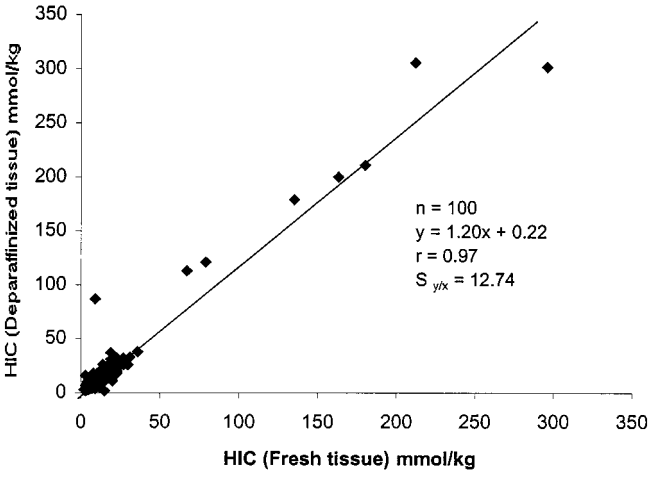

Fig. 1. Correlation of HIC measured in fresh and deparaffinized liver biopsy specimens.

dard reference material 1577b, bovine liver that has a certified iron concentration of 3.29 ± 0.27 mmol/kg dry weight. The mean liver iron concentration obtained was 3.36 mmol/kg (n = 30), with a CV of 4.2%. The precision of liver iron determination on embedded tissue was assessed by cutting a number of different size tissue samples from a single, relatively large histological sample. The smaller the piece of deparaffinized liver tissue used for the analysis, the more imprecise were the results for that piece (Table 1). When we interpolated from the data in Table 1, we found a CV of ~10% for a dry weight of 0.4 mg. This dry weight of paraffin-embedded tissue is the same as that defined by Olynyk et al. *(2)* as the weight below which results agreed poorly with results on samples of fresh liver specimens.

HIC was determined in both fresh and deparaffinized liver specimens (n = 100). There was a significant linear relationship between HIC determined in fresh and deparaffinized tissue (Fig. 1). We observed a positive bias for HIC determined in deparaffinized tissue when the HIC was >100 mmol/kg; however, only eight samples fell into this category. When the deparaffinized HIC result was <100 mmol/kg (n = 92), no significant difference between HIC determined in deparaffinized and fresh tissue from the same patient was observed using the Wilcoxon signed-rank test. For 85% of the HIC measurements on deparaffinized specimens, the result was either within 20% of the fresh result or both results fell within the reference interval of 5–24 mmol/kg dry weight (92% for 65 samples with dry weights ≥0.4 mg and 71% for 35 specimens with dry weights <0.4 mg). We calculated the HII for a group of 51 patients with HIC of 2–306 mmol/kg dry wt. HII values ranged from 0.1 to 5.6. Determination of the HII from deparaffinized specimens correctly classified 96% of patients (49 of 51) as GH or non-GH, using an HII value >1.9 as diagnostic of GH. For specimens weighing ≥0.4 mg, 98% (44 of 45) of patients were classified correctly, with one patient having borderline results (deparaffinized HII = 1.9; fresh tissue HII = 1.1).

In summary, determination of the HII of the deparaffinized specimens provided a high diagnostic accuracy when the sample weight was ≥0.4 mg. In contrast to other methods *(2, 3)*, in most cases it is not necessary to use the entire histological specimen in our method. When iron overload is suspected after histological examination of a fixed liver sample, it is possible to quantify the iron concentration on the same material. Moreover, material from the archives of anatomic pathology departments can be used for chemical iron determination many years after biopsy or autopsy.

References

1. George PM, Conaghan C, Angus HB, Walmsley TA, Chapman BA. Comparison of histological and biochemical hepatic iron indexes in the diagnosis of genetic haemochromatosis. J Clin Pathol 1996;49:159–63.
2. Olynyk JK, O'Neill R, Britton RS, Bacon BR. Determination of hepatic iron concentration in fresh and paraffin-embedded tissue: diagnostic implications. Gastroenterology 1994;106:674–7.
3. Van Deursen C, de Metz M, Koudstaal J, Brombacher P. Measurement of liver iron content in paraffin-embedded biopsies. J Clin Chem Clin Biochem 1988;26:689–91.

Anthocyanins Are Detected in Human Plasma after Oral Administration of an Elderberry Extract, *Guohua Cao[1,2] and Ronald L. Prior[1*]* ([1] US Department of Agriculture, Agriculture Research Service, Jean Mayer Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111, and [2] Nutritional Science Department, University of Connecticut, Storrs, CT 06269; * address correspondence to this author at: USDA, ARS, HNRCA, 711 Washington St., Boston, MA 02111; fax 617-556-3299, e-mail prior_us@hnrc.tufts.edu)

Anthocyanins are a group of natural antioxidants *(1–4)* widely distributed in fruits and vegetables. Anthocyanins have two absorbance peaks, at 270–280 nm and 510–540 nm, respectively. The intake of anthocyanins in humans has been estimated to be 180–215 mg/day in the US *(5)*, which is much higher than the intake (23 mg/day) of other flavonoids, including quercetin, kaempferol, myricetin, apigenin, and luteolin *(6)*. Various biological and pharmacological activities of anthocyanins have been reported using crude fruit extracts, which are rich in anthocyanins *(7)*. However, the absorption of dietary anthocyanins has never been shown clearly in humans, although one substance with an absorbance spectrum similar to those of the anthocyanins was reported in the plasma of nonsupplemented human subjects *(8)*, and anthocyanin-like compounds have been found in human urine *(9)*. We report here direct evidence of the absorption of anthocyanins in humans, which was obtained by combining an octadecylsilane (ODS) solid-phase extraction procedure for plasma sample preparation and an HPLC system with diode array for anthocyanin separation and detection.

One male subject (one of the authors), 35 years of age, consumed 25 g of elderberry extract containing 1.5 g of

total anthocyanins after fasting overnight. The elderberry extract is commercially available as a nutritional supplement for humans. Its main constituents are anthocyanins, mainly cyanidin 3-glucoside and cyanidin 3-sambubioside; other polyphenols were very limited *(10)*. Blood samples were obtained before and 30 and 60 min after anthocyanin consumption. The EDTA blood samples were centrifuged at 500*g* for 10 min at 4 °C, and the plasma was quickly removed. A 1.0-mL aliquot of plasma was diluted immediately with 200 μL of 0.44 mol/L trifluoroacetic acid (TFA) aqueous solution and then frozen at −80 °C for storage before HPLC analysis for

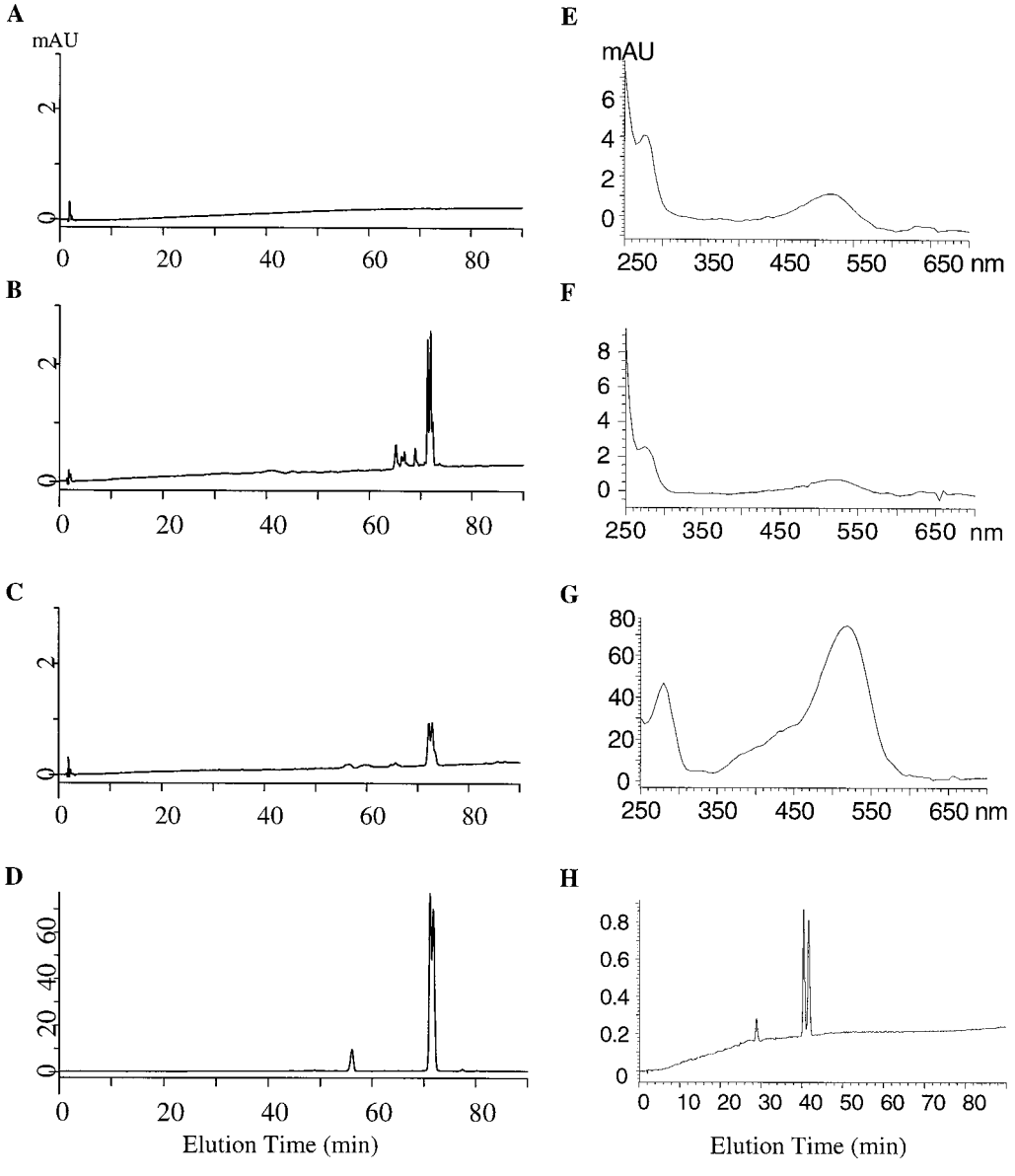

Fig. 1. HPLC chromatograms (*A–D, H*) and peak spectra (*E–G*) of an elderberry extract and plasma samples collected from one human subject before and after consumption of 25 g of the elderberry extract.

The absorbance was recorded at 520 nm. (*A*), chromatogram of plasma collected before consumption of the elderberry extract; (*B*), chromatogram of plasma collected 30 min after consumption of the elderberry extract; (*C*), chromatogram of plasma collected 60 min after consumption of the elderberry extract; (*D*), chromatogram of elderberry extract containing 1.2 μg of anthocyanins (cyanidin 3-glucoside equivalent); (*E*), spectrum of the peak eluted at ~71.3 min in plasma collected 30 min after consumption of the elderberry extract (see *panel B*); (*F*), spectrum of the peak eluted at ~71.3 min in plasma collected 60 min after consumption of the elderberry extract (see *panel C*); (*G*), spectrum of the peak eluted at ~71.3 min in the elderberry extract (see *panel D*); (*H*), chromatogram of elderberry extract containing 12.0 ng of anthocyanins. The spectrum of the peak eluted at ~71.9 min in *panels B–D* was very similar to that of the peak that eluted at ~71.3 min (data not shown). A binary linear gradient method was used for samples *A–D* as follows: 0–50 min, 0–24% B ; 50–110 min, 24–38% B; 110–120 min, 38–100% B. Mobile phase A: 25 mmol/L sodium acetate in water. Mobile phase B: 25 mmol/L sodium acetate in methanol. Both mobile phases were adjusted to pH 1.5 with TFA. The flow rate was maintained at 1.0 mL/min. Sample in *panel H* was analyzed using modified mobile phases: 0–20 min, 0–26% B; 20–80 min, 26–33% B.

anthocyanins. The anthocyanins in the plasma were extracted using an ODS solid-phase extraction cartridge (Sep-Pak C_{18}).[3] The cartridge was washed with 10 mL of methanol and equilibrated with 10 mL of 0.44 mol/L TFA before use. One milliliter of the plasma diluted with 200 μL of 0.44 mol/L TFA was centrifuged, and the supernatant was applied to the cartridge. Water-soluble compounds, polar lipids, and neutral lipids were eluted from plasma samples with 10 mL of 0.44 mol/L TFA, 10 mL of dichloromethane, and 10 mL of benzene, respectively. Samples were eluted finally with 5 mL of 0.44 mol/L TFA in methanol. The methanol phase was collected and should have contained anthocyanins, if they were present in the plasma samples. The methanol phase was evaporated to dryness with a rotary evaporator and redissolved in 200 μL of 0.44 mol/L TFA aqueous solution. After the removal of any possible precipitates by centrifugation, 80 μL of the TFA aqueous solution, which contained the redissolved anthocyanins, was injected into an HPLC system to determine anthocyanin concentrations. The HPLC system included a BSA PM-80 pump, a Zorbax SB-C18 column (4.6 $\times$ 250 nm), and an HP diode array detector.

No peaks were detected at 520 nm in the plasma samples collected before the consumption of the elderberry extract (Fig. 1A). However, two main peaks at 520 nm were revealed in the plasma samples collected 30 min (Fig. 1B) or 60 min (Fig. 1C) after the consumption of the elderberry extract. The elution times (~71.3 min and 71.9 min, respectively) and spectra of these two peaks in the plasma samples were the same as for the two anthocyanins detected in the elderberry extract (Fig. 1, D–G), indicating that anthocyanins can be absorbed in their glycosidic forms in humans. The plasma concentration of anthocyanins at 30 min after the consumption of the elderberry extract was at least 100 μg/L. The two main anthocyanin peaks were not separated completely in Fig. 1, B–D. However, these anthocyanins peaks were separated by modifying the mobile phases (Fig. 1H). The two main anthocyanins eluted at ~42 min when the modified mobile phases were used. However, only the elderberry extract and the plasma collected 60 min after consumption of the elderberry extract had sufficient volume to be reanalyzed. The data shown in Fig. 1H were from the elderberry extract.

As can be seen in Fig. 1, E–G, the anthocyanins have two absorbance peaks, one at 280 nm ($A_{280\ nm}$) and another at 520 nm ($A_{520\ nm}$), which was exactly the same as those of pure cyanidin 3-glucoside (data not shown). However, the $A_{280\ nm}/A_{520\ nm}$ ratio calculated for the two anthocyanins contained in the elderberry extract was obviously different from that for the two anthocyanins detected in the plasma. This difference may be a result of sample preparation or a changed pH environment because the $A_{280\ nm}/A_{520\ nm}$ ratio calculated for the two anthocyanins detected in the plasma was similar to that for anthocyanins we purified from wild blueberries, also using a C_{18} column and organic solvents (data not shown).

The substance found by Paganga and Rice-Evans *(8)* in human plasma also had two absorbance peaks (at 280 nm and 520 nm, respectively). Its $A_{280\ nm}/A_{520\ nm}$ ratio was also similar to the ratio we report here for the two anthocyanins measured in human plasma after the consumption of elderberry extract. However, the peak at 280 nm reported by Paganga and Rice-Evans *(8)* was very broad (250–350 nm), which is different from what we found for either pure anthocyanins (250–300 nm) or anthocyanins detected in plasma (250–300 nm). The "anthocyanins" reported by Lapidot et al. *(9)* in human urine after the consumption of red wine were not anthocyanins in their original forms. They were likely metabolic products of the red wine anthocyanins. The spectra of those reported "anthocyanins" were quite different from those of purified anthocyanins in that they had at least three absorbance peaks (at 280 nm, 430 nm, and 520 or 550 nm, respectively), with the main peak at 430 nm *(9)*. Anthocyanins should not have an absorbance peak around 430 nm.

In conclusion, by combining an ODS solid-phase extraction procedure for sample preparation and a diode array-HPLC system for anthocyanin separation and detection, we have successfully obtained direct evidence of the absorption of anthocyanins in humans for the first time.

[3] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

References

1. Sichel G, Corsaro C, Scalia M, Bilio AJD, Bonomo RP. In vitro scavenger activity of some flavonoids and melanins against $O_2^{\cdot-}$. Free Radic Biol Med 1991;11:1–8.
2. Tsuda T, Shiga K. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. Biochem Pharmacol 1996;52:1033–9.
3. Wang H, Cao G, Prior RL. The oxygen radical absorbing capacity of anthocyanins. J Agric Food Chem 1997;45:304–9.
4. Van Acker SABE, van den Berg DJ, Tromp MNJL. Structural aspects of antioxidant activity of flavonoids. Free Radic Biol Med 1996;20:331–42.
5. Kühnau J. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev Nutr Diet 1976;24:117–91.
6. Hertog MGL, Hollman PCH, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. Nutr Cancer 1993;20:21–9.
7. Morazzoni P, Bombardelli E. *Vaccinium myrtillus* L. Fitoterapia 1996;67:3–29.
8. Paganga G, Rice-Evans CA. The identification of flavonoids as glycosides in human plasma. FEBS Lett 1997;401:78–82.
9. Lapidot T, Harel S, Granit R Kanner J. Bioavailability of red wine anthocyanins as detected in human urine. J Agric Food Chem 1998;46:4297–302.
10. Abuja PM, Murkovic M, Pfannhauser W. Antioxidant and prooxidant activities of elderberry (*Sambucus nigra*) extract in low-density lipoprotein oxidation. J Agric Food Chem 1998;46:4091–6.