Phytochemical Analysis
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A Single HPLC-PAD-APCI/MS Method for the Quantitative Comparison of Phenolic Compounds Found in Leaf, Stem, Root and Fruit Extracts of *Vaccinium angustifolium*

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Abstract: A method was developed for the analysis of *Vaccinium angustifolium* Ait. (Lowbush blueberry), which is a widely used natural health product, particularly for the treatment of diabetic symptoms. While the anthocyanin content of the fruit has been well characterized, the chemistry of the vegetative parts used in supportive therapy for diabetes has been largely ignored. Using a metabolomics-based approach for compound identification with an emphasis on phenolic metabolites, a single HPLC-PAD-APCI/MS method was developed for the separation and quantitation of the major metabolites found in the 95% ethanol extracts of leaf, stem, root and fruit. The leaf extract contained high concentrations of chlorogenic acid (~100 μg/mg extract) and a variety of quercetin glycosides that were also detected in the fruit and stem extracts. Flavan-3-ol monomers (+)-catechin and (–)-epicatechin were found in all plant parts but their procyanidin dimers were exclusively identified in the stem and root. The accuracy and precision of the presented method were corroborated by low intra- and inter-day variations in quantitative results in all plant part extracts. Further validation of the extraction and analytical protocols focused on identified compounds with reputed anti-diabetic activity, revealing recoveries greater than 80% and detection limits of 0.12–2.73 μg/mL. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: HPLC-PAD-APCI/MS; quantitative determination; chlorogenic acid; quercetin glycosides; procyanidins; antidiabetic plants; *Vaccinium angustifolium*; Ericaceae.

INTRODUCTION

Various members of the genus *Vaccinium* have been used as traditional medicines for the treatment of diabetic symptoms (Jellin *et al.*, 2005, Leduc *et al.*, 2006) and possess putative anti-diabetic activity (Cignarella *et al.*, 1996; Blumenthal, 1998; Chambers and Camire, 2003). Although *V. myrtillus* L (European blueberry or bilberry) and *V. macrocarpon* Ait. (American cranberry) are recognized as sources of anti-diabetic phytochemicals, recent surveys have identified *V. angustofolium* Ait. (Canadian lowbush blueberry) as highly recommended by Quebec traditional practitioners and Cree Elders of Eeyou Istchee for treatment of diabetic symptoms and complications (Haddad *et al.*, 2003; Leduc *et al.*, 2006).

Lowbush blueberry fruits are collected and consumed throughout regions of north-eastern North America and possess well-documented antioxidant properties (Costantino *et al.*, 1992; Kalt *et al.*, 1999; Wang and Jiao, 2000; Kay and Holub, 2002; Rimando *et al.*, 2004). By virtue of its popular consumption as a food, the phytochemistry of *V. angustifolium* fruits is relatively well described, particularly with regard to anthocyanin content (Kalt *et al.*, 2001; Prior *et al.*, 2001; Wu and Prior, 2005). The traditional medicinal uses of the plant, however, include the leaves, stems and roots in addition to the fruits to treat different diabetic symptoms. Moreover, in a recent *in vitro* study, extracts of *V. angustifolium* leaf, stem, root and berry each elicited a different spectrum of anti-diabetic activities (Martineau *et al.*, 2006). Few, if any, studies have investigated or compared the chemistry of these sources.

The present study describes the development and validation of an HPLC-PAD-APCI/MS method for analysing the phytochemical content of *V. angustifolium* with an emphasis on phenolic compounds in different plant parts. Phenolic compounds were emphasised because of their abundance in closely related *Vaccinium*

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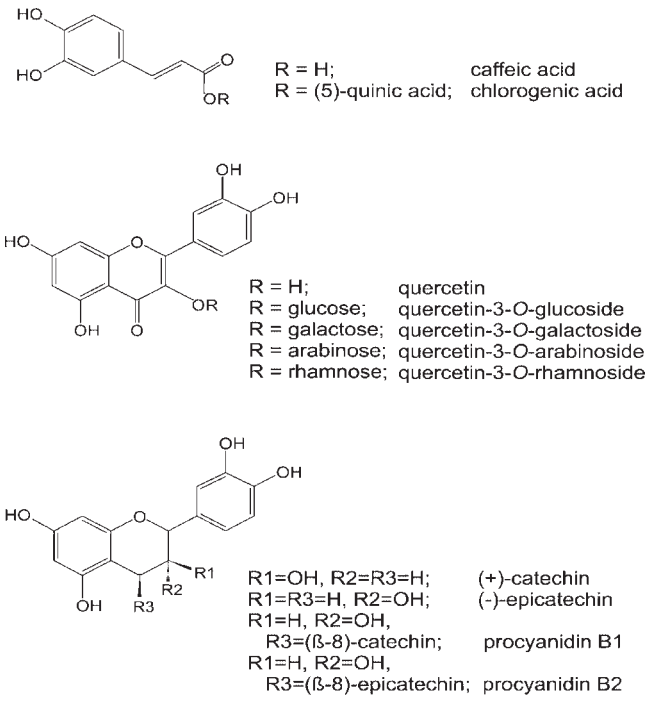

species (Witzell *et al.*, 2003) and because many different phenolics have demonstrated *in vivo* anti-diabetic activities (Kim *et al.*, 2003; Coskun *et al.*, 2005). Using a metabolomics-based approach for preliminary compound identification and reference standards for confirmation, chemical profiles for each organ were determined and quantitative comparisons conducted.

EXPERIMENTAL

Materials. HPLC solvents and analytical-grade trifluoroacetic acid (TFA) were purchased from EMD (Darmstadt, Germany) and J. T. Baker (Phillipsburg, NJ, USA), respectively. Standards of (+)-catechin and quercetin-3-*O*-glycoside were obtained from Extrasynthase (Lyon, France), quercetin, chlorogenic acid and caffeic acid from Sigma (Oakville, Canada) and (–)-epicatechin from Fluka (Buchs, Switzerland). Procyanidins were graciously provided by Dr. D. Ferreira (University of Mississippi, USA) and the remaining standards (quercetin glycosides and compounds comprising the phenolic library) were isolated and purified 'in house' (>95% purity as determined by HPLC; Lozoya *et al.*, 1994).

Sample preparation and extraction. Fresh samples of wild *Vaccinium angustifolium* Ait. were collected near La Vérendrye Wildlife Reserve, Quebec, Canada on 27 August 2005. Voucher specimens were stored at the University of Ottawa (UO 19190). After harvesting, the plant material was washed and manually separated into organs: leaves, stems, roots and fruits.

Fresh leaves were preserved in 95% ethanol then filtered, crushed and re-extracted three times with fresh ethanol (95%) at room temperature for 12 h. The four ethanolic phases were recovered, pooled, dried at 40°C on a rotary evaporator and lyophilised. Roots and stems were air-dried and stored at room temperature in the dark prior to extraction. The fruits were frozen at –80°C. A Wiley mill with mesh size 40 was used for grinding each source and the resulting powder extracted three times in ethanol at room temperature for 12 h. The ethanolic phase was then dried and lyophilised as above. All extracts were stored at 4°C.

HPLC-PAD-APCI/MS analyses. HPLC-MS analyses were conducted on leaf, stem, root and fruit extracts. Since the anthocyanin content of fruit has been extensively characterized (Kalt *et al.*, 2001; Prior *et al.*, 2001; Rimando *et al.*, 2004; Wu and Prior, 2005), this family of compounds was not examined in detail. Analyses were performed on an Agilent (Palo Alto, CA, USA) 1100 LC MSD VL APCI system consisting of an autosampler, quaternary pump, photodiode array detector (PAD), and an online APCI/MS with mass range of 50–15,000 amu. A Waters (Mississauga, Canada) YMC ODS-AM column (100 × 2 mm i.d.; 3 μm particle size), maintained at 50°C, was employed at a flow rate of 0.3 mL/min. The elution conditions were optimized with a mobile phase of aqueous TFA (0.05%), pH 3.4 (solvent A) and methanol (solvent B) as follows: initial conditions 92:8 (A:B) maintained 0–5 min, followed by four linear gradients of 8–13% B in 2 min, 13–30% B in 14 min, 30–60% B in 3 min, and 60–100% B in 2 min. The column was then washed with 100% B for 2 min, returned to the initial conditions (92:8) in 2 min, and re-equilibrated for 6 min, resulting in a total run time of 36 min. An aliquot (1 μL) of each extract was injected through the autosampler for each run and the subsequent elution profiles were monitored on-line at 325 nm and 280 nm (PAD) and by total-ion current (TIC). A monitoring wavelength of 520 nm was also used for qualitative analysis of anthocyanins in the fruit extract.

The mass spectrometer was tuned in dual polarity mode at the outset of all experiments. MS detection was performed in both positive and negative ionization modes. For positive ionization mode, the optimized spray chamber conditions were: drying gas flow rate of 6.0 L/min, nebulizer pressure of 40 psig, drying gas temperature of 300°C, vaporizer temperature of 400°C, capillary voltage of 3000 V and corona current of 3.0 μA. For negative ionization mode, the conditions were: drying gas flow rate of 6.0 L/min, nebulizer pressure of 60 psig, drying gas temperature of 350°C, vaporizer temperature of 400°C, capillary voltage of –3000 V, and corona current of 15.0 μA. APCI was conducted at 300°C with the vaporizer at 400°C; nebulizer pressure, 40 psig; nitrogen (drying gas) flow

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rate, 6.0 L/min; fragmentation voltage, 20 V; capillary voltage, 3000 V; corona current, 3.0 μA. The MS was operated in scan mode within 100–800 amu with fragmentation voltages of 20 and −160 V for positive and negative ionization, respectively.

Metabolomics-based compound identification. Over 120 purified phenolic compounds were injected in the HPLC system where the UV absorption spectrum was scanned and saved into a searchable library of reference standards. These spectra were used for comparison with the absorption spectra of the unknown compounds in the test extracts. A preliminary match to a library entry was defined by at least 95% similarity between the spectrum of an unknown peak and the library entry, as determined by the Chemstation software. Matches were further corroborated by visual inspection of the spectral match and by the presence of a major ion in the MS of the unknown peak's mass spectrum corresponding to the entry molecule or its major ion fragment (e.g. an ion peak corresponding to a monomeric procyanidin, along with a second peak corresponding to a dimer, would support a match to a procyanidin library entry).

Co-chromatography of standards of the identified compounds spiked into plant extracts was then performed to confirm matches, with retention time serving to distinguish between isomers (e.g. quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside).

Compound quantification. Identified metabolites were quantified on the basis of area under the peak of HPLC-PAD chromatograms using calibration curves produced using pure standards analysed on the same day. Phenolic acids and flavonols were quantified at 325 nm and catechins and procyanidins were quantified at 280 nm. Mean quantities were calculated from freshly dissolved triplicates of each extract quantified on three separate days (minimum $n = 9$).

Method validation. Validation was performed for HPLC-DAD-APCI/MS in the positive ionisation mode using the organic phase gradient programme described above. Recovery experiments were undertaken by injecting aliquots of epicatechin and quercetin-3-*O*-glucoside standards (0.5, 1.0, 1.5 and 2.0 mg/mL) to stem material and aliquots of chlorogenic acid standard (2.5, 5.0, 7.5 and 10 mg/mL) to leaf material. Spiked and unspiked samples were extracted as described above. Experiments were carried out in triplicate and coefficients of variation determined accordingly.

The limit of detection (LOD) and limit of quantification (LOQ), respectively defined as a 3:1 and 10:1 peak-to-noise ratio, were determined for selected compounds by analysis of serially diluted leaf, stem, root and fruit extracts.

Statistical analysis. All statistical analyses were performed with Systat software version 10.0 (SPSS, Chicago, IL, USA); ANOVA analysis and subsequent Tukey *post-hoc* tests were used to compare plant-part specific metabolite levels. Recovery data were analysed by simple linear regression.

RESULTS AND DISCUSSION

Chromatographic separations of extracts and compound identification

The ethanolic extraction of *V. angustifolium* leaves, stems, roots and fruits produced yields of 30, 9, 5 and 50% extract, respectively. Based on our previous work with phenolic-rich extracts (Bily *et al.*, 2004), it was established that an aqueous mobile phase containing 0.05% TFA produced high-quality peak shape and separation. Linear gradients of methanol and acetonitrile were individually optimized as the organic phase. Although compounds eluted more quickly from all extracts in acetonitrile, the resolution of early and late eluting peaks suffered and lower signal-to-noise ratios were observed during analysis of stems and roots, presumably due to high procyanidin/tannin content. While this noise was still apparent using methanol as the organic phase, subsequent optimization (as described in Experimental) with a 28 min methanol gradient programme yielded major peaks from all plant parts with sufficient separation for quantitation. Attempts to improve the separation of late eluting peaks by decreasing flow rate or by isocratic elution resulted in broader peaks. The improvement provided by extended gradient programs was marginal. MS results were also improved using methanol but only when the column was maintained at high temperature (50°C). Note that the described methods were not optimized for separation of anthocyanins.

Metabolomics-based compound identification yielded several preliminary matches that corresponded to caffeic acid derivatives, quercetin derivatives, flavan-3-ols and procyanidins. The identities of 10 compounds were confirmed within the leaf extract, 12 within the stem extract and seven within the root extract. Excluding anthocyanin peaks, eight compounds were identified in the fruit extract. Additional tentative identifications were noted but not definitively confirmed and amounts were not quantitated (Table 1). While conclusive identifications could not be made for all major peaks, the metabolomics approach proved successful as a guide. Owing to the presence of many closely related compounds, similar UV absorption spectra and/or ion fragmentation patterns highlighted the need for tri-corroboration of these measures and retention time with those of standards. In cases where all criteria were not met adequately, confirmation was made (or

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Table 1 List of the compounds identified in *Vaccinium angustifolium* leaf, stem, root and fruit extracts by metabolomics-based HPLC-PAD-APCI/MS analysis

Peak	R_t[a] (min)	$[M]^+$ and fragments (m/z)	Compound	Content (mg/g dry matter) ± SEM[b]			
				Leaf	Stem	Root	Fruit
1	4.0	355/195/163	Chlorogenic acid isomer*	√	√		
2	5.3	579/291	Procyanidin B1		0.81 ± 0.02	0.04 ± 0.007	
3	6.6	291/139	Catechin	6.16 ± 0.52	1.25 ± 0.05	0.05 ± 0.009	trace
4	8.3	169	Vanillic acid		trace	0.01 ± 4e^{-4}	
5	9.2	195/179/163	Caffeic acid	0.36 ± 0.02	0.03 ± 0.007		
6	10.5	355/195/163	Chlorogenic acid	31.19 ± 0.55	0.09 ± 0.008	0.03 ± 0.007	1.54 ± 0.01
7	11.2	579/291	Procyanidin B2		0.98 ± 0.03	0.21 ± 0.01	
8	13.7	291/139	Epicatechin	7.25 ± 0.35	2.90 ± 0.10	0.70 ± 0.04	trace
9	13.9	577/291	Procyanidin*		√	√	
10	14.9	579/291	Procyanidin*		√	√	
11	15.4	579/291	Procyanidin*		√	√	
12	24.2	465/303	Quercetin-3-*O*-galactoside	1.96 ± 0.05	0.45 ± 0.01		0.31 ± 0.02
13	24.8	465/303	Quercetin-3-*O*-glucoside	3.49 ± 0.08	0.13 ± 0.01		0.41 ± 0.02
14	25.2	465/303	Quercetin-hexoside*	√	√		√
15	25.8	435/303	Quercetin-3-*O*-arabinoside	2.74 ± 0.06	0.67 ± 0.02		trace
16	26.3	435/303	Quercetin-pentoside*	√	√		√
17	26.6	449/303	Quercetin-3-*O*-rhamnoside	1.46 ± 0.05	0.01 ± 7e^{-4}		trace
18	26.8	595/449/287	Kaempferol-3-*O*-rutinoside*	√			
19	27.6	303	Quercetin	1.24 ± 0.04	0.01 ± 6e^{-4}		0.03 ± 2e^{-4}

* indicates tentative identification without confirmation relative to a pure standard.
√ indicates the detection but not quantification of a tentatively identified compound.
[a] R_t, retention time.
[b] SEM, standard error of the mean.

rejected) by spiking the extract with a suitable amount of standard.

Distinct root and leaf compounds were identified while the stem extract appeared to be intermediate between leaf and root. Chlorogenic acid was identified as the most abundant phenolic in leaves consistent with previous analyses of leaves from *V. myrtillus* (Witzell and Shevtsova, 2004). Beyond this early peak, most peaks in the leaf chromatograph corresponded to quercetin glycosides [Fig. 1(A)]. The identity of four of these glycosides was confirmed (quercetin-3-*O*-galactoside, Q-3-gal; quercetin-3-*O*-glucoside, Q-3-glu; quercetin-3-*O*-arabinoside, Q-3-ara; quercetin-3-*O*-rhamnoside, Q-3-rham) and two additional quercetin glycosides were identified, one containing a hexose and the other a pentose, according to their MS fragmentation pattern and absorption spectra [Table 1, Fig. 1(C)]. The remaining identified peaks corresponded to an isomer of chlorogenic acid (peak 1), (+)-catechin (catechin, peak 3), caffeic acid (peak 5), and (–)-epicatechin (epicatechin, peak 8). Small peaks eluting at retention times of 14.4 and 26.8 min, tentatively identified as *p*-coumaroyl quinic acid and kaempferol-3-*O*-rutinoside respectively, were seen only in the leaf chromatogram.

By contrast, stem extracts contained only low levels of chlorogenic acid. The same quercetin glycosides found in the leaf extract were identified in stems though in lower amounts and at different relative abundances [Fig. 2(A)]. In addition to the monomeric flavan-3-ols detected in leaf material (catechin and epicatechin), a variety of peaks corresponding to dimers (procyanidins) were eluted from the stem extract during the first 15 min of the gradient.

These procyanidin peaks were also seen in the root extract (Fig. 3), revealing a chemical profile similar to that of the stem but unlike that of the leaf or fruit. The presence of procyanidins B1 and B2 was confirmed in both stem and root chromatographs. Peaks 9–11, each with catechin-matching absorption spectra, represent additional procyanidin species, as supported by MS data indicating two dimers (peaks 10 and 11) and a double-linked dimer [see Table 1, Fig. 2(C); Nakamura *et al.*, 2003]. Similarly, the compounds eluting from stem and root extracts between 19 and 21 min again produced catechin-like UV spectra and shared a common ion fragment (m/z = 249). To gain greater insight into the diversity of procyanidin oligomers occurring in the stem and root, an acetone extraction should be considered and a broader mass range employed during ion scanning. Finally, small amounts of vanillic acid were exclusively detected in the root extract (peak 4).

In this study, the fruit chromatograph strongly resembled the leaf chromatograph; excluding

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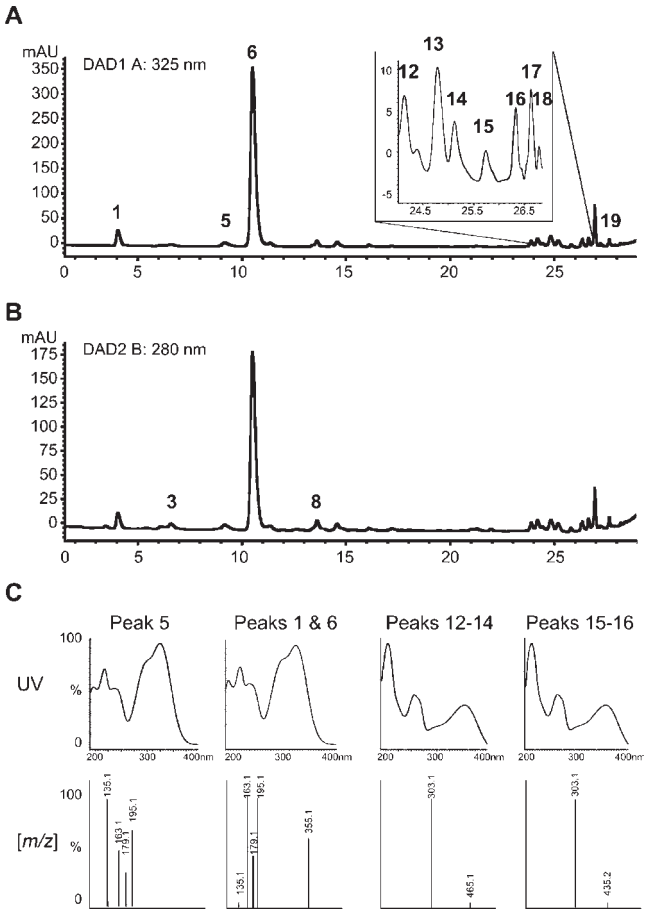

Figure 1 HPLC chromatograms of a leaf extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1. The UV absorption and mass spectral data obtained from selected peaks identified multiple caffeic acid and quercetin derivatives (C, [*m/z*] data reported for ions with relative abundances >15%).

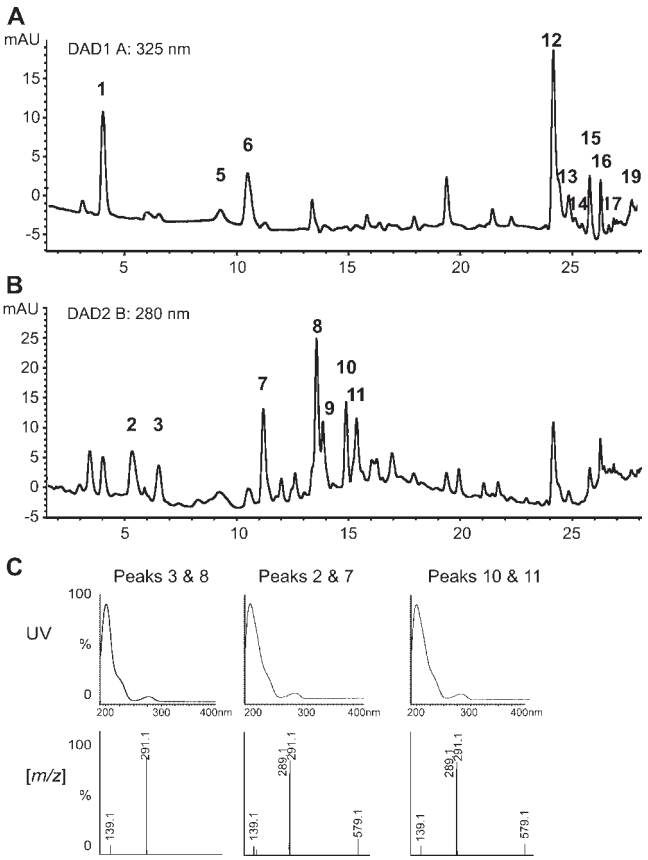

Figure 2 HPLC chromatograms of a stem extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1. The UV absorption and mass spectral data obtained from selected peaks identified several closely related compounds representing procyanidin species (C, [*m/z*] data reported for ions with relative abundances >15%).

anthocyanin peaks, the peaks in the fruit chromatograph were representative of chlorogenic acid, quercetin glycosides and catechins (Fig. 4). These findings are consistent with the compounds previously identified from lowbrush blueberries (Taruscio *et al.*, 2004). PAD and MS data for the quercetin hexosides indicated that these compounds eluted independently of anthocyanins. In the case of Q-3-ara and Q-3-rham, co-elution with anthocyanins prevented accurate quantitation and confirmation by MS data [Fig. 4(B)]. Interfering anthocyanins (eluting between 26 and 27 min) were tentatively identified as malvidin glycosides, producing ion fragments of 331 *m/z* (data not shown). Separation of anthocyanins improved drastically, resolving 20 peaks, with a 5% formate aqueous phase during chromatography, as previously reported (Prior *et al.*, 2001).

Although low levels of procyanidins (3 μg/g dry wt) have been reported in lowbush blueberry fruits (Prior

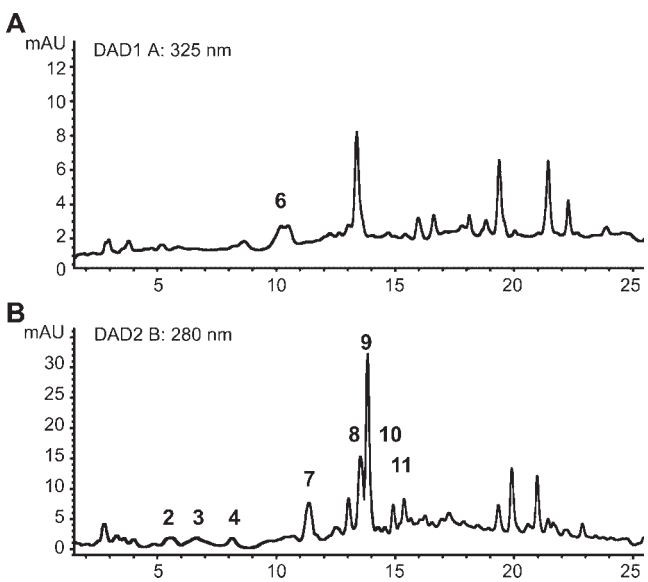

Figure 3 HPLC chromatograms of a root extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1.

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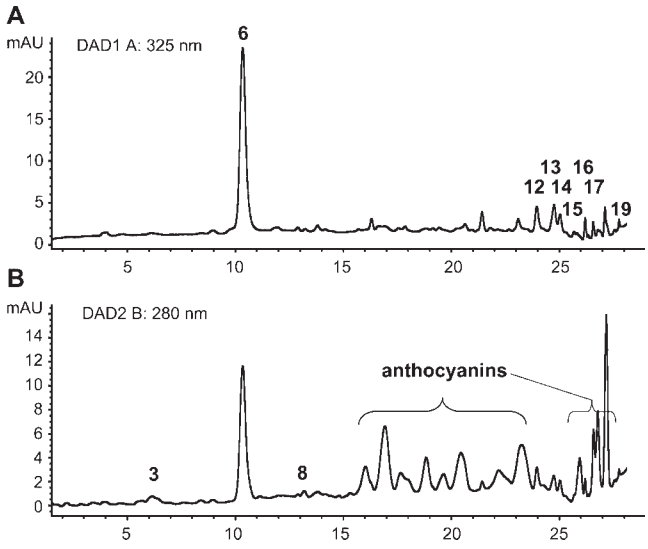

Figure 4 HPLC chromatograms of a fruit extract of *Vaccinium angustifolium* with photodiode array detection (PAD) at 325 nm (A) and 280 nm (B). Numbered peaks represent identified compounds as reported in Table 1.

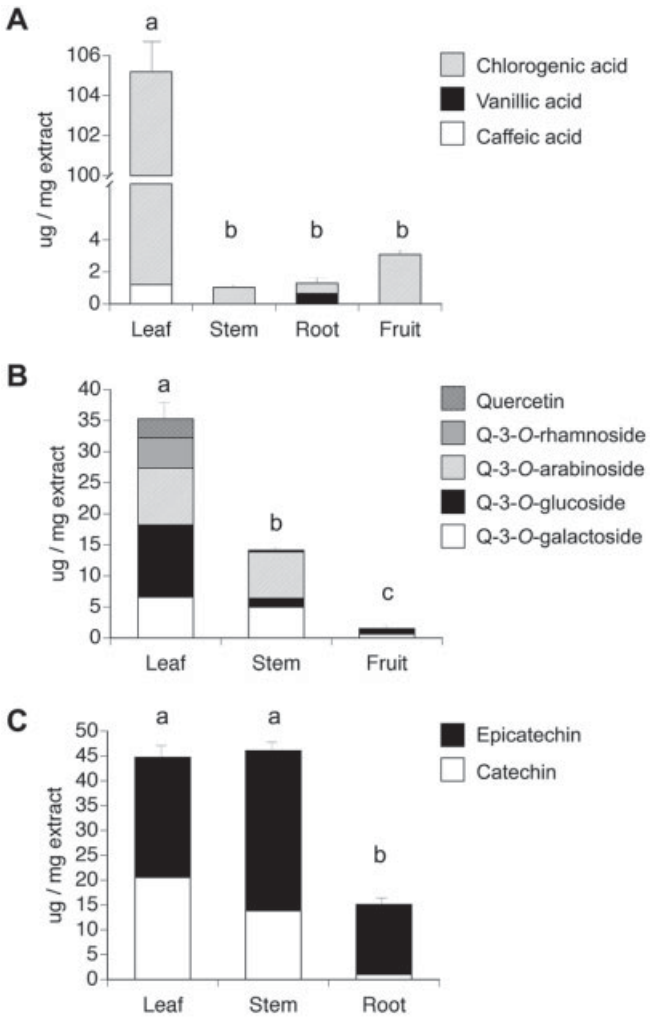

Figure 5 Quantitative comparisons of commonly identified metabolites in leaf, stem, root and/or fruit extracts. Chlorogenic acid was the most abundant phenolic acid in all extracts (A). The total quantified quercetin derivatives were highest in the leaf extract (B). Total monomeric procyanidin content was similar in leaf and stem extracts but was significantly lower in the root (C). Significant differences ($p < 0.05$), as determined by one-way analysis of variance with Tukey *post hoc* test, are designated by letters (a, b, c).

et al., 2001), only trace amounts of monomeric catechin and epicatechin were detected using the presented methods. Higher procyanidin yields can be achieved with an acetone extraction but phenolic content of each plant part is also subject to both temporal and environmental variation (Witzell *et al.*, 2003; Witzell and Shevtsova, 2004), which may contribute to variation between reports. Development of a validated method for constituent identification and quantification is therefore a necessary step if comparisons are to be made.

Whereas certain compounds (catechin, chlorogenic acid and epicatechin) were common to all plant organs, none was ubiquitously abundant and most were restricted in distribution. In this study, chlorogenic acid, the predominant phenolic acid derivative detected, was 30 times more concentrated in leaf extract than in fruit and >100 times more concentrated than in stem or root extracts [Fig. 5(A)]. The distribution of identified quercetin derivatives likewise differed significantly between plant parts. While Q-3-glu and Q-3-rham accounted, respectively, for 36 and 18% of the quantified quercetin glycosides in leaf extract, they represented just 10 and 1% in stem extract. In the fruit extract, where glycoside species were present in similar ratios to those seen in the leaf, the content was approximately ten fold less [Fig. 5(B)]. With regard to flavan-3-ols, leaf and stem extracts had similar quantities but the concentration in roots was significantly lower [Fig. 5(C)]. The detected ratio of epicatechin to catechin was more variable, roughly 1:1 in the leaf extracts, 2:1 in the stem and 14:1 in the root. Although the stem extract contained higher levels of procyanidins B1 and B2, the area of peak 9 [Figs 2(B) and 3(B)], corresponding to an unknown procyanidin dimer, was substantially greater in the root.

Considering that, in a separate pharmacological study, each extract (leaf, stem, root and fruit) elicited a range of *in vitro* anti-diabetic activities (Martineau *et al.*, 2006), it is interesting that all the identified classes of phenolic compounds, chlorogenic acid, quercetin derivatives, procyanidins and anthocyanins, have demonstrated anti-diabetic potential *in vitro* and *in vivo* (Hemmerle *et al.*, 1997; Rodriguez de Sotillo and Hadley, 2002; Kim *et al.*, 2003; Tsuda *et al.*, 2003; Pinent *et al.*, 2004; Ajay *et al.*, 2005; Coskun *et al.*, 2005; Dias *et al.*, 2005; Jayaprakasam *et al.*, 2006). Validation of the extraction and analytical protocols

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therefore focused on those compounds with reputed anti-diabetic activity. However, since *V. angustifolium* contains additional secondary metabolites that may possess biological activity, further chemical analyses and assay-guided fractionation are required to identify the active constituents.

Method validation of marker compounds

Dual PAD monitoring of UV absorption at wavelengths of 325 and 280 nm provided optimal detection of all identified metabolites except anthocyanins. Moreover, detection of anthocyanins in the fruit extract was severely dampened at 325 nm, simplifying the detection of quercetin derivatives (Fig. 4). Should anthocyanin characterization be required, a wavelength of 520 nm would identify these compounds [Fig. 4(B)].

The production of reference calibration curves during compound quantification revealed linear response profiles (R^2 > 0.999) for all quantified compounds at minimum ranges of 6.6–330 μg/mL (Q-3-ara) to 7.7–1440 μg/mL (chlorogenic acid; data not shown).

To assess the accuracy and precision of the presented method, variation in the results for each quantified compound were calculated within and between days. Expressed as the co-efficient of variation (CV, standard deviation/mean × 100%), variation between successive trials (intraday) was minimal on days 1 and 2 (Table 2). The slightly more pronounced variability on

Table 2 Intraday and interday variation in quantitative results

	Intraday variation (SD[a]/mean amount × 100%)			Interday variation
	Day 1	Day 2	Day 3	Days 1–3
Leaf				
Catechin	0.63	1.56	6.45	5.27
Caffeic acid	14.32	7.26	6.40	21.71
Chlorogenic acid	0.14	0.94	1.39	3.23
Epicatechin	0.12	8.61	12.74	7.71
Quercetin-3-*O*-galactoside	0.36	0.54	2.74	3.85
Quercetin-3-*O*-glucoside	0.23	0.64	3.65	3.49
Quercetin-3-*O*-arabinoside	0.23	1.40	8.25	9.71
Quercetin-3-*O*-rhamnoside	0.19	2.16	0.91	0.79
Quercetin	0.77	2.84	6.40	6.38
Stem				
Procyanidin B1	0.78	0.20	13.40	6.43
Catechin	1.00	0.77	4.26	7.84
Chlorogenic acid	2.71	1.23	5.20	9.83
Procyanidin B2	0.85	2.29	8.76	7.41
Epicatechin	4.00	4.98	10.94	9.58
Quercetin-3-*O*-galactoside	1.29	1.23	0.69	2.10
Quercetin-3-*O*-glucoside	1.33	0.32	10.32	12.82
Quercetin-3-*O*-arabinoside	0.63	0.41	9.77	6.51
Quercetin-3-*O*-rhamnoside	1.50	0.70	14.07	5.09
Quercetin	1.16	2.72	9.82	22.30
Root				
Procyanidin B1	5.25	1.00	5.79	7.05
Catechin	0.91	2.46	7.89	5.49
Vanillic acid	4.32	2.04	17.66	11.1
Chlorogenic acid	9.15	0.29	13.02	8.04
Procyanidin B2	9.92	0.53	7.90	8.72
Epicatechin	5.61	5.20	4.25	4.54
Fruit				
Chlorogenic acid	1.61	0.61	4.68	2.07
Quercetin-3-*O*-galactoside	7.12	24.32	4.47	2.86
Quercetin-3-*O*-glucoside	4.73	0.43	3.25	2.08
Quercetin	1.18	2.11	3.57	0.88

[a] SD, standard deviation.

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Table 3 Methodological limits of detection and quantification and their corresponding minimum concentrations for detection in the leaf, stem, root and fruit extracts of *V. angustifolium*

Compound	Limit of detection (μg/mL ± SEM)	Limit of quantification (μg/mL ± SEM)
Procyanidin B1	1.63 ± 0.30	5.42 ± 0.99
Catechin	2.73 ± 0.40	9.11 ± 1.12
Chlorogenic acid	0.33 ± 0.04	1.10 ± 0.11
Procyanidin B2	2.03 ± 0.19	6.77 ± 0.49
Epicatechin	1.77 ± 0.15	5.91 ± 0.44
Quercetin-3-*O*-galactoside	0.42 ± 0.06	1.38 ± 0.19
Quercetin-3-*O*-glucoside	0.44 ± 0.06	1.46 ± 0.18
Quercetin	0.12 ± 0.02	0.39 ± 0.09

Table 4 Recovery of selected compounds from leaves and stems of *V. angustifolium*

	CV[a] of yield (%)	Recovery (% recovered)					
		Level 1	Level 2	Level 3	Level 4	Mean[b]	R^{2c}
Leaf		(+2.5 mg)	(+5.0 mg)	(+7.5 mg)	(+10.0 mg)		
Chlorogenic acid	6.1	88.0	84.0	100.4	107.7	103.8	0.985
Stem		(+0.5 mg)	(+1.0 mg)	(+1.5 mg)	(+2.0 mg)		
Epicatechin	4.8	100.3	89.6	87.8	92.3	91.0	0.962
Quercetin-3-*O*-glucoside	4.8	77.8	83.3	78.1	101.4	80.1	0.970

[a] CV, coefficient of variation; standard deviation/mean × 100%.
[b] As determined from slope of regression analysis of injected and recovered quantities.
[c] R^2, *R*-squared value of linear regression.

the third day was probably due to column degradation as this third trial was conducted several months following the first two. Nevertheless, with the exception of a few compounds detected at levels near or below 1 μg/mg extract, interday variation was generally less than 10% (Table 2).

Analysis of serially diluted samples and subsequent peak to noise ratio calculation generated LOD and LOQ values for a subset of identified compounds (Table 3). LOD and LOQ values are reported as the lowest detectable and quantifiable amounts of a compound present in a 1 mL sample (i.e. total content of the sample, regardless of extract concentration) and did not differ significantly between plant parts. The LOD values of chlorogenic acid, Q-3-gal, Q-3-glu and quercetin were 329, 415, 437 and 116 ng, respectively, while the catechins and procyanidins were not detectable until concentrations exceeding 1.5 μg/mg extract. LOQ values were consistently lower for compounds quantified at 325 nm than those quantified at 280 nm; to increase sensitivity to flavan-3-ols, a lower monitoring wavelength can be employed (230 nm), but baseline drift and/or noise will also increase. The recommended extract concentrations for quantifiable detection of major metabolites is 10 mg/mL for leaf extract and 25 mg/mL for stem, root and fruit extracts.

Recovery analyses were restricted to three representative compounds spiked into either leaf or stem material prior to extraction. Plotting recovered amounts vs spiked amounts, the corresponding regression line provides the mean recovery across concentrations (the slope), the linearity of recovery over the range of spiking (R^2) and the approximate content of unspiked samples (*y*-intercept). Despite spiking high amounts of chlorogenic acid into leaves, all were recovered during the extraction procedure (Table 4). Considering that separate extractions conducted on separate samples produced a yield with a CV of 6.1%, a mean recovery of 104% is reasonable. While epicatechin and Q-3-glu were recovered well with mean recoveries of 91 and 80%, respectively, and R^2 values greater than 0.96 (Table 4), more complex procyanidins were probably recovered less efficiently. In general, the best recoveries were observed from extracted leaves, which were preserved fresh. We therefore recommend using 80% ethanol for extraction of dried samples.

Following these results, the validated method is currently being used to evaluate the phytochemical content and corresponding medicinal properties of the plant from population to population and from season to season. The wide distribution and range of habitats occupied by the species suggests that, as

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V. angustifolium becomes more popular as a medicinal plant and commercial products are made available, the value of a method for rapid analysis of marker compounds increases.

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