Table 1**). In 10 repeated injections of mirtoselect, the retention times of the individual anthocyanins had coefficients of variation between 0.33 and 1.68, and their retention indices, as compared with C3G, displayed coefficients of variation between 0.19 and 1.15 (**Table 1**). Analysis of spiked human plasma (1 mL) furnished lower limits of detection (LOD; S/N > 3) and quantitation (LOQ; S/N > 10) of 0.5 and 1.5 ng/mL (1 and 3 nM), respectively. Extraction from spiked urine or aqueous solution gave LOD and LOQ values of 0.25 and 1.0 ng/mL (0.5 and 2 nM), respectively. These numbers suggest good sensitivity when compared to previously published methods of extraction of anthocyanins from bilberry (*10*). The LOD and LOQ values determined here for urinary anthocyanins are comparable to those described before for anthocyanins ingested with foodstuffs other than bilberries (*18*, *19*). Detection was linear over the range 1−1000 ng/mL ($r^2 = 0.999$) for C3G in plasma and urine; accuracy was 100 ± 9 and 100 ± 6% for plasma and urine, respectively.

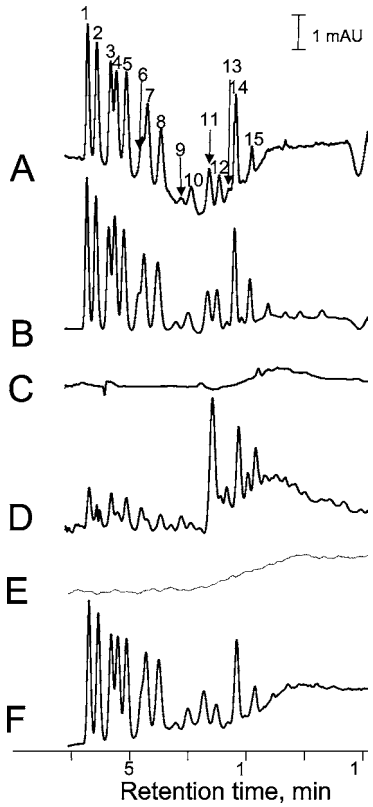

Figure 3. Analysis by HPLC of extracts containing bilberry anthocyanins (mirtoselect) from the following matrices: (**A**) aqueous solution of mirtoselect (10 μg/mL) prior to SPE, (**B**) aqueous solution of mirtoselect (350 ng/mL), (**C**) human urine sample collected prior to mirtoselect consumption, (**D**) urine sample collected 6 h after a bolus dose of 4.5 g of mirtoselect, (**E**) human plasma, and (**F**) human plasma spiked with 350 ng/mL mirtoselect. Extracts B–F underwent SPE. Urine and plasma collection and anthocyanin extraction and separation were as described in the Materials and Methods. Peaks 1–15 are those described in **Figure 2**. Chromatograms are representative of three separate analyses.

In order to demonstrate the practical application of the techniques described above, urine from a healthy volunteer who had consumed mirtoselect was investigated for anthocyanin content. Analysis of urine by HPLC yielded 19 peaks (**Figure 3D**). The majority of these peaks had retention times comparable to those of parent anthocyanins found in mirtoselect, but the relative ratios of individual anthocyanins were different from

Table 1. Repeatability of Retention Time (t_R) and Retention Index (i_R) Relative to C3G for Anthocyanins in a Standardized Extract of Bilberry[a]

peak	anthocyanin	*n*	$t_R \pm$ SD (min)	CV	$i_R \pm$ SD	CV
		C3G standard				
5	C3G	10	4.64 ± 0.06	1.23	n/a	n/a
		bilberry extract				
1	delphinidin-3-galactoside	10	3.01 ± 0.04	1.30	0.65 ± 0.00	0.68
2	delphinidin-3-glucoside	10	3.40 ± 0.05	1.34	0.74 ± 0.00	0.52
3	cyanidin-3-galactoside	10	3.94 ± 0.05	1.28	0.86 ± 0.00	0.22
4	delphinidin-3-arabinoside	10	4.20 ± 0.06	1.39	0.91 ± 0.00	0.19
5	C3G	10	4.60 ± 0.06	1.28	1.00 ± 0.00	0.00
6	petunidin-3-galactoside	10	5.29 ± 0.06	1.08	1.15 ± 0.01	0.78
7	cyanidin-3-arabinoside	10	5.47 ± 0.06	1.04	1.19 ± 0.01	0.38
8	petunidin-3-glucoside	10	6.09 ± 0.07	1.19	1.32 ± 0.01	0.53
9	peonidin-3-galactoside	10	6.89 ± 0.12	1.68	1.50 ± 0.02	1.14
10	petunidin-3-arabinoside	10	7.42 ± 0.08	1.06	1.61 ± 0.01	0.78
11	peonidin-3-glucoside	10	8.21 ± 0.06	0.74	1.78 ± 0.02	0.97
12	malvidin-3-galactoside	10	8.60 ± 0.05	0.57	1.87 ± 0.02	1.02
13	peonidin-3-arabinoside	10	9.02 ± 0.04	0.45	1.96 ± 0.02	1.07
14	malvidin-3-glucoside	10	9.34 ± 0.04	0.38	2.03 ± 0.02	1.09
15	malvidin-3-arabinoside	10	10.0 ± 0.03	0.33	2.18 ± 0.03	1.15

[a] *n*, number of samples; SD, standard deviation; CV, coefficient of variation {(SD/mean) × 100}.

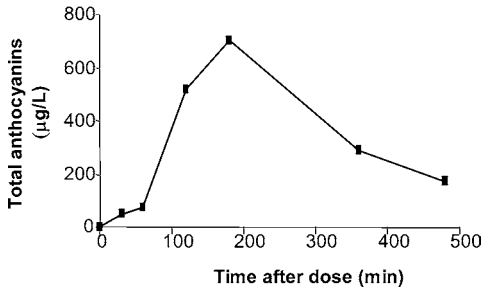

Figure 4. Time-dependent excretion of anthocyanin species in the urine of a human who ingested a single oral dose of 4.5 g of mirtoselect. Analysis was by HPLC as described in the Materials and Methods, and concentrations of all anthocyanin species are calculated based on standards of authentic C3G. Results are the means of three replicates.

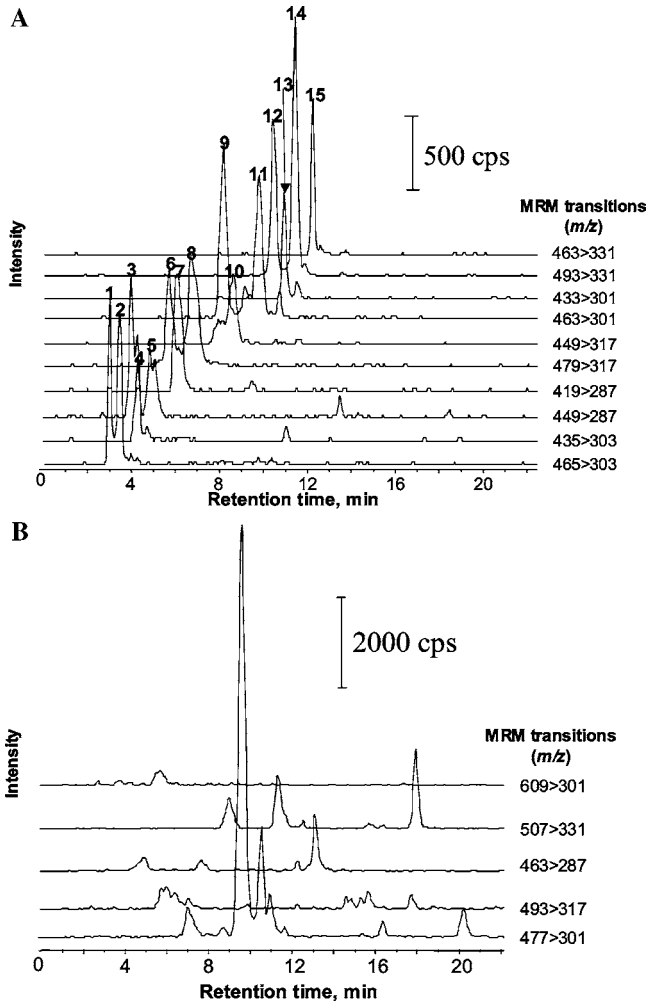

Figure 5. Analysis for anthocyanin species by LC/MS/MS of samples of urine from a human 3 h after ingestion of 4.5 g of mirtoselect. (**A**) Spectra of unchanged anthocyanins contained in mirtoselect; (**B**) spectra of anthocyanin metabolites peonidin arabinoside glucuronides (609 > 301), malvidin glucuronides (507 > 331), cyanidin glucuronides (463 > 287), petunidin glucuronides (493 > 317), and peonidin glucuronides (477 > 301). Analyses were performed as described in the Materials and Methods using MRM; spectra are representative of at least three analyses.

those observed with authentic mirtoselect (**Figure 3A**,**B**). The retention times of the predominant anthocyanins in human urine are indicative of methylated cogeners, peonidin and malvidin glycosides. The largest peak had a retention time similar to that of peonidin-3-glucoside (**Figure 3D**). In contrast, in the urine of mice, which had received mirtoselect in their diet, malvidin-3-galactoside was the predominant anthocyanin species (*20*). Levels of total anthocyanins excreted in urine (**Figure 4**) were consistent with those previously observed in humans taking elderberry or grape anthocyanins (*17*, *18*, *21*−*23*). In the individual studied here, maximal excretion was slightly later than that described in earlier studies of elderberry and grape extracts where t_{max} was between 1 and 2 h (*16*, *17*, *20*−*22*).

Identification of Anthocyanin Species in Human Urine. Urine obtained from a human who had consumed mirtoselect was investigated by LC/MS/MS in MRM mode. The presence of unmodified parent compounds was unequivocally demonstrated (**Figure 5A**), and the predominant anthocyanin species was identified as a glucuronide of peonidin (**Figure 5B**), consistent with the results obtained by HPLC (**Figure 3D**) Given the complicated mixture of anthocyanins in mirtoselect, LC/MS/MS analysis of human urine after consumption of mirtoselect detected several anthocyanin metabolites (**Figure 5B**). Upon the basis of their MRM transitions, the predominant metabolites at all time points were glucuronides of the following species: peonidin arabinoside (*m*/*z* 609 > 301), malvidin (*m*/*z* 507 > 331), cyanidin (*m*/*z* 463 > 287), petunidin (*m*/*z* 493 > 317), and peonidin (*m*/*z* 477 > 301) (**Figure 5B**). Metabolites of bilberry anthocyanins observed at the limits of detection were delphinidin glucuronide (*m*/*z* 479 > 303), malvidin arabinoside glucuronide (*m*/*z* 639 > 331), peonidin glycoside glucuronides (*m*/*z* 639 > 301), malvidin glycoside glucuronides (*m*/*z* 669 > 331), and petunidin glycoside glucuronides (*m*/*z* 655 > 317). Further studies are required to unequivocally identify these glucuronide metabolites. The results are in agreement with previous investigations of anthocyanin bioavailability in humans, according to which anthocyanins are excreted unchanged (*12*, *17*−*18*, *21*−*23*). The metabolite profile in rodents, which have ingested anthocyanins, has been reported to be dominated by methylated and glucuronidated metabolites (*19*, *24*−*26*). The occurrence of anthocyanidin glucuronides in the urine of humans, who have ingested anthocyanins, described here and previously (*18*, *23*), is consistent with the notion that anthocyanins can undergo deconjugation to their aglycons, which are then further biotransformed. The intermediate metabolic forma-

tion of anthocyanidins from anthocyanins has been buttressed by analytical results in rodents (*19*, *25*). Anthocyanidins are suspected to exert more potent pharmacological activity than their glycosidic precursors (*2*).

In conclusion, the methods described here seem suitable for application in the analysis of anthocyanins in bilberry extract and in mammalian biomatrices after ingestion of dietary anthocyanins. The advantages of these methods over previously published ones are speed and resolution of analytes and lack of utilization of organic solvents. These methods may therefore be useful in the clinical evaluation of the effects of anthocyanins on health.

ACKNOWLEDGMENT

We thank Indena SpA (Milan, Italy) for the provision of Mirtoselect and the staff of the Biomedical Services Unit, University of Leicester, for their help with animal husbandry.

LITERATURE CITED

(1) Andriambeloson, E.; Magnier, C.; Haan-Archipoff, G.; et al. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J. Nutr.* **1998**, *128*, 2324−2333.

(2) Cooke, D.; Steward, W. P.; Gescher, A. J.; Marczylo, T. H. Anthocyans from fruits and vegetables-does bright colour signal cancer chemopreventive activity? *Eur. J. Cancer* **2005**, *41*, 1931−1940.

(3) Morazzoni, P.; Bombardelli, E. Vaccinium myrtillus L. *Fitoterapia* **1996**, *67*, 3−29.

(4) Renaud, S.; de Logeril, M. Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(5) Mazza, G. Health aspects of natural colors. In *Natural Food Colorants: Science and Technology*; Lauro, G. J., Francis, F. J., Eds.; Marcel Dekker Inc.: New York, 2000; pp 289−314.

(6) Trevithick, J. R.; Mitton, K. P. Antioxidants and diseases of the eye. In *Antioxidant Status, Diet, Nutrition and Health*; Papas, A. M., Ed.; CRC Press: New York, 1999; pp 545−566.

(7) Tsuda, T.; Watanabe, M.; Ohshima, K.; et al. Antioxidative activity of the anthocyanin pigments cyaniding 3-O-β-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407−2410.

(8) Kuhnau, J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. *World Rev. Nutr. Diet* **1976**, *24*, 117−191.

(9) Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutr. Cancer* **1993**, *20*, 21−29.

(10) Nyman, N. A.; Kumpulainen, J. T. Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 4183−4187.

(11) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731−7737.

(12) 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.

(13) Zhang, Z.; Kou, X.; Fugal, K.; McLaughlin, J. Comparison of HPLC methods for determination of anthocyanins and anthocyanidins in bilberry extracts. *J. Agric. Food Chem.* **2004**, *52*, 688−691.

(14) Ichiyanagi, T.; Kashiwada, Y.; Ikeshiro, Y.; et al. Complete assignment of bilberry (*Vaccinium myrtillus L.*) anthocyanins separated by capillary zone electrophoresis. *Chem. Pharm. Bull.* **2004**, *52*, 226−229.

(15) Wu, X.; Prior, R. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 2589−2599.

(16) Schwarz, M.; Hillebrand, S.; Habben, S.; et al. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochem. Eng. J.* **2003**, *14*, 179−189.

(17) Bitsch, R.; Netzel, M.; Frank, T.; Strass, G.; Bitsch, I. Bioavailability and biokinetics of anthocyanins from red grape juice and red wine. *J. Biomed. Biotechnol.* **2004**, *5*, 293−298.

(18) Frank, T.; Sonntag, S.; Strass, G.; Bitsch, I.; Bitsch, R.; Netzel, M. Urinary pharmacokinetics of cyanidin glycosides in healthy young men following consumption of elderberry juice. *Int. J. Clin. Pharm. Res.* **2005**, *25*, 47−56.

(19) Talavera, S.; Felgines, C.; Texier, O.; et al. Anthocyanin metabolism in rats and their distribution to digestive area, kidney and brain. *J. Agric. Food. Chem.* **2005**, *53*, 3902−3908.

(20) Cooke, D.; Schwarz, M.; Boocock, D.; Winterhalter, P.; Steward, W. P.; Gescher, A. J.; Marczylo, T. H. Effect of cyanidin-3-glucoside and an anthocyanin mixture from bilberry on adenoma development in the APCmin mouse model of intestinal carcinogenesis-Relationship with tissue anthocyanin levels. *IJC* **2006**, in press.

(21) Cao, G.; Muccitelli, H. U.; Sanchez-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.

(22) Milbury, P. E.; Cao, G. H.; Prior, R. L.; Blumberg, J. Bioavailability of elderberry anthocyanins. *Mech. Ageing Dev.* **2002**, *123*, 997−1006.

(23) Bitsch, R.; Netzel, M.; Sonntag, S.; Strass, G.; Frank, T.; Bitsch, I. Urinary excretion of cyanidin glucosides and glucuronides in healthy humans after elderberry juice ingestion. *J. Biomed. Biotechnol.* **2004**, *5*, 343−345.

(24) Ichiyanagi, T.; Shida, Y.; Mamunur Rahman, M.; Hatano, Y.; Konishi, T. Extended glucuronidation is another major path of cyanidin 3-O-β-D-glucopyranoside metabolism in rats. *J. Agric. Food Chem.* **2005**, *53*, 7312−7319.

(25) Wu, X.; Pittman, H. E.; Prior, R. L. Pelargonidin is absorbed and metabolized differently than cyanidin after marionberry consumption in pigs. *J. Nutr.* **2004**, *134*, 03−2610.

(26) Ichiyanagi, T.; Shida, Y.; Mamunur Rahman, M.; et al. Metabolic pathway of cyanidin 3-O-β-D-glucopyranoside in rats. *J. Agric. Food Chem.* **2005**, *53*, 145−150.

Received for review June 5, 2006. Revised manuscript received July 21, 2006. Accepted July 21, 2006. We received a program grant from the UK Medical Research Council.

JF061562Q