

diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin Chem* 1993;39:66–71.

6. Hill JB, Palmer P. Filter paper blood collection and punching as a means of quantification. *Clin Chem* 1969;15:381–9.
7. Levy HL, Barkin E. Comparison of amino acid concentrations between plasma and erythrocytes. Studies in normal human subjects and those with metabolic disorders. *J Lab Clin Med* 1971;78:517–23.

**Determination of Hepatic Iron Concentration in Fresh and Paraffin-embedded Tissue,** John P. Beilby,<sup>1\*</sup> Alex W. Prins,<sup>1</sup> and Nigel R. Swanson<sup>2</sup> (<sup>1</sup>Clinical Pathology, Path-Centre, Nedlands Western Australia 6009, Australia, and <sup>2</sup>University Department of Medicine, Sir Charles Gairdner Hospital, Nedlands, Western Australia 6009, Australia; \* author for correspondence: fax 61 8 93463882, e-mail John.Beilby@health.wa.gov.au)

Serum iron, transferrin, transferrin saturation, and ferritin are used to determine the iron status of an individual. When iron overload is suspected because of abnormal iron indices, the accurate determination of hepatic iron content (HIC) is important for the diagnosis of genetic hemochromatosis (GH). The measurement of HIC and the calculation of the hepatic iron index (HII), defined as HIC/age, enables the discrimination between patients with homozygous GH and those with either the heterozygous form of the disease or secondary causes of hepatic iron deposition. Despite the advent of DNA genotyping for the diagnosis of GH, liver biopsies are sometimes required for histological grading of liver disease and biochemical analysis to quantify the HIC. Genotyping will now identify many homozygotes before the development of clinical symptoms and an increased HII, and may reduce the need for obtaining a liver biopsy in some patients. However, in our experience the number of specimens submitted for HIC analysis has not changed over an 8-month period since the introduction of genotyping for GH.

Two techniques are used for the determination of liver iron content. The more common technique is histological grading of stainable iron, using Prussian blue dye; the other technique is the quantitative chemical determination of iron. The latter requires the liver sample to be homogenized or digested with acid before iron determination by either colorimetry or atomic absorption spectroscopy. The diagnosis of GH can be established reliably

using the HII calculated from the hepatic iron concentration determined by atomic absorption spectroscopy. Although an age-corrected histological HII may be derived from histological staining for iron, it does not reliably distinguish GH from other causes of increased HIC (1). Quantitative HIC is usually determined on fresh tissue submitted to the laboratory at the time of biopsy. Occasionally, only the paraffin-embedded (histological) liver biopsy specimens are available for quantitative iron determination because the analysis was not anticipated at the time of biopsy and only deemed necessary after histological examination. In addition, in some instances the fresh sample submitted might be too small for analysis or was contaminated. Olynyk et al. (2) used a colorimetric assay to determine HIC and reported that HIC in deparaffinized specimens agreed with that in fresh tissue in a series of 44 biopsies. The aim of our study was to extend the findings of Olynyk et al. in a larger series of samples, using graphite furnace atomic absorption spectroscopy.

Liver biopsies were obtained during routine clinical evaluation from 100 patients. The biopsies were taken percutaneously with a Tru-cut needle. A portion of the tissue (~5 mm length) was sent fresh to the laboratory in a clean, dry container and stored at 4 °C until analysis (within 1 week). The other piece was fixed in formaldehyde (40 g/L) and embedded in paraffin as part of routine histological processing. For this study, embedded tissue was dissected from the paraffin block, and the wax removed by washing (2 × 30 min) in xylene. Only ~50% of the embedded tissue was removed from the paraffin block, thus enabling the fixed specimen to be returned to the archives for future reference. The fresh and deparaffinized specimens were dried at 80 °C for 24 h and weighed. The dry weights were 0.31–3.30 mg (median, 0.80 mg) for the fresh tissue and 0.13–1.18 mg (median, 0.50 mg) for the deparaffinized tissue. The dried tissue (fresh and deparaffinized) was digested with a mixture of nitric acid (200 µL) and hydrogen peroxide (400 µL) at 100 °C in a glycol bath for 15–20 min. A stock digest was made by diluting each digested sample to 5 mL with deionized water. The iron concentration was determined by graphite furnace atomic absorption spectroscopy on a Varian SpectraAA300 spectrometer with Zeeman background correction using pyrolytically coated graphite tubes. The stock digest was diluted to bring the iron concentration within the calibration range. Dilutions were performed with deionized water, with nitric acid added to maintain an acid concentration of 40 mL/L. Calibrators were also prepared in 40 mL/L nitric acid; standard additions were not used. Triplicate analyses were performed on each solution. The furnace settings were as follows: for drying, ramp to 85 °C (5 s), ramp to 95 °C (40 s), and ramp to 120 °C (10 s); for ashing, ramp to 800 °C (5 s) and hold at 800 °C (3 s); for atomization, ramp to 2300 °C (1.1 s, read signal), hold at 2300 °C (2 s, read signal), and hold at 2300 °C (2 s, tube clean). The absorbance was determined at 372 nm.

The interassay imprecision (CV) was determined using the National Institute of Standards and Technology stan-

**Table 1. Precision of HIC from paraffin-embedded tissue.**

Sample length, <sup>a</sup> mm	Dry weight, <sup>b</sup> mg	HIC, <sup>b</sup> mmol/kg dry wt	SD	CV, %
4	1.64	18.3	0.50	2.7
2	0.67	18.5	1.29	7.0
1	0.32	17.3	2.22	13
0.5	0.12	20.0	3.16	16

<sup>a</sup> Approximate lengths.

<sup>b</sup> Mean values of four replicates.

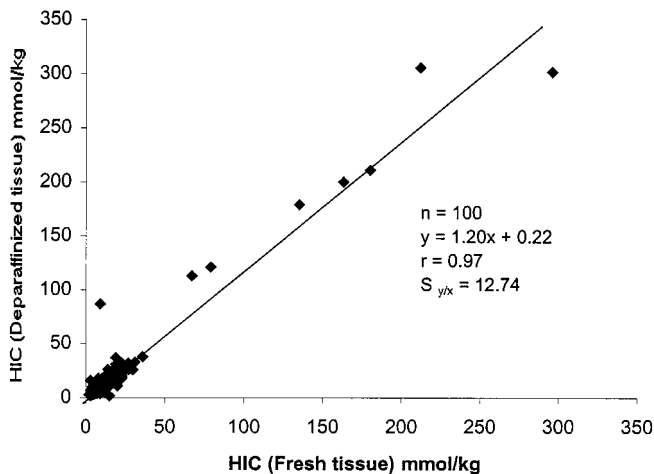


Fig. 1. Correlation of HIC measured in fresh and deparaffinized liver biopsy specimens.

standard reference material 1577b, bovine liver that has a certified iron concentration of  $3.29 \pm 0.27$  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  $\sim 10\%$  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  $\geq 0.4$  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  $\geq 0.4$  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  $\geq 0.4$  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<sup>1,2</sup> and Ronald L. Prior<sup>1\*</sup>** (<sup>1</sup> US Department of Agriculture, Agriculture Research Service, Jean Mayer Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111, and <sup>2</sup> 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