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Ellagitannins, Flavonoids, and Other Phenolics in Red Raspberries and Their Contribution to Antioxidant Capacity and Vasorelaxation Properties

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Analysis of extracts of Glen Ample raspberries (*Rubus idaeus* L.) by gradient, reverse phase HPLC with diode array and tandem mass spectrometry identified eleven anthocyanins, including cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside), and pelargonidin-3-glucoside. Significant quantities of an ellagitannin, sanguiin H-6, with an M_r of 1870 were detected along with lower levels of a second ellagitannin, lambertianin C, which has an M_r of 2804. Other phenolic compounds that were detected included trace levels of ellagic acid and its sugar conjugates along with one kaempferol- and four quercetin-based flavonol conjugates. Fractionation by preparative HPLC revealed that sanguiin H-6 was a major contributor to the antioxidant capacity of raspberries together with vitamin C and the anthocyanins. Vasodilation activity was restricted to fractions containing lambertianin C and sanguiin H-6.

KEYWORDS: Raspberries; ellagitannins; anthocyanins; vitamin C; flavonols; HPLC; tandem mass spectrometry; antioxidant capacity; vasodilation activity

INTRODUCTION

Raspberries (*Rubus idaeus* L.) have a high free radical scavenging capacity and are rich in both vitamin C and total phenolics (*1*). They contain a distinct spectrum of anthocyanins (*2*). The major component is cyanidin-3-sophoroside with smaller quantities of other anthocyanins, including cyanidin-3-(2^G-glucosylrutinoside), cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-sophoroside, pelargonidin-3-(2^G-glucosylrutinoside), and pelargonidin-3-glucoside (*2*−*4*). Some varieties of raspberries contain very high levels of ellagitannins, which on hydrolysis release ellagic acid (*5*), a compound that has been reported to have antiviral activity (*6*) and provide protection against cancers of the colon (*7*), lung, and esophagus (*8*). Raspberries also contain a wide variety of quercetin and kaempferol-based flavonol conjugates with the major components being quercetin-3-glucuronide and quercetin-3-glucoside (*5*, *9*). In addition, raspberry juice is reported to contain catechins (*10*).

This paper reports on the phenolic compounds in Glen Ample raspberries which are cultivated extensively on a commercial basis in Scotland. Extracts were analyzed by gradient, reverse phase HPLC with diode array detection followed by tandem mass spectrometry. Preparative HPLC was carried out to identify compounds in raspberries possessing antioxidant activity and vasorelaxation ability. Natural dietary phenolics can cause endothelium-dependent vasodilation in human and rodent arteries (*11*), via the release of nitric oxide from vascular endothelium and subsequent increases in cGMP levels (*11*−*13*). Previous studies have shown that red wine- and grape-derived products are potent vasodilators in vitro. This cardioprotective effect has been attributed to the phenolic component, especially the high grape skin-derived anthocyanin content, of red wine. To date,

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there is no published information about the vasodilatory activity of raspberries.

MATERIALS AND METHODS

Chemicals. Cyanidin-3-glucoside was purchased from Apin Chemicals (Abingdon, Oxford, U.K.). Methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, U.K.). All other chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, U.K.).

Plant Material. One kilogram of ripe field-grown raspberries cv. Glen Ample, hand picked at Blairgowrie, Perthshire, U.K., was frozen in liquid nitrogen within 3 h of harvest and thereafter stored at −80 °C prior to processing.

Extraction of Raspberries. Forty grams of raspberries were macerated in an ice-cold pestle and mortar, and the resulting homogenate was centrifuged at 2000*g* for 30 min at 4 °C. The supernatant was decanted and the pellet homogenized and extracted with acidified (0.1% HCl) methanol, after which it was recentrifuged. The two supernatants were combined, and made up to a known volume with acidified methanol. This was then subdivided into 2 mL aliquots and stored in microcentrifuge tubes at −80 °C prior to analysis.

For preparative studies, the same procedure was used with 150 g of raspberries, after which the acidified extract in 1% methanol was applied to a 50 mm × 20 mm (inside diameter) ion-exchange column (Diaion HP-20) which was eluted with 100 mL of acidified methanol and water (1:99, v/v) to remove sugars and other contaminants. It was next washed with 100 mL of 100% acidified methanol to elute phenolic and flavonoid compounds. The methanol fraction was then passed through a 50 mm × 20 mm (inside diameter) column containing a 40 μm C_{18} silica gel support that was further eluted with 100 mL of acidified methanol. The methanolic eluates were combined and reduced to dryness in vacuo prior to preparative HPLC.

Qualitative Analysis of Phenolic Compounds in Raspberries by HPLC with Absorbance and MS Detection. Methanolic extracts of raspberries were analyzed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler and with detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo-Finnigan, San Jose, CA). Separation was carried out using a RP-MAX 4 μm, 250 mm × 4.6 mm (inside diameter), C_{12} reverse phase column (Phenomenex, Torrance, CA) maintained at 40 °C and eluted at a flow rate of 1.0 mL/min with a 60 min gradient from 5 to 30% acetonitrile in water containing 1% formic acid. After being passed through the flow cell of the diode array detector, the column eluate was split and 50% directed to an LCQ Duo mass spectrometer (Thermo-Finnigan) with an electrospray interface operating in full scan data dependent MS/MS mode from 150 to 2000 amu. Each sample was analyzed twice, first in positive ion mode and then in negative ion mode.

Analysis of Vitamin C by HPLC with Absorbance Detection. The vitamin C contents of fractions collected after preparative HPLC of a raspberry extract were analyzed by HPLC (*14*) using a Nucleosil ODS 5 μm, 250 mm × 4.6 mm (inside diameter) column (Jones Chromatography, Glamorgan, U.K.) eluted isocratically at a flow rate of 0.6 mL/min using a Gilson model 305 liquid chromatograph with a cooled autoinjector and a 231 absorbance detector operating at 263 nm. Data were recorded on a Gilson 715 data system.

Preparative Reverse Phase HPLC. The acidic, partially purified methanolic extract from 150 g of raspberries was redissolved in 100 mL of 5% methanol in water containing 1% formic acid and a 10 mL aliquot fractionated into 60 separate 5 mL samples using a Gilson 305 gradient liquid chromatograph with a Rheodyne preparative injector and a 10 mL sample loop. Separation was carried out using a 150 mm × 20 mm (inside diameter), 5 μm ODS-H optimal column (Capital HPLC, Broxburn, U.K.) maintained at 40 °C in a Shimadzu (Kyoto, Japan) CTO-6A column oven. Samples were eluted at a flow rate of 10 mL/min with a 30 min gradient from 5 to 25% acetonitrile in 1% formic acid followed by an 80% wash. The column eluate was directed first to a Shimadzu SPD-10A*vp* UV−vis absorbance monitor operating at 520 nm before being directed to a Gilson FC 203 microfraction collector. Collection of column eluate began 3 min after injection of the sample, with successive fractions collected every 0.5 min thereafter. Signals from the absorbance monitor were processed by a Reeve Analytical 27000 data system. Each fraction was then analyzed by HPLC−MS/MS as described above. The antioxidant capacity, total phenol content, and anthocyanin content of each fraction were also determined as outlined below. The vitamin C content of fractions 1−6 was determined by HPLC using methodology that is also outlined above.

Determination of the Total Phenol Content. The total phenol contents of raspberry extracts were determined in gallic acid equivalents using the Folin−Ciocalteu method (*15*).

Colorimetric Analysis of the Total Anthocyanin Content. The anthocyanin contents of raspberry extracts were estimated using a pH shift method (*16*). Anthocyanins were quantified as cyanidin-3-glucoside equivalents, one of the three major anthocyanins in raspberries, using an extinction coefficient ϵ of 29 600.

Measurement of Antioxidant Capacity by Electron Spin Resonance Spectroscopy. The antioxidant capacity of raspberry extracts was determined by their ability to reduce the Fremy's salt (potassium nitrosodisulfonate) (*17*). The extracts were diluted to 5% (v/v) with ethanol and water (12:88, v/v). Three 1.0 mL aliquots were reacted with an equal volume of 1 mM Fremy's radical in ethanol and water (12:88, v/v). The electron spin resonance spectra of the low field resonance of the Fremy's radical were obtained after 20 min, by which time the reaction was complete. The signal intensity was obtained by double integration and the concentration calculated by comparison with a control reaction using ethanol and water (12:88, v/v) without raspberry extract. Spectra were obtained at 21 °C on a Bruker ECS 106 spectrometer equipped with a cylindrical (TM110 mode) cavity and operating at ca. 9.5 GHz (X-band frequency). The microwave power and modulation amplitude were set at 2 mW and 0.01 mT, respectively.

Vasorelaxation Assay. Male New Zealand rabbits, weighing ca. 3.5 kg, were anesthetized by intravenous administration of pentobarbitone (100 mg/kg) with 1000 IU of heparin into the marginal ear vein. The descending thoracic aorta was then carefully removed, cleaned of adhering fat and connective tissue, and cut into transverse ring segments ca. 3−5 mm in length. The rings were suspended in 10 mL organ baths filled with Krebs buffer solution [118.4 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 11 mM D-glucose (pH 7.4)] continuously oxygenated with 16% O_2, 5% CO_2, and 79% N_2 and maintained at 37 °C to mimic the internal environment. Rings were placed under an optimal resting tension of 2 g. This tension was kept constant throughout the course of the experiment. Vessel contraction and/or relaxation was assessed via an isometric force transducer, linked in turn to a data handling system.

After an equilibration period of 45 min, vessels were maximally contracted twice with KCl (50 mM). After each contraction, vessels were washed out with Krebs solution. Vessel tone was then raised in all vessels with phenylephrine (10^{-7} M), and relaxed with acetylcholine, to test for functional and intact endothelium. Vessels were then washed thoroughly with Krebs solution.

After washing had been carried out and the vessels had been returned to initial baseline tension, aortic rings with functional endothelium were precontracted submaximally with phenylephrine (10^{-7} M). After precontraction of the aortic rings, the effect of the raspberry fractions on vascular tone was studied. Once a stable plateau had been reached, 25 μL of each raspberry fraction concentrate was added to the organ bath to induce vasodilation in the aortic ring segments. All results were calculated and expressed as the percentage vasorelaxation induced by each raspberry fraction following precontraction with phenylephrine.

RESULTS

HPLC−Diode Array−MS/MS Analysis of Raspberry Phenolics. An extract of Glen Ample raspberries was analyzed by reverse phase HPLC using a 60 min, 5 to 30% acetonitrile gradient with the column eluate being directed first to a diode array absorbance monitor and then to a mass spectrometer with an electrospray interface operating in full scan MS/MS mode. The sample was analyzed twice, in positive ion mode and then in negative ion mode. The 10−25 min absorbance trace obtained

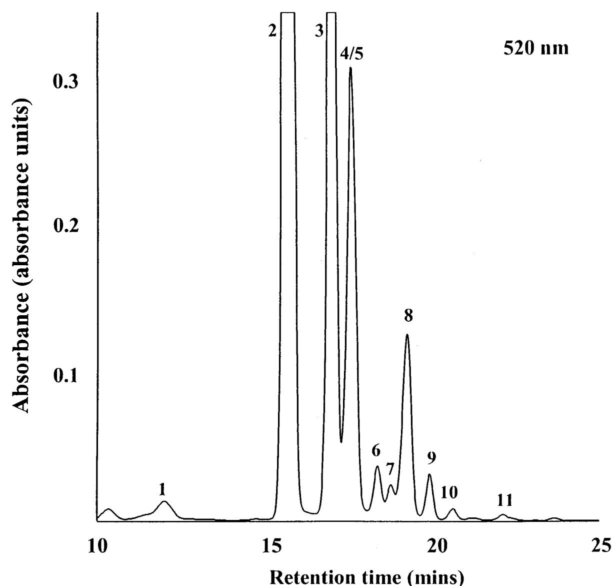

Figure 1. Gradient reverse phase HPLC of an extract of Glen Ample raspberries. The 10–25 min segment of a 60 min gradient from 5 to 30% acetonitrile in water containing 0.1% formic acid is illustrated with detection at 520 nm. The numbering of the peaks refers to their subsequent identification by tandem mass spectrometry.

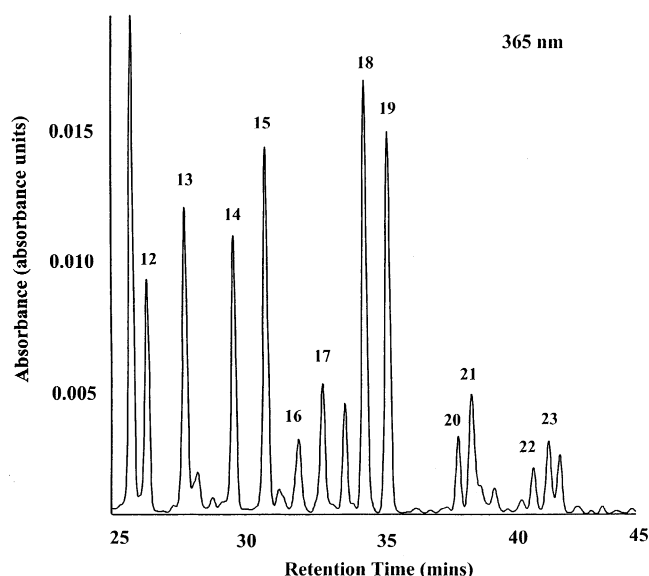

Figure 2. Gradient reverse phase HPLC of an extract of Glen Ample raspberries. The 25–45 min segment of a 60 min gradient from 5 to 30% acetonitrile in water containing 0.1% formic acid is illustrated with detection at 365 nm. The numbering of the peaks refers to their subsequent identification by tandem mass spectrometry.

at 520 nm is illustrated in **Figure 1** in which the 11 peaks, on which positive ion MS data were obtained, are labeled. Further peaks were detected when the 25−45 min segment of the chromatogram was examined at 365 nm (**Figure 2**). The peaks labeled 12−23 in **Figure 2** are those that yielded negative ion MS data. The identifications of peaks 1−23, based on MS/MS data and λ_{max}, are summarized below and outlined in **Table 1**. Identification of 11 anthocyanins, all of which had previously been detected in raspberries, though not in a single variety, in a TLC-based study by Barritt and Torre (*18*), was assisted by data on the MS fragmentation patterns of anthocyanins and their aglycones and the m/z losses associated with cleavage of various sugars and other substituent groups presented by Giusti et al. (*19*). Likewise, the data of Rommel and Wrolstad (*5*) and Zafrilla et al. (*20*) aided the MS/MS-based identifications of flavonols and ellagic acid sugar conjugates, respectively. Compounds in addition to those listed in **Table 1** were identified by MS/MS, but they were invariably present in trace amounts close to or cochromatographing with larger peaks which precluded accurate analysis by HPLC with diode array detection. Compounds falling in this category included (−)-epicatechin ($M^+ - m/z$ 291) which had a retention time (t_R) of 21.8 min and a catechin dimer ($M^+ - m/z$ 579) which had a t_R of 18.5 min.

Peak 1 (t_R = 12.3 min) was an anthocyanin with a λ_{max} of 519 nm. When analyzed by MS/MS, it yielded an M^+ at m/z 611 which fragmented to produce a m/z 449 ion (M − 162, loss of a glucosyl group) and m/z 287 which equates with cyanidin produced from m/z 449 by cleavage of a second glucosyl fragment. On the basis of this evidence, it is concluded that this compound is cyanidin-3,5-diglucoside.

Peak 2 (t_R = 15.7 min) had a λ_{max} of 519 nm, indicating that it was an anthocyanin which on the basis of an M^+ at m/z 611 that produced a fragment ion at m/z 287 (M − 324, loss of a sophorosyl unit) was identified as cyanidin-3-sophoroside which is a major anthocyanin in raspberries (see **Figure 2**).

Peak 3 (t_R = 17.1 min) was another major anthocyanin and was identified as cyanidin-3-(2^G-glucosylrutinoside) on the basis of its λ_{max} at 519 nm and a mass spectrum comprising an M^+ at m/z 757 which fragmented to produce a minor ion at m/z 611 (M − 146, loss of a rhamnosyl group) and a major ion at m/z 287 (− 324, loss of two glucosyl moieties).

Peak 4 (t_R = 17.5 min) was cyanidin-3-glucoside. Its properties corresponded with those of an authentic standard with a λ_{max} of 519 nm and an MS/MS spectrum consisting of an M^+ at m/z 449 which on loss of a glucosyl unit (M − 162) yielded a secondary fragment at m/z 287 corresponding to cyanidin.

Peak 5 (t_R = 17.7 min) was identified as cyanidin-3-sambubioside on the basis of its λ_{max} at 519 nm and a mass spectrum comprising an M^+ at m/z 581 which fragmented to produce a minor ion at m/z 449 (M − 132, loss of a xylosyl group) and a major ion at m/z 287 (M − 162, loss of a glucosyl unit).

Peak 6 (t_R = 18.4 min) had a λ_{max} at 503 nm which is 16 nm lower than the λ_{max} of the cyanidin conjugates. The MS/MS spectrum, with an M^+ at m/z 595 that fragments with a loss of m/z 324 to produce an m/z 271 ion, corresponds with pelargonidin-3-sophoroside which has an M_r that is 16 mass units smaller than that of cyanidin-3-sophoroside in peak 2.

Peak 7 (t_R = 18.8 min) had a λ_{max} at 519 nm and on the basis of its MS/MS spectrum [m/z 727 (M^+), 581 (M − 146, loss of a rhamnosyl moiety), and 287 (− 294, loss of xylosyl and glucosyl groups)] was identified as cyanidin-3-xylosylrutinoside.

Peak 8 (t_R = 19.3 min) had an absorbance spectrum with a λ_{max} at 519 nm, and MS/MS analysis yielded an M^+ at m/z 595 which fragmented to give an m/z 287 ion (M − 308, cleavage of a rutinosyl unit). Peak 7 is therefore cyanidin-3-rutinoside.

Peak 9 (t_R = 20.0 min) had a λ_{max} of 503 nm, and the MS/MS spectrum was identical to that of cyanidin-3-(2^G-glucosylrutinoside) in peak 3 except that the ions were at m/z values that were 16 amu lower [$M^+ - m/z$ 741 with fragment ions at m/z 595 (M − 146) and 271 (M − 324)]. Peak 8 is therefore pelargonidin-3-(2^G-glucosylrutinoside).

Peak 10 (t_R = 20.7 min) had a λ_{max} of 503 nm, and the mass spectrum contained an M^+ at m/z 433 which fragmented to yield

Table 1. Summary of the Properties of Compounds Detected in Extracts of Ample Raspberries Following Analysis by HPLC with Diode Array and MS/MS Detection[a]

peak	t_R (min)	λ_{max} (nm)	compound	$M^{+/-}$ (m/z)	fragment ions (m/z)
1	12.3	519	cyanidin-3,5-diglucoside	625^+	449 (M^+ – Glc), 287[Cyan] (M^+ – Glc – Glc)
2	15.7	519	cyanidin-3-sophoroside	611^+	287[Cyan] (M^+ – Soph)
3	17.1	519	cyanidin-3-(2^G-glucosylrutinoside)	757^+	611 (M^+ – Rham), 287[Cyan] (M^+ – Rham – Glc – Glc)
4	17.5	519	cyanidin-3-glucoside	449^+	287[Cyan] (M^+ – Glc)
5	17.7	519	cyanidin-3-sambubioside	581	449 (M^+ – Xyl), 287[Cyan] (M^+ – Xyl – Glc)
6	18.4	503	pelagonidin-3-sophoroside	595^+	271[Pel] (M^+ – Soph)
7	18.8	519	cyanidin-3-xylosylrutinoside	727^+	581 (M^+ – Rham), 287[Cyan] (M^+ – Rham – Xyl – Glc)
8	19.3	519	cyanidin-3-rutinoside	595^+	287[Cyan] (M^+ – Rham – Glc)
9	20.0	503	pelagonidin-3-(2^G-glucosylrutinoside)	741^+	595 (M^+ – Rham), 271[Pel] (M^+ – Rham – Glc – Glc)
10	20.7	503	pelagonidin-3-glucoside	433^+	271[Pel] (M^+ – Gluc)
11	22.1	503	pelagonidin-3-rutinoside	579^+	271[Pel] (M^+ – Rham – Glc)
12	26.3	250	lambertianin C	$[1401^-]2$	1250 ({$[1401^-]$2-HHDP}/2), 935[Casu/Pot]
13	27.8	250	sanguiin H-6	1869^-	1567 (M^- – HHDP), 1265 (M^- – HHDP – HHDP), 935 (M^- – HHDP – HHDP – Glc – Galloyl)
14	29.7	361	ellagic acid–pentose conjugate	433^-	301[HHDP] (M^- – Pent)
15	31.0	365	ellagic acid–pentose conjugate	433^-	301[HHDP] (M^- – Pent)
16	32.1	nd	ellagic acid	301^-	
17	33.0	365	quercetin-3-rutinoside (rutin)	609^-	301[Q] (M^- – Rham – Glc)
18	34.7	365	quercetin-3-glucoside	463^-	301[Q] (M^- – Glc)
19	35.5	365	quercetin-3-glucuronide	477^-	301[Q] (M^- – GlcAC)
20	38.2	365	methylquercetin–pentose conjugate	447^-	315[Iso] (M^- – Pent)
21	38.7	360	ellagic acid acetylxyloside	475^-	301[HHDP] (M^- – XylAc)
22	41.1	365	kaempferol glucuronide	461^-	285[K] (M^- – GlcAC)
23	41.6	360	ellagic acid acetylarabinoside	475^-	301[HHDB] (M^- – AraAc)

[a] Peak numbers and retention times refer to the numbers given in **Figures 1** and **2**. Cyan, cyanidin; Pel, pelargonidin; Q, quercetin; Iso, isorhamnetin; K, kaempferol; Glc, glucosyl; Sop, sophorosyl; Rham, rhamnosyl; Pent, pentosyl; XylAc, acetylxyloside; AraAc, acetylarabinoside; GlcAC, glucuronosyl; Casu/Pot, casuarictin/potentillin; HHDP, hexahydroxyphenoyl; $M^{+/-}$, molecular ion.

m/z 271 (M − 162) which is in keeping with the properties of pelargonidin-3-glucoside.

Peak 11 (t_R = 22.2 min) also had a λ_{max} of 503 nm and on the basis of the MS data that were obtained, an M^+ at m/z 579 giving rise to a secondary fragment at m/z 271 (M − 308), is identified as pelargonidin-3-rutinoside.

Peak 12 (t_R = 26.3 min) had a λ_{max} of 250 nm and when subjected to acid hydrolysis yielded ellagic acid. The mass spectrum of this compound was complicated as the M_r was greater than the 2000 amu upper mass limit of the mass spectrometer. The exact mass of 2804 was determined from a doubly charged ion at m/z 1401. Another doubly charged fragment was observed at m/z 1250 [M − 302, loss of a hexahydroxyphenoyl (HHDP) unit]. The first singly charged ion at m/z 933 corresponds with the ellagitannin isomers casuarictin/potentillin. On the basis of this fragmentation pattern, the compound is identified as lambertianin C, which is a trimer of casuarictin/potentillin, previously identified in raspberry leaves (*21*).

Peak 13 (t_R = 27.8 min) had a λ_{max} of 250 nm and when subjected to acid hydrolysis yielded ellagic acid. The MS/MS spectrum had an M^- at m/z 1869 which fragmented to produce m/z 1567 (M − 302, loss of an HHDP unit), 1265 (M − 302, a further loss of HHDP), 1103 (M − 162, loss of a glucosyl group), 933 (M − 170, loss of a gallic acid), and 631 (M − 302, loss of HHDP). On the basis of the MS/MS spectrum and previously published data (*21*−*23*), this compound is identified as sanguiin H-6 which is a dimer of casuarictin/potentillin.

Peaks 14 and 15 (t_R = 29.7 and 31.0 min, respectively) had λ_{max}s of 361 nm and produced a mass spectrum with an M^- at m/z 433 which yielded a fragment ion at m/z 301 (M − 132), which corresponds to ellagic acid. This is indicative of the presence of pentose conjugates of ellagic acid. One of these compounds may be ellagic acid arabinoside, the presence of which has been reported in raspberries (*20*).

Peak 16 (t_R = 32.1 min) also had a λ_{max} of 361 nm. It was identified as ellagic acid on the basis of the mass spectral data as it produced an M^- at m/z 301, but was at such a low concentration no MS/MS data were obtained. This peak cochromatographed with an authentic ellagic acid standard.

Peak 17 (t_R = 33.0 min) had a λ_{max} of 365 nm and is quercetin-3-*O*-rutinoside (rutin) on the basis of cochromatography with an authentic standard and a mass spectrum with ions at m/z 609 (M^-) and 301 (M − 308).

Peak 18 (t_R = 34.7 min) had a λ_{max} of 365 nm and was identified as quercetin-3-glucoside on the basis of cochromatography with an authentic standard and an MS/MS spectrum with a molecular ion at m/z 463 and a fragment ion at m/z 301 (M − 162, loss of a glucosyl unit).

Peak 19 (t_R = 35.5 min) was also a flavonol with a λ_{max} of 365 nm. The mass spectrum had an m/z 477 molecular ion that yielded an M − 176 (cleavage of a glucuronosyl unit) fragment ion at m/z 301, indicating the presence of quercetin-3-glucuronide which has been detected previously in raspberries (*24*).

Peak 20 (t_R = 38.2 min) was characterized by a λ_{max} of 365 nm and an MS/MS spectrum with an m/z 447 molecular ion which ionized to produce an M − 132 fragment (loss of a pentosyl unit) at m/z 315, suggesting the presence of a methylquercetin−pentose conjugate, probably a xyloside.

Peaks 21 and 23 (t_R = 38.7 and 41.6 min, respectively) had λ_{max}s of 360 nm, and the mass spectra of both had an m/z 475 molecular ion and a fragment ion at m/z 301 (M − 174, loss of an acetylpentose unit). Both components are therefore tentatively identified as ellagic acid−acetylpentose conjugates. On the basis of their HPLC elution order and the data of Zafrilla et al. (*20*), peak 21 may be ellagic acid acetylxyloside and peak 23 ellagic acid acetylarabinoside.

Peak 22 (t_R = 41.1 min) also had a λ_{max} of 365 mn and a mass spectrum with an m/z 461 molecular ion that yielded an MS/MS fragment at m/z 285 (M − 176, loss of a glucuronosyl

group), indicating the existence of a kaempferol glucuronide, the presence of which has previously been reported in raspberries (*9*, *24*).

Fractionation of a Raspberry Extract. After removal of the sugars and after having been run through a low-pressure reverse phase column, an extract aliquot corresponding to 15 g of raspberries was separated into 60 fractions by preparative, gradient elution reverse phase HPLC. The fractions were then analyzed colorimetrically for total phenolics and anthocyanin content as well as for antioxidant and vasodilation activity. The data that were obtained are presented in **Figure 3**. The highest concentration of phenolics was centered in fraction 38. There were also smaller peaks in fractions 2−4 and fraction 7 as well as a broad band of phenolics, which corresponded to the anthocyanins in fractions 23−30. There was a close match between profiles obtained for the total phenolics and the antioxidant capacity with zones of antioxidant activity being detected in fractions 2−4, 23−32, and 36−39 with most activity being present in fraction 38. There were no peaks of antioxidant activity eluting after fraction 40 in fractions that contained the ellagic acid derivatives and flavonols listed in **Table 1**. Vasodilation assays were carried out on fractions 20−40, and this revealed a major peak of activity in fractions 35−39.

Analysis of the individual fractions by LC−MS/MS established that sanguiin H-6 was concentrated in fraction 38, with smaller amounts in fractions 37 and 39, while lambertianin C was present in fractions 35−37. Consistent with the data obtained by colorimetric assay, LC−MS/MS showed that fractions 23−30 contained anthocyanins with the major components being distributed as follows: cyanidin-3,5-diglucoside in fractions 22 and 23, cyanidin-3-sophoroside in fractions 23−25, cyanidin-3-(2^G-glucosylrutinoside) in fractions 25−27, cyanidin-3-glucoside in fractions 27−29, and cyanidin-3-rutinoside in fractions 29 and 30. No recognizable ions were detected when fractions 2−4 and 7 were analyzed by LC−MS/MS. Vitamin C eluted as a void volume peak under the HPLC conditions used for MS/MS. However, analysis by HPLC, using the procedure outlined in Materials and Methods, revealed the presence of vitamin C in fractions 2−4.

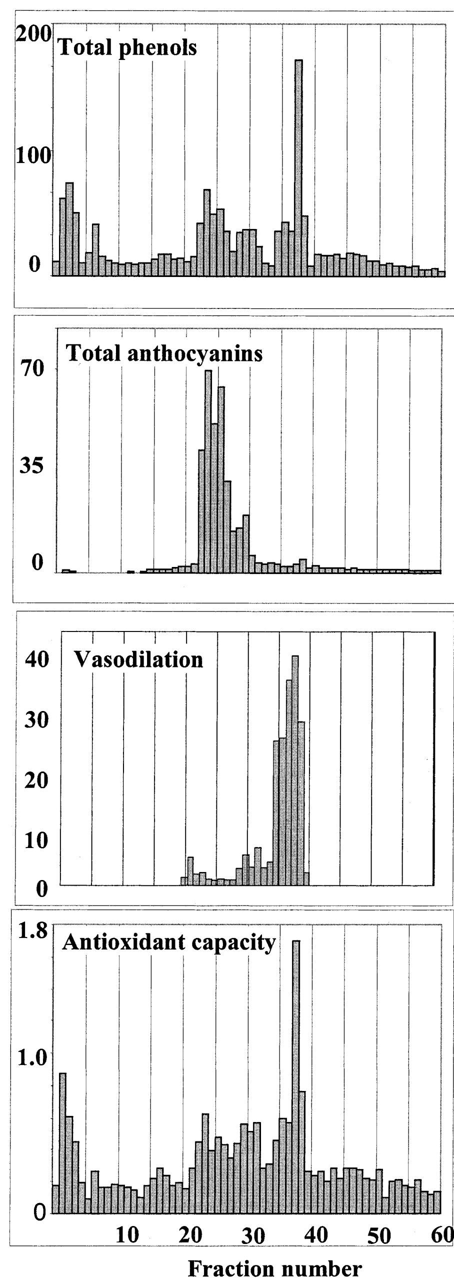

Figure 3. Preparative reverse phase HPLC of an extract of Glen Ample raspberries. The column was eluted with a 30 min gradient from 5 to 25% acetonitrile in water containing 1% formic acid followed by an 80% acetonitrile wash. Successive 30 s fractions were collected after 3 min and concentrated and aliquots assayed for total phenolics, anthocyanins, antioxidant capacity, and vasodilation activity. Data are expressed as follows: total phenolics, micrograms of gallic acid equivalents per gram fw; anthocyanins, micrograms of cyanidin-3-glucoside equivalents per gram fw; vasodilation activity, % relaxation; and antioxidant capacity, number of Fremy's radicals reduced ($\times 10^{17}$) per gram fw.

DISCUSSION

LC−MS/MS analysis identified 11 anthocyanins in Glen Ample raspberries (**Table 1**). The main components were cyanidin-3-sophoroside, cyanidin-3-(2^G-glucosylrutinoside), and cyanidin-3-glucoside with smaller amounts of cyanidin-3-rutinoside, pelargonidin-3-sophoroside, and pelargonidin-3-(2^G-glucosylrutinoside) and trace levels of cyanidin-3,5-diglucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside, pelargonidin-3-sophoroside, pelargonidin-3-glucoside, and pelargonidin-3-rutinoside. The flavonols quercetin-3-rutinoside, quercetin-3-glucoside, and quercetin-3-glucuronide were identified along with a kaempferol glucuronide conjugate and a putative xyloside conjugate of methylquercetin. Two ellagitannins were also detected. The first, lambertianin C, which has an M_r of 2804, consists of six HHDP, three galloyl, and three glucosyl moieties. The second ellagitannin, sanguiin H-6, which was present in substantial amounts, has an M_r of 1870 and comprises four HHDP, two galloyl, and two glucosyl units. Trace levels of ellagic acid were also present as well as two pentose conjugates of ellagic acid that eluted from the HPLC column immediately before ellagic acid. One of these compounds may be ellagic arabinoside which was detected in low concentrations in raspberries and raspberry jams (*20*). In addition, these investigators also characterized ellagic acid acetylxyloside and ellagic acid acetylarabinoside which were also detected in our study.

The data obtained in this study indicate that raspberries do not contain high levels of ellagic acid per se and that the high levels of this compound that have been detected after hydrolysis of extracts (*1*, *5*) are likely to have been derived primarily from sanguiin H-6 and lambertianin C.

Preparative reverse phase HPLC of a raspberry extract separated three zones of antioxidant capacity in fractions 2−4

which contained vitamin C, fractions 23−32 where anthocyanins were present, and fractions 36−39 where lambertianin C and sanguiin H-6 were detected (**Figure 3**). The major peak of antioxidant activity was in fraction 38 which contained sanguiin H-6. However, calculation of the overall antioxidant activity in each of the three zones reveals that the vitamin C zone in fractions 2−4 reduced 1.6×10^{17} radicals/g fresh weight (fw), the anthocyanins in fractions 23−32, 3.8×10^{17} radicals/g fw, and the ellagitannins in fractions 36−39, 3.2×10^{17} radicals g^{-1} fw. Thus, although no single anthocyanin makes a contribution to the antioxidant capacity of the raspberries equivalent to that of sanguiin H-6, the combined contribution of the 11 anthocyanins in fractions 23−32 is slightly higher than that of the ellagitannins in fractions 36−39. The antioxidant activity in fractions 2−4, which contain vitamin C, is half that of the ellagitannins. The lack of antioxidant activity after fraction 40 indicates that ellagic acid and its sugar conjugates and flavonols do not make a significant contribution to the antioxidant capacity of Glen Ample raspberries.

Vasodilation assays were carried out on fractions 20−40. The anthocyanins in fractions 23−32 exhibited little activity, while a major zone of activity in fractions 35−39 was closely associated with the presence of lambertianin C and sanguiin H-6. Many plants, especially fruits and vegetables, contain extractable compounds that cause endothelium-dependent vasorelaxation in vitro (*25*). In this study, raspberry fractions associated with the presence of lambertianin C and sanguiin H-6 have been shown to be potent vasodilators of rabbit aortic vessels with intact endothelium. However, a previous study has shown that anthocyanins derived from red wine, unlike those from raspberries, induced vasorelaxation (*11*). These anthocyanins were subsequently found to be more potent dilators than oligomeric condensed tannins that are also present in red wine. Additional work with anthocyanins has demonstrated that delphinidin, but not malvidin or cyanidin, is able to elicit endothelium-dependent vasorelaxation in vascular tissue (*12*). Delphinidin-induced vasorelaxation, which was comparable to that of the original red wine polyphenolic extract, was mediated via the release of nitric oxide (*11*). Therefore, from the data obtained with grapes and other red fruits, it is evident that only specific anthocyanin structures may be able to induce vasorelaxation. The exact anthocyanins from red wine that produce a cardioprotective vasodilatory effect remain to be determined.

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