JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Bilberry Adulteration Using the Food Dye Amaranth

KERRY G. PENMAN,[†] CLYNTON W. HALSTEAD,[†,‡] ANITA MATTHIAS,[†] JAMES J. DE VOSS,[§] JULIA M. U. STUTHE,[§] KERRY M. BONE,[†,#] AND REGINALD P. LEHMANN*[,†,§]

MediHerb Research Laboratories, 3/85 Brandl Street, Eight Mile Plains, Brisbane, Queensland 4113, Australia; University of Western Sydney, Penrith South DC, New South Wales 1797, Australia; School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Queensland 4072, Australia; and School of Health, University of New England, Armidale, New South Wales 2351, Australia

Vaccinium myrtillus or bilberry fruit is a commonly used herbal product. The usual method of determining the anthocyanin content is a single-wavelength spectrophotometric assay. Using this method, anthocyanin levels of two extracts were found to be 25% as claimed by the manufacturers. When high-performance liquid chromatography (HPLC) was used, however, one extract was found to contain 9% anthocyanins probably not derived from *V. myrtillus* but from an adulterant. This adulterant was subsequently identified, using HPLC, mass spectroscopy, and nuclear magnetic resonance, as amaranth, that is, 3-hydroxy-4-[(4-sulfo-1-naphthalenyl)azo]-2,7-naphthalenedisulfonic acid trisodium salt—a synthetic dark red sulfonic acid based naphthylazo dye. As described in this study, if deliberate adulteration occurs in an extract, a single-wavelength spectrophotometric assay is inadequate to accurately determine the levels of compounds such as anthocyanins. Detection of deliberate adulteration in commercial samples thus requires the use of alternative, more sophisticated, methods of analysis such as HPLC with photodiode array detection as a minimum.

KEYWORDS: Bilberry; amaranth; adulteration

INTRODUCTION

Vaccinium myrtillus L. or bilberry fruit is a commonly used herbal product. Clinical trials have demonstrated the benefits of bilberry in the management of visual disorders such as retinopathy and vascular conditions such as venous insufficiency and capillary fragility (*1*, *2*). These trials were conducted using bilberry preparations with a standardized anthocyanin content as the quality marker of the herbal product. Anthocyanins are highly colored natural pigments that are responsible for the red-blue-purple color of ripe bilberries. These anthocyanins in bilberry have been shown to be bioavailable (*3*) and are considered to be the main contributors to its therapeutic activity.

Traditionally, herbs were harvested and processed by the herbalist. Now, product manufacturers often purchase extracts which have been prepared from the herb in a way that should prevent degradation of the therapeutic components. Unfortunately, this lends itself to the possibility of deliberate adulteration that can be detected only if rigorous testing/analysis is undertaken.

For bilberry, the pharmacopoeial method for determination of the anthocyanin content is by a single-wavelength (528 nm) spectrophotometric assay with the percentage anthocyanin content calculated using cyanidin-3-glucoside chloride specific absorbance values (*4*). If there is no adulteration, this method is adequate to determine the levels of anthocyanins in a given extract. If deliberate adulteration has taken place, however, the spectrophotometric method will yield a false-positive result with the adulterant masquerading as the anthocyanins. Reliance on simple detection methods can thereby be disadvantageous, as was highlighted by our evaluation of a commercial bilberry extract that contained relatively low levels of anthocyanins. As described in this study, detection of deliberate adulteration in commercial samples requires more sophisticated methods of analysis.

MATERIALS AND METHODS

Chemicals. The adulterated standardized dry extract powder was obtained from China through an Australian distributor. The authentic bilberry standardized dry extract powder from the fruit was of European origin (Indena, Italy). Both extracts are claimed to be standardized to 25% anthocyanins by the manufacturer. Amaranth was purchased from Sigma (Sydney, Australia). Cyanidin chloride was purchased from Extrasynthase (Genay, France). Solvents of high-performance liquid chromatography (HPLC) grade or better were purchased from Merck. All other reagents were of analytical reagent grade and purchased from Merck, BDH, or Scharlau.

Spectrophotometry. Spectrophotometric measurements and scans were acquired at room temperature on a Varian Cary-50 spectrophotometer controlled by Cary WinUV software.

* Corresponding author (telephone +61 7 3423 6521; fax +61 7 3423 6599; e-mail reg@mediherb.com.au).
[†] MediHerb Research Laboratories.
[‡] University of Western Sydney.
[§] University of Queensland.
[#] University of New England.

10.1021/jf061387d CCC: $33.50
Published on Web 08/29/2006

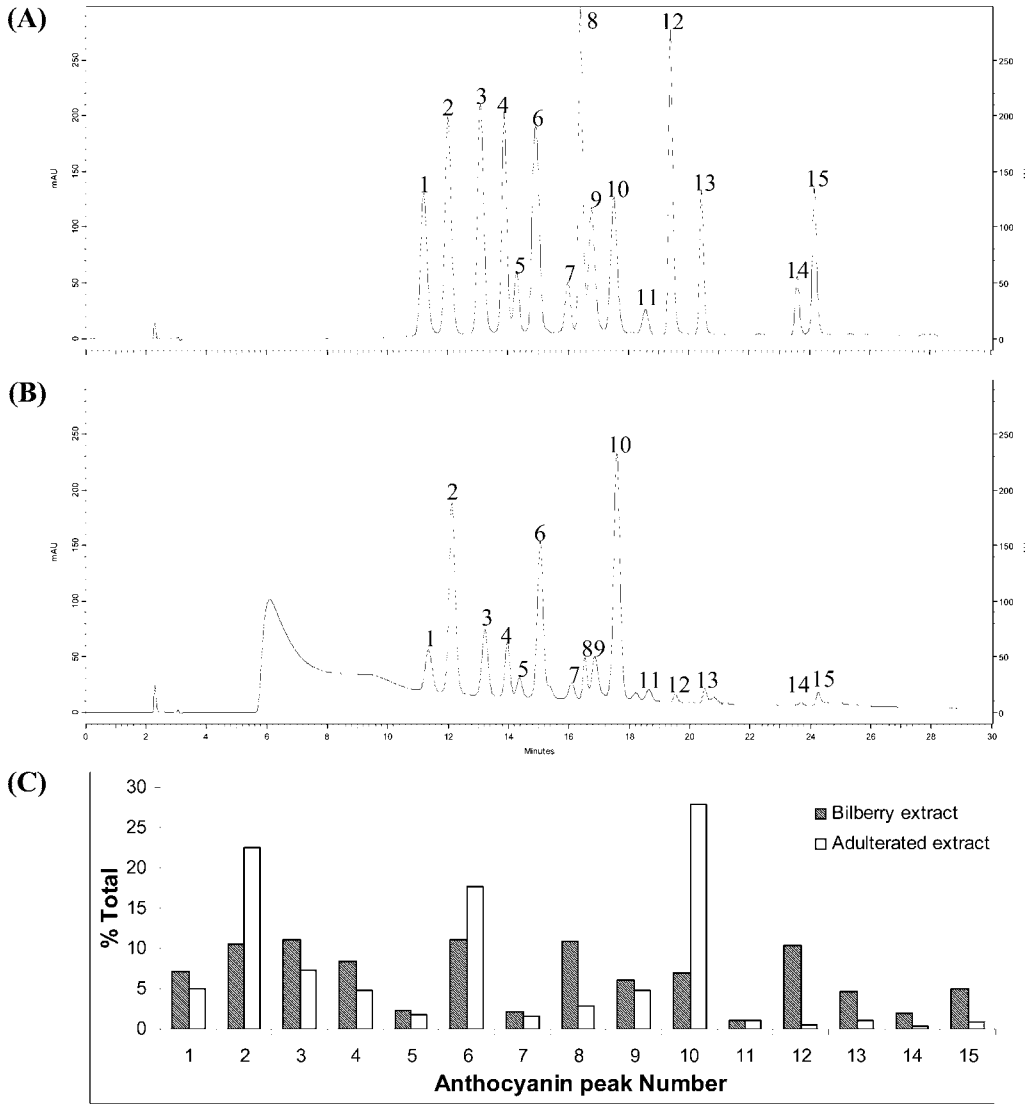

Figure 1. HPLC traces at 540 nm for two purported bilberry extracts: (**A**) bilberry extract; (**B**) adulterated extract; (**C**) comparison of percent of total for each anthocyanin present in both extracts calculated from peak areas. Peaks: 1, delphinidin 3-*O*-galactoside; 2, delphinidin 3-*O*-glucoside; 3, cyanidin 3-*O*-galactoside; 4, delphinidin 3-*O*-arabinoside; 5, cyanidin 3-*O*-glucoside; 6, petunidin 3-*O*-galactoside; 7, cyanidin 3-*O*-arabinoside; 8, petunidin 3-*O*-glucoside; 9, peonidin 3-*O*-galactoside; 10, petunidin 3-*O*-arabinoside; 11, peonidin 3-*O*-glucoside; 12, malvidin 3-*O*-galactoside; 13, peonidin 3-*O*-arabinoside; 14, malvidin 3-*O*-glucoside; 15, malvidin 3-***O***-arabinoside. Peaks have been identified by comparison with previously published HPLC profiles (*11*).

A modification of the British Pharmacopoeia method (*4*) was used. Essentially, samples were dissolved in methanol and then diluted using a methanol/0.1% hydrochloric acid mixture, and absorbance was measured at 528 nm. The percentage anthocyanins was determined according to the equation

$$(A \times V)/(\mathrm{SA} \times m)$$

where A = absorbance at 528 nm, V = final volume (mL), SA = 718, which is the specific absorbance of cyanidin 3-glucoside at 528 nm, and m = mass of sample being examined (g).

The Institute for Nutraceutical Advancement (INA) method (*5*) was also used to calculate the anthocyanin content in bilberry extracts. Basically, concentrated stock solutions of the extracts in water were prepared. These were then diluted 1:25 in buffers with pH ranging from 1 to 13. For the INA method, the pH 1 buffer consists of potassium chloride and hydrochloric acid (HCl), whereas the pH 4.5 buffer is a sodium acetate solution adjusted with HCl. Buffers at pH 3, 5, and 7 consisted of a mixture of 0.1 M citric acid and 0.1 M sodium citrate combined to give the correct pH, whereas pH 9, 11, and 13 buffers were a combination of hydrochloric acid and sodium hydroxide. The percentage of anthocyanins was determined according to the equation

$$[((A_{510,\mathrm{pH1.0}}) - (A_{700,\mathrm{pH1.0}})) - ((A_{510,\mathrm{pH4.5}}) - (A_{700,\mathrm{pH4.5}}))] \times \mathrm{MW} \times \mathrm{DF} \times V \times 100/\epsilon \times \mathrm{L} \times m$$

where A = absorbance, MW = molecular weight of cyanidin 3-glucoside (449.2), DF = dilution factor, V = final volume (mL), ϵ = extinction coefficient of cyanidin 3-glucoside (26900), L = cell path length, and m = mass of sample being examined (g).

Chromatographic Analysis. Extracts (0.1 g) were dissolved in 25 mL of 100% methanol and sonicated. Hydrolyzed samples were prepared by refluxing the extract (0.1 g) with 40 mL of 100% methanol, 7.5 mL of concentrated HCl, and 2.5 mL of water. All samples were filtered using 0.45 μm hydrophobic fluoropore membranes prior to HPLC analysis.

Anthocyanin concentrations in samples were determined by peak area using a gradient HPLC system (Shimadzu LC10AT) equipped with a photodiode array detector (PDA). Ten microliter samples were injected onto an Alltech Alltima C18 (5 μm) 150 × 4.6 mm i.d. column. The mobile phase was a mixture of 50 mM phosphoric acid and acetonitrile at a flow rate of 0.8 mL/min. The gradient increased from 10 to 35% acetonitrile over the first 30 min and then to 100% over the next 7 min, where it remained for the next 5 min. The acetonitrile content

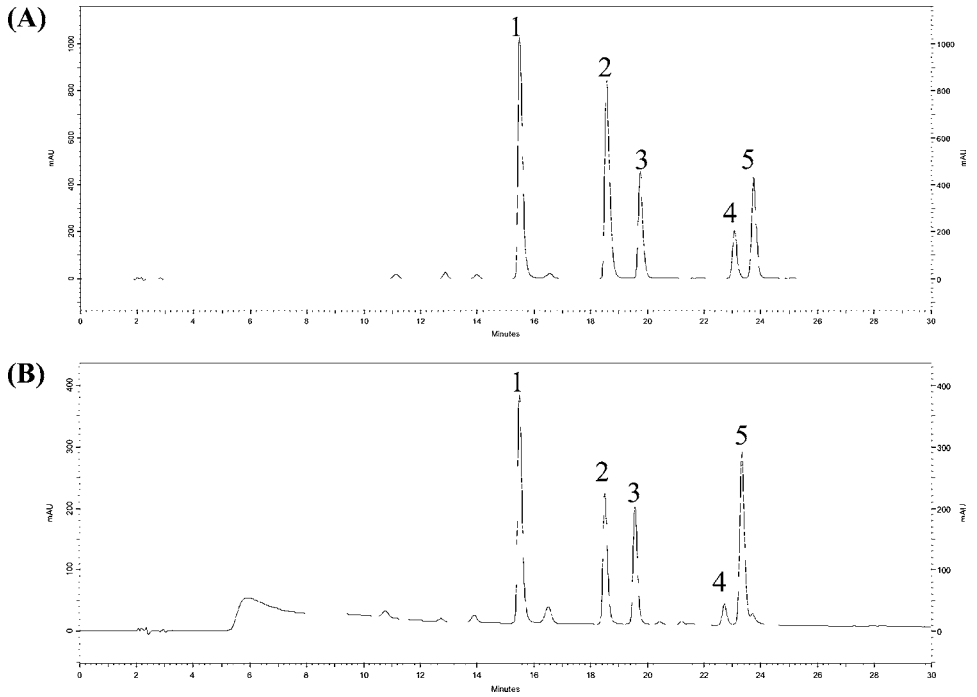

Figure 2. HPLC traces at 540 nm for two hydrolyzed bilberry extracts: (**A**) bilberry extract; (**B**) adulterated extract. Peaks: 1, delphinidin; 2, cyanidin; 3, petunidin; 4, peonidin; 5, malvidin. Peaks have been identified using a cyanidin standard and by comparison with previously published HPLC profiles (*12*).

was then returned to 10% over the next 5 min, and this was followed by a 5 min re-equilibration period at 10% acetonitrile prior to the next injection.

Further analysis was done using a gradient HPLC system coupled to a quadrupole mass spectrometer (Shimadzu QP8000α) operating in both positive and negative ion modes using an atmospheric pressure chemical ionization (APCI) interface. Samples were analyzed on a Phenomenex Luna C18 (3 μm) 100 × 2.00 mm column. The mobile phase was a mixture of water and acetonitrile at a flow rate of 0.3 mL/min with a gradient profile similar to that used for the HPLC.

Adulterant Isolation. The adulterant in the sample was isolated by column chromatography on silica gel 60, 0.06−0.2 mm, for column chromatography (70−230 mesh ASTM) as the stationary phase. The silica gel (50 g) was slurry packed using chloroform and then conditioned with methanol. The extract (0.5 g) was dissolved in methanol (5 mL) and applied to the column. Five 50 mL fractions (three with 100% methanol followed by two with 50% methanol) were collected. Fraction 2 (in 100% methanol) was concentrated by evaporation and then further fractionated on a second column prepared similarly as above, and four 50 mL fractions were collected (one with 100% methanol followed by two with 50% methanol and one with water). The second fraction isolated off this column yielded ≈18 mg of the pure adulterant as determined by HPLC.

NMR Analysis. ^{1}H NMR spectra were acquired on a Bruker AV500 (txi 5 mm probe) instrument, and ^{13}C NMR spectra were recorded on a Bruker AV400 (BBO 5 mm probe) instrument. Samples were prepared in CD_3OD or in a CD_3OD/D_2O 50:50 solvent mixture. Spectra were calibrated using the signals of the residual methanol in the CD_3OD (^{1}H, δ 3.30; ^{13}C, δ 49.0) as internal reference.

RESULTS AND DISCUSSION

When two commercial bilberry extracts were initially analyzed using the simple spectrophotometric specified in the *British Pharmacopoeia* (BP; *4*), the total measured anthocyanin content of both was found to be 24%. This is very similar to that claimed by the manufacturers (25%). Upon further analysis using more complex spectrophotometric as well as chromatographic methods, differences in the total anthocyanin content of the two extracts were found. One extract was authenticated to be bilberry with an anthocyanin content of 24% (bilberry extract), whereas the other was found to contain only 9% anthocyanins (adulterated extract) and an adulterant that was subsequently identified as amaranth. Furthermore, the difference in the relative amounts of the anthocyanins observed in the adulterated extract compared with those in the authentic one suggested that they were possibly not even derived from bilberry (*V. myrtillus*).

Both samples displayed similar chromatographic profiles (**Figure 1**) with a good correlation for both spectra and retention times for most of the 15 anthocyanins, although those in the adulterated extract (**Figure 1B**) were present at lower concentrations. On further examination, it was realized that the anthocyanin profile of the two extracts differed markedly in the relative ratios of the individual components as emphasized when each anthocyanin was expressed as a percentage of the total present (**Figure 1C**). Whereas the relative profile of these peaks in the bilberry extract was similar to previously published profiles (*6*, *7*), that for the adulterated extract was not. This marked alteration in the proportions of the anthocyanins in the HPLC profile suggests that the adulterated extract does not in fact contain any bilberry (*V. myrtillus*). Different anthocyanin profiles have been reported for other *Vaccinium* species (*8*) and for unrelated species such as red kidney beans (*9*), but none of these match the profile found in this adulterated bilberry extract.

In addition to these differences in the anthocyanins, the adulterated extract also contained a broad, early eluting, poorly shaped peak (**Figure 1B** at a retention time of 6−8 min). Routine HPLC quantification methods for bilberry use an acid hydrolysis step that removes the sugar units and converts the anthocyanins to the corresponding anthocyanidin aglycone species. The unknown peak in the adulterated extract remained unchanged by this acid hydrolysis step (**Figure 2**). After isolation of the corresponding compound using column chromatography, data that identified structural features of this unknown material were obtained. LC-MS indicated a molecule

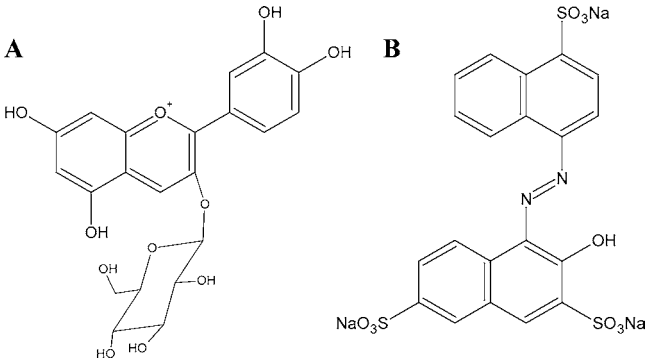

Figure 3. Representative structures of the anthocyanin cyanidin 3-glucoside (**A**) and amaranth (**B**).

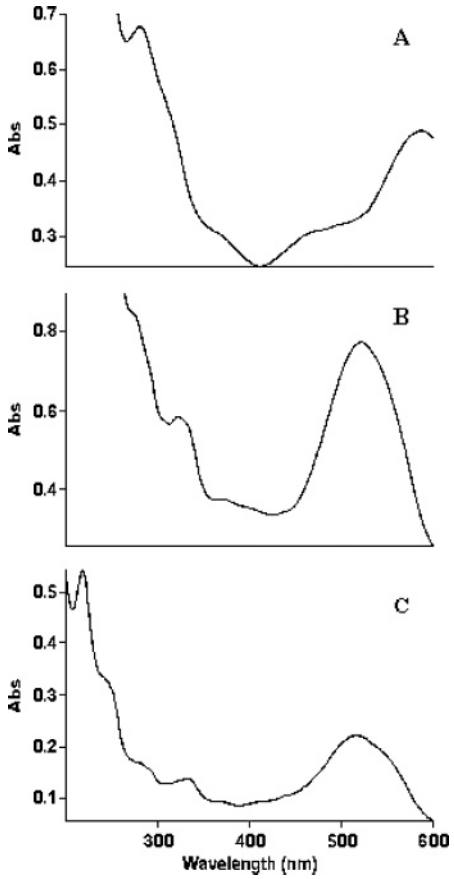

Figure 4. Wavelength spectrum of the different preparations at a pH of 5: (**A**) bilberry extract; (**B**) adulterated extract; (**C**) amaranth.

in the range of 500−550 atomic mass units, and various observed ions indicated losses compatible with sulfonates (see Supporting Information). NMR suggested the presence of aromatic systems, most likely naphthyl-like structures that are not found in anthocyanins. Careful analysis of the ^{1}H and ^{13}C NMR data suggested that the adulterant may be amaranth, [3-hydroxy-4-[(4-sulfo-1-naphthalenyl)azo]-2,7-naphthalenedisulfonic acid trisodium salt]. Subsequent comparison of the isolated adulterant with authentic amaranth yielded identical chromatographic and spectral data (see Supporting Information).

Amaranth is a sulfonic acid based naphthylazo dye used as a coloring agent, FD&C Red No. 2, and is very different structurally from the anthocyanins (**Figure 3**). It was banned by the U.S. Food and Drug Administration in 1976 for use in foods, drugs, and cosmetics due to suspected carcinogenic activity. The presence of amaranth in this extract implies that the safety profile that has been established for bilberry over years of traditional use cannot be relied upon in this instance.

Table 1. Comparison of Anthocyanin Content of the Two Bilberry Extracts Using Different Analysis Methods[a]

	% anthocyanins (w/w)		
sample	BP	HPLC	INA
unadulterated bilberry	24	24	25
adulterated bilberry	24	9	7

[a] BP, British Pharmacopoeia single-wavelength spectroscopic method (*3*); HPLC, high-performance liquid chromatography method; INA, INA dual-wavelength spectroscopic method (*4*).

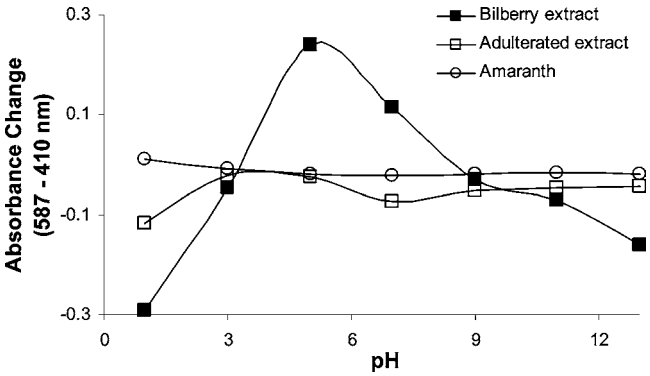

Figure 5. Differential absorbance for the three preparations at the different pH values. From the spectrum obtained for the bilberry extract (see **Figure 3A**), two wavelengths were chosen (a minimum of 410 and a maximum of 587), and the difference in absorbance between the two wavelengths was plotted for each preparation at the different pH values.

When the simple BP spectrophotometric method is used, amaranth has UV−visible spectral characteristics similar to those found for anthocyanins. This results in the false 24% anthocyanin content result obtained for the adulterated extract. The significant absorbance of amaranth at a pH of 5 (**Figure 4**) over the range of the detection wavelengths used in different analysis methods contributes to the total absorbance and, hence, anthocyanin content, measured for the adulterated extract. One method of analysis, from the INA (*5*), targets the known color and absorbance differences found for anthocyanins at different pH values. At pH 1.0, anthocyanins exist as the intensely colored oxonium or flavylium forms, whereas at pH 4.5, they exist as colorless carbinols (*10*). Using the INA pH differential method, total anthocyanin content is proportional to the difference in absorbance at 510 nm from the same sample prepared at both pH 1.0 and 4.5. An anthocyanin content of 7% was found for the adulterated extract, which is close to that calculated using HPLC (9% anthocyanins in the adulterated extract) (**Table 1**). These values are far less than the value of 24% obtained when the pharmacopoeial single-wavelength spectrophotometric method was used.

Measurement of the absorbance of bilberry anthocyanins over a range of pH values (a modification of the INA method) displayed marked differences depending on the pH of the solution as would be expected. The differences between the bilberry pH dependence and the lack of change with pH for both the adulterated bilberry extract and amaranth have been depicted in **Figure 5**, where for each pH the change in absorbance between 587 and 410 nm has been graphed. These two wavelengths were chosen from **Figure 4**, where at a pH of 5 bilberry has maximum at 587 nm and a minimum at 410 nm. This results in the marked differences seen over the pH range for each of the samples examined.

This work has demonstrated the necessity for more than a simple one-wavelength spectroscopic assay to assess the components present in a herbal extract. Adulteration is far too easily achieved when such an assay is relied upon to analyze marker compound levels such as the anthocyanin levels in purported bilberry extracts.

Supporting Information Available: Mass spectral data and figure showing the total ion spectra for the isolated adulterant and amaranth; NMR data with figures showing ^{1}H and ^{13}C spectra and a table comparing the ^{13}C chemical shifts for the isolated adulterant and amaranth. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Canter, P. H.; Ernst, E. Anthocyanosides of *Vacinnium myrtillus* (bilberry) for night vision—a systematic review of placebo-controlled trials. *Surv. Ophthalmol.* **2004**, *49*, 38−50.

(2) Mills, S.; Bones, K. Bilberry fruit (*Vaccinium myrtillus L.*). In *Principles and Practices of Phytotherapy: Modern Herbal Medicine*; Churchill, Livingstone: Edinburgh, Scotland, 2000; pp 297−302.

(3) Morrazoni, P.; Livio, S.; Scilingo, A.; Malandrino, S. *Vaccinium myrtillus* anthocyanosides pharmacokinetics in rats. *Arzneimittelforschung* **1991**, *41*, 128−31.

(4) British Pharmacopoeia Commission Office. Bilberry fruit. In *British Pharmacopoeia*; London, U.K., 2004; Vol. 1, p 258.

(5) Anthocyanin content in bilberry by pH-differential spectrophotometry; Institute of Nutraceutical Advancement (INA) method 116.000 (http://www.nsf.org/business/ina/bilberry.asp).

(6) Baj, A.; Bombardelli, E.; Gabetta, B.; Martinelli, E. M. Qualitative and quantitative evaluation of *Vaccinium myrtillus* anthocyanins by high-resolution gas chromatography and high-performance liquid chromatography. *J. Chromatogr.* **1983**, *279*, 365−372.

(7) Nakajima, J.-I.; Tanaka, I.; Seo, S.; Yamazaki, M.; Saito, K. L/C/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries. *J. Biomed. Biotechnol.* **2004**, *5*, 241−247.

(8) Prior, R. L.; Lazarus, S. A.; Cao, G.; Muccitelli, H.; Hammerstone, J. F. Identification of procyanidins and anthocyanins in blueberries and cranberries (*Vaccinium spp.*) using high performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 1270−1276.

(9) Choung, M.-G.; Choi, B.-R.; An, Y.-N.; Chu, Y.-H.; Cho, Y.-S. Anthocyanin profile of Korean cultivated kidney bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **2003**, *51*, 7040−704.

(10) Brouillard, R.; Delaporte, B. Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton transfer, hydration and tautomeric reactions of malvidin 3-glucoside. *J. Am. Chem. Soc.* **1977**, *99*, 8461−8468.

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

(12) 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.

Received for review May 17, 2006. Revised manuscript received July 24, 2006. Accepted July 26, 2006.

JF061387D