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Identification and Quantification of Short Oligomeric Proanthocyanidins and Other Polyphenols in Boysenberry Seeds and Juice

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ABSTRACT: Proanthocyanidins and other polyphenols in the seeds and juice of boysenberry were quantitatively analyzed. Polyphenolic extracts were prepared from the waste seeds and commercial juice by chromatographic fractionation. Compositional analysis revealed that both extracts contained six polyphenolic classes: flavanol monomers, proanthocyanidins, anthocyanins, ellagic acid, ellagitannins, and flavonol glycosides. Ellagitannins were the most abundant polyphenols in both extracts. Proanthocyanidins were present as short oligomers consisting of dimeric and trimeric procyanidins and propelargonidins, with the most abundant component being procyanidin B4 in both extracts. Quantification by high-performance liquid chromatography−mass spectrometry (HPLC−MS) revealed that the seeds contained a 72-fold higher amount of proanthocyanidins than the juice. These results indicate that boysenberry fruits contain short oligomeric proanthocyanidins along with flavanol monomers and the seeds represent a good source of short oligomeric proanthocyanidins.

KEYWORDS: Boysenberry, proanthocyanidin, polyphenol, seeds, juice, HPLC−MS/MS analysis

INTRODUCTION

Proanthocyanidins (PAs), which are also known as condensed tannins, are oligomeric or polymeric flavanols common in many plant-derived foods. Dietary PAs have attracted increasing attention because of their structural diversity and potential health benefits.[1] Those in cacao,[2] apple,[3] and grape[4] have been elucidated and have been shown to have unique profiles of flavanol units and degree of polymerization (DP). These structural characteristics are assumed to be associated with their reported physiological activities, such as antioxidant, antibacterial, anti-inflammatory, and vasorelaxing activities.[1] Dietary PAs may also play an important role in the prevention of cardiovascular diseases[5] and various cancers.[6] Berry fruits are rich sources of dietary polyphenols, and as such, berry polyphenols are the best studied; they have been reported to have a wide range of bioactivities.[7,8] The chemical structures of representative polyphenols from berries are shown in Figure 1 and include flavanol monomers, PAs, anthocyanins, flavonol glycosides, ellagic acid, and ellagitannins. Studies of boysenberry polyphenols to date have focused on the major anthocyanins in the juice.[9] However, recent work has found that berry fruits also contain considerable amounts of PAs with structural and compositional diversity that is dependent upon the berry species. For example, the total PA contents of six Manitoba berries were found to range from 276 to 505 mg/100 g and consist of a series of oligomers and polymers,[10] and PA contents from 15 berries in Finland ranged from 32.9 to 1880 mg/100 g.[11] To date, no study has been reported on the PA content of boysenberry juice or seeds.

Boysenberry is a hybrid *Rubus* berry, considered to be derived from a cross between *Rubus loganobaccus* and *Rubus baileyanus* Britt., and there are currently several cultivars available as boysenberry types.[12] Boysenberry fruits are used in a number of different forms in the human diet. Fruit processing for juice and puree typically removes the pomace and seeds as waste, which is used as animal feed. Because boysenberry juice is known to be rich in a unique mixture of polyphenols, including four anthocyanins[12] and four ellagitannins,[13] the waste products may also be rich in polyphenols, with potential health benefits, and could represent a possible source of new value-added products from boysenberries. This potential is further supported by evidence that the PA content of seeds has been reported to be higher than for the flesh in both apples[3] and grapes.[4] In light of these observations, the major objectives of this study were to identify and quantify the polyphenols, including PAs, present in extracts of boysenberry seeds and juice and to compare the polyphenolic profiles of these extracts.

MATERIALS AND METHODS

Reagents and Materials. Commercial juice concentrate (65° Brix) and waste seeds of boysenberry comprised of a mixture of cultivars were obtained from Berryfruit Export New Zealand, Ltd. (Nelson, New Zealand). Authentic (+)-catechin (CA), (−)-epicatechin (EC), procyanidin (PC) B1 (EC-4β → 8-CA), and PC B2 (EC-4β → 8-EC), quercetin-3-*O*-glucoside (Q3Glc), and cyanidin-3-*O*-glucoside (C3Glc) were purchased from Extrasynthese (Genay Cedex, France). A grape seed extract, Gravinol, was obtained from Kikkoman Corp. (Noda, Japan). An ellagitannin standard, punicalagin A&B, was purchased from ChromaDex (Irvine, CA). PC B3 (CA-4α → 8-CA) and PC C2 (CA-4α → 8-CA-4α → 8-CA) were kindly provided by K. Suzuki

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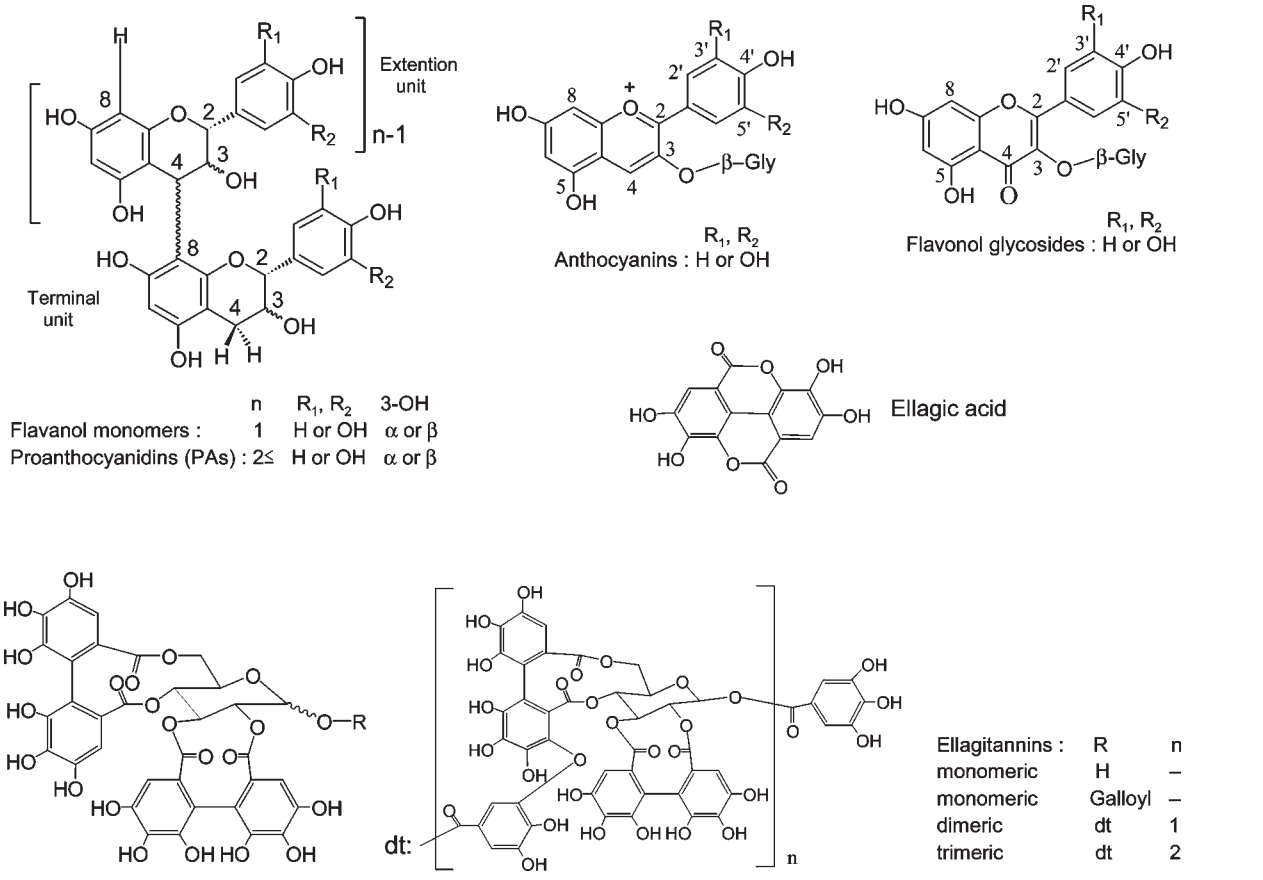

Figure 1. Chemical structures of six classes of polyphenols in berry fruits: flavanol monomers, PAs, anthocyanins, flavonol glycosides, ellagic acid, and ellagitannins.

(Tokyo Institute of Technology).[14] Chromatographic separation was conducted using the phenolic absorbent resin of Amberlite XAD-7HP (Organo, Tokyo, Japan) and Sephadex LH-20 (18−111 μm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). All of the other reagents and solvents used in this study were of analytical or high-performance liquid chromatography (HPLC) grade.

Extraction of Polyphenols from Boysenberry Seeds and Juice. Finely ground boysenberry seeds (940 g) were defatted with hexane (3.0 L), and the resulting powder was extracted with 60% aqueous ethanol (3.0 L, 2 times). The extract was condensed *in vacuo* to remove the ethanol, and the aqueous solution was applied to an Amberlite XAD-7HP column (250 × 45 mm inner diameter). After the column was washed with water (500 mL), it was eluted with ethanol (1.0 L), followed by 60% aqueous acetone (1.0 L), to give a polyphenolic fraction. Ethanol and acetone were evaporated *in vacuo* to afford a boysenberry seed XAD extract (Bs-XAD; 17.0 g). A solution of Bs-XAD (2.50 g) in ethanol (20 mL) was charged on a Sephadex LH-20 column (250 × 25 mm inner diameter) and eluted with ethanol (300 mL), followed by 70% aqueous acetone (300 mL). Evaporation of the former fraction yielded a dark purple residue (0.91 g), and the latter gave boysenberry seed LH-20 extract (Bs-LH; 1.30 g). Similar fractionation of boysenberry juice (13° Brix; 500 mL) with an Amberlite XAD-7HP column gave a boysenberry juice XAD extract (Bj-XAD; 4.40 g), and successive treatment with a Sephadex LH-20 column afforded a boysenberry juice LH-20 extract (Bj-LH; 0.42 g).

Reversed-Phase (RP) HPLC−Diode Array Detector (DAD) and HPLC−Tandem Mass Spectrometry (MS/MS) Analyses. Qualitative and quantitative analyses of polyphenolic components were performed using a Shimadzu Prominence UFLC system (Shimadzu Corp., Kyoto, Japan) equipped with a DAD and a triple quadrupole mass spectrometer, API 3200 (Applied Biosystems/MDS SCIEX, Foster City, CA), to obtain the MS and MS/MS data. PA and polyphenol separations were performed using a RP L-column2 column (150 × 2.1 mm inner diameter, 3 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan) at a flow rate of 0.2 mL/min at 40 °C. The mobile phase consisted of 0.1% (v/v) formic acid/methanol (solvent B) in 0.1% (v/v) formic acid/H_2O (solvent A), 10−20% B for 30 min, 20−100% B from 30 to 50 min, 100% B from 50 to 55 min, and then returning to the initial concentration from 55 to 65 min. The DAD was set at 280, 350, and 520 nm to monitor the UV−vis absorption, and the UV−vis spectra were recorded from 190 to 650 nm. Total ion chromatograms were recorded over a mass range of m/z 100−1800 using a scan duration of 0.1 amu. Peaks giving m/z values corresponding to possible PAs in total ion chromatograms were further investigated by the product ion scan. Individual precursors and product ions were measured automatically with the negative-ion mode of −4500 V of ion spray voltage and conditions as follows: curtain gas (N_2), 20 arbitrary units; collision gas (N_2), 3 arbitrary units; drying gas (N_2), heated to 400 °C. The product ion scan analysis was conducted by monitoring the precursor ion and two characteristic product ions, fragmented by quinone-methide cleavage of the interflavan bond.[15] Co-chromatographic analyses of boysenberry extracts were performed in this HPLC−MS system using standard samples of flavanol monomers (CA and EC), PC dimers (B1, B2, and B3), a PC trimer (C2), and Gravinol containing PC dimers (B1, B2, B3, and B4).[16]

Normal Diol Phase (NP) HPLC−Fluorescence Analyses. Further HPLC analyses were carried out on a Shimadzu 113 HPLC system equipped with a RF-10AXL fluorescence detector and a Develosil

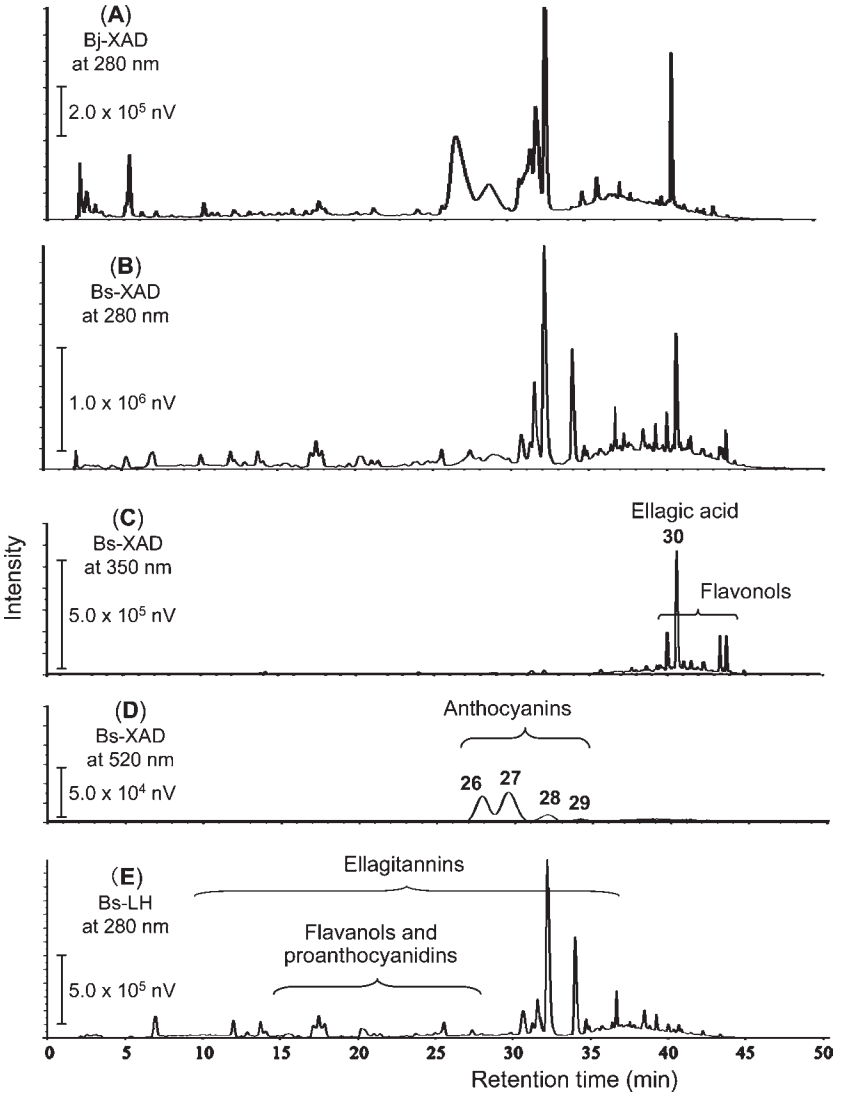

Figure 2. RP HPLC−DAD profiles at (A, B, and E) 280 nm, (C) 350 nm, and (D) 520 nm of Amberlite XAD-7HP (Bj-XAD and Bs-XAD) and Sephadex LH-20 (Bs-LH) extracts from boysenberry juice and seeds. Peaks classified are numbered and referenced to Table 1.

100 Diol-5 column (250 × 4.6 mm inner diameter; Nomura Chemical Co., Ltd., Seto, Japan) under the modified chromatographic conditions published by Kelm et al.[2] A flow rate of 1.0 mL/min at 35 °C, with the HPLC gradient consisting of 2% acetic acid/acetonitrile (solvent A) and 2% acetic acid/3% water/methanol (solvent B) as follows: 0−37.6% B for 60 min, 37.6−100% B from 60 to 60.1 min, 100% B from 60.1 to 70 min, and then returning to the initial concentration from 70 to 80 min. Fluorescence detection was conducted with an excitation wavelength of 230 nm and emission at 321 nm. All samples were prepared by dissolution in methanol, followed by filtering through 0.45 μm polytetrafluoroethylene syringe filters for subsequent HPLC injection.

Thiolysis and HPLC Analysis. Thiolysis and subsequent HPLC analysis were carried out in triplicate according to a modified Guyot method.[17] Each XAD and LH-20 extract (45 μg) from boysenberry juice and seeds in methanol (45 μL) was mixed with 3.3% (v/v) hydrochloric acid in methanol (60 μL) and 5% (v/v) benzyl-α-thiol in methanol (120 μL), and the mixture was kept at room temperature for 90 min. The resulting thiolytic products were analyzed by RP HPLC. The HPLC parameters were the same as for the PA analyses, and elution conditions were as follows: elution solvents A [2.5% (v/v) acetic acid in water] and B (100% acetonitrile) using a gradient program of 10−14% B from 0 to 20 min, 14−25% B from 20 to 25 min, 25−45% B from 25 to 45 min, 45% B from 45 to 50 min, 45−100% B from 50 to 55 min, 100% B from 55 to 60 min, and then a post-run with 10% B for 10 min to equilibrate the column for the next injection. The proportions of constituent flavan-3-ols and average DP were calculated using procyanidin dimers B2 and B3 as standards for the quantification of EC-benzylthioether (BTE) and CA-BTE after thiolysis, respectively. Afzelechin-BTEs were quantified as CA-BTE equivalent.

Matrix-Assisted Laser Desorption Ionization−Time-of-Flight−Mass Spectrometry (MALDI−TOF−MS). The Bs-LH extract was mixed in a 1:1 ratio with 0.1 M dihydroxybenzoic acid in 90% methanol, and 2 μL of the mixture was spotted onto a ground stainless-steel MALDI target for MALDI analysis using the dry droplet method. Bruker Reflex III MALDI−TOF−MS (Billerica, MA) equipped with a N_2 laser (337 nm) was used in the MALDI analysis, and all of the data were obtained in positive-ion reflectron TOF mode.

Quantification of Polyphenols. Quantification of polyphenol components was performed from the peak areas recorded on HPLC by injection of sample solutions (5 μL), prepared by dissolving boysenberry extracts (10 mg) in methanol (10 mL). Flavanol monomers and PAs were analyzed using LC−MS/MS and NP HPLC as described above,

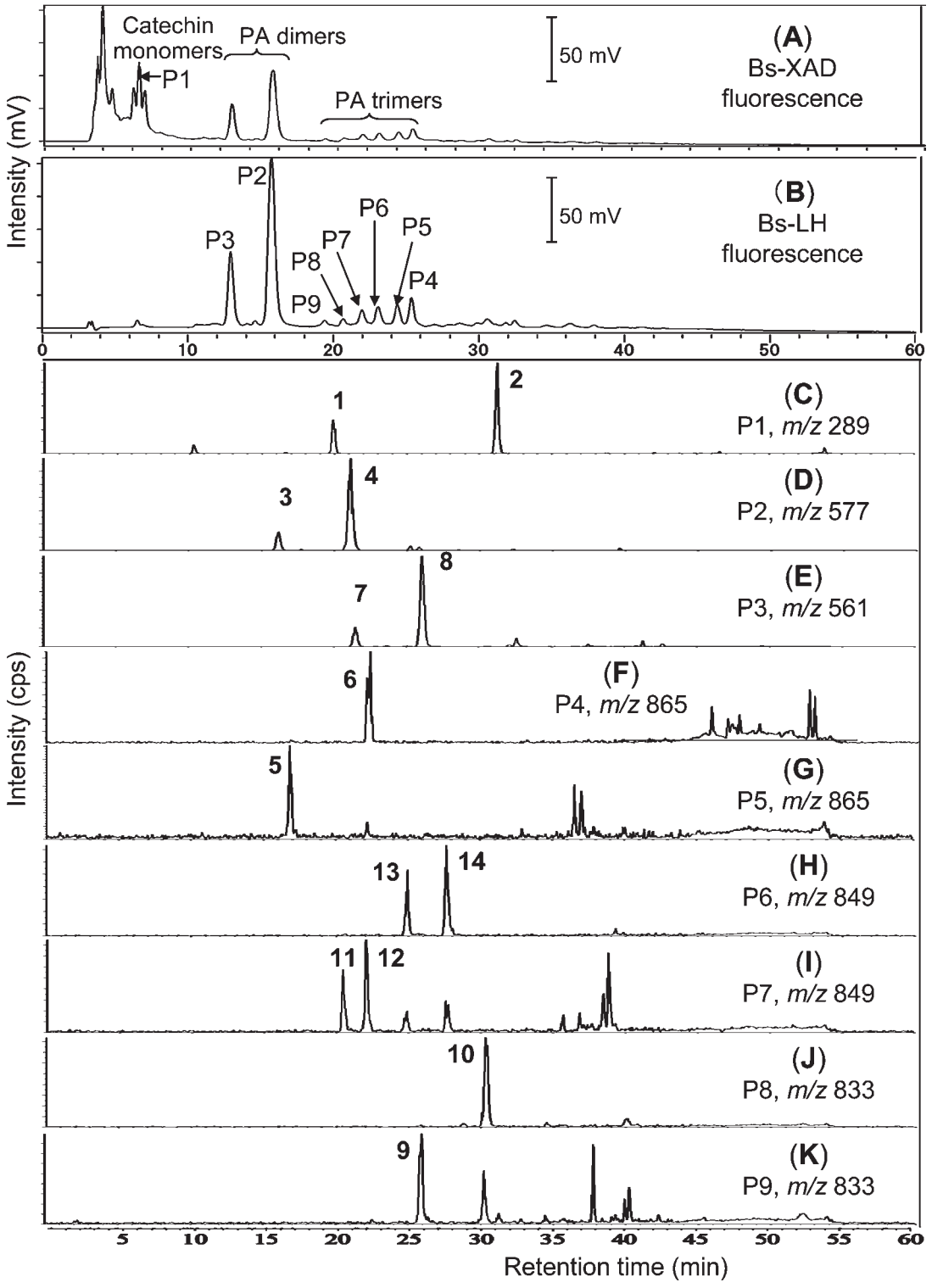

Figure 3. PA profiles of Amberlite XAD-7HP (Bs-XAD) and Sephadex LH-20 (Bs-LH) extracts from boysenberry seeds using (A and B) NP HPLC−fluorescence and (C−K) HPLC−MS. Components characterized are numbered and referenced to Table 1.

and concentrations of monomers, PA dimers, and trimers were calculated from HPLC−MS chromatograms using authentic CA, PC B2, and PC C2 as reference compounds, respectively. Anthocyanins were quantified on RP HPLC at 520 nm using C3Glc as an external reference, and flavonol-3-*O*-glycosides were similarly quantified at 350 nm using Q3Glc. Ellagitannins were analyzed at 280 nm, and the concentrations were calculated using punicalagin A&B as an external reference, using the sum of the two isomeric peak areas.[18] Ellagic acid was quantified with an ellagic acid standard.

RESULTS

Polyphenolic Profiles of Boysenberry Seed and Juice Extracts. Polyphenolic components were extracted from

defatted boysenberry seed powder with 60% ethanol, then concentrated, and treated with Amberlite XAD-7HP particles to remove sugars and organic acids. The bulk of polyphenols was eluted with ethanol followed by 60% aqueous acetone to give Bs-XAD. In a similar manner, boysenberry juice concentrate was used to prepare Bj-XAD.

The RP HPLC absorption spectra of both Bj-XAD and Bs-XAD at 280 nm contained many peaks (panels A and B of Figure 2). These were preliminarily classified into flavonol glycosides, ellagic acid, anthocyanins, PAs, and ellagitannins, by a comparison of their ultraviolet−visible (UV−vis) spectra and retention times (t_R) to those of the corresponding available standards, Q3Glc, ellagic acid, C3Glc, PC B2, and punicalagin A&B, respectively. Absorption profiles of Bs-XAD at 350 and 520 nm indicated the presence of flavonols and ellagic acid and anthocyanins, respectively (panels C and D of Figure 2). Polyphenolic components in the XAD extracts were further fractionated using a Sephadex LH-20 column eluted with ethanol followed by 70% aqueous acetone. The ethanol fractions had strong absorption maxima at 350 and 520 nm, but the 70% aqueous acetone fraction (Bs-LH) exhibited absorption at only 280 nm. Under this fractionation regime, the PAs and ellagitannins were found to be partially purified in the LH-20 extracts. The RP HPLC profile of Bs-LH at 280 nm is shown in Figure 2E.

DP of Boysenberry PAs. The presence of PAs in Bj-LH and Bs-LH extracts was confirmed by NP HPLC−fluorescence chromatography, and the constituent compounds were separated according to their DP, as shown in panels A and B of Figure 3. Individual peaks (P_1−P_9) were fractionated, and the components were analyzed by RP HPLC−MS/MS. The major negative ions containing structural information for peaks P_1, P_2, P_3, P_4, P_5, P_6, P_7, P_8, and P_9 were m/z 289, 577, 561, 865, 865, 849, 849, 833, and 833, respectively (panels C−K of Figure 3). These MS chromatograms reveal structural information of not only 16 mass number differences between the dimers (m/z 577 and 561) and trimers (m/z 833, 849, and 865) but also isomeric constituents with the same molecular weights. From these data, the major PAs in boysenberry juice and seeds were found to be short-chain PA oligomers, with interlinkage units consisting of (epi)catechin [(E)CA] and (epi)afzelechin [(E)AF], that is, flavanol monomers (P_1; compounds **1** and **2**), PC dimers (P_2; compounds **3** and **4**), propelargonidin (PP) dimers (P_3; compounds **7** and **8**), PC trimers (P_4 and P_5; compounds **5** and **6**), PP trimers with one (E)AF unit (P_6 and P_7; compounds **11**−**14**), and PP trimers with two (E)AF units (P_8 and P_9; compounds **9** and **8**).

Structural Characterization of Boysenberry PAs. Five PA components (**1**, **2**, **3**, **4**, and **5**) were identified using RP HPLC−MS/MS as CA, EC, PC B3, PC B4, and PC C2, respectively, by co-chromatography with authentic samples. Interlinked units of other PAs were investigated by MS/MS and thiolysis. MS/MS analysis revealed quinone-methide cleavage[15] in these PAs (**6**−**14**), resulting in characteristic product ions with different m/z ratios, which enabled fragment ions to be distinguished from terminal and extension units. Representative precursor ion and product ion spectra of a dimer **8** were shown in panels A and B of Figure 4. This dimer was identified as (E)AF → (E)CA based on m/z 289.4 {[(E)CA − 1]$^-$} and 271.4 {[(E)AF − 3]$^-$} fragmented by the quinone-methide cleavage. As shown in Table 1, a characteristic product ion of m/z 289 was only detected in the PAs, indicating that PAs in boysenberries have terminal (E)CA and that (E)AF moieties interlinked in extension units.

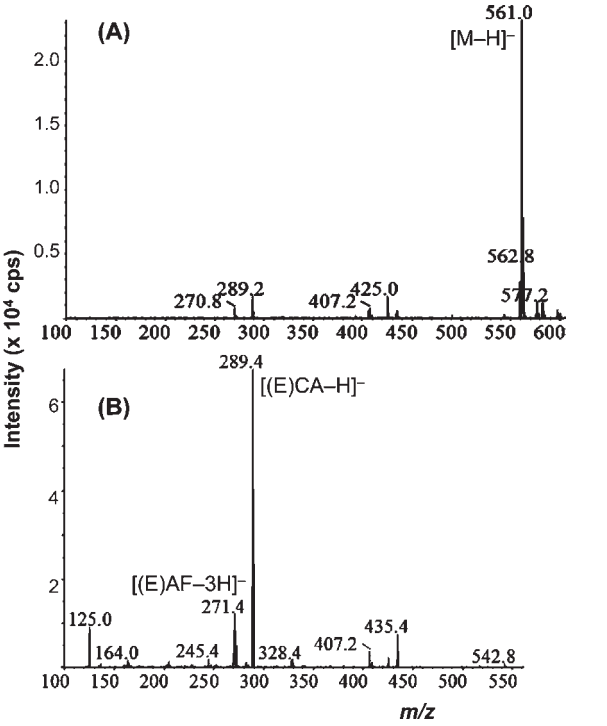

Figure 4. (A) Precursor ion and (B) product ion spectra of a propelargonidin dimer **8**, (E)AF → (E)CA, on negative-ion mode HPLC−MS/MS.

The interlinkages and mean degree of polymerization (mDP) were confirmed by thiolysis and subsequent HPLC analysis. Treatment of Bs-XAD, Bs-LH, Bj-XAD, and Bj-LH extracts with benzyl-α-thiol successfully cleaved the interlinkages, yielding degradation products. Analytical data of these products, obtained by RP HPLC, are summarized in Table 2 and indicate that the terminal units of PAs in both seed and juice extracts are CA and EC and that the extension units consist of CA, EC, and (E)AF. Quantification of the linkages also indicated that the content of extensional (E)AF units was higher in seed PAs (12.7 ± 0.3 and 14.2 ± 0.1% for XAD and LH extracts, respectively) compared to juice PAs (1.5 ± 0.0 and 1.8 ± 0.1%, respectively) and mDP in seed PAs (2.8 ± 0.3 and 2.7 ± 0.0%, respectively) was greater than juice PAs (1.9 ± 0.1 and 2.1 ± 0.0%, respectively). Results of the analysis using thiolysis were consistent with the analytical data obtained by RP HPLC−MS/MS and NP HPLC−fluorescence in Figure 2.

Other Polyphenolic Components of Boysenberry Extracts. In addition to PAs, other polyphenols present in Bs-XAD and Bj-XAD were analyzed by RP HPLC−MS/MS. The presence of ellagic acid in both extracts was confirmed by retention time and MS data (m/z 301). The presence of flavonol glycosides was also suggested by their UV spectra (data not shown), which were similar to that of Q3Glc. Furthermore, the molecular ions [M − 1]$^-$ of compounds **30**, **31**, and **32** were observed at m/z 433, 477, and 593, and the product ions, [aglycon −1]$^-$, were detected at m/z 301, 301, and 285, respectively. These results and previously published data[18] support the identification of compounds **30**, **31**, and **32** as quercetin arabinoside, quercetin glucuronide, and kaempferol coumaroylglucoside, respectively, although the regiochemistry of glycosyl moieties remains unassigned. Four broad peaks detected

Table 1. Contents and Analytical Data of Polyphenols in Waste Seeds (Bs) and Commercial Juice (Bj) of Boysenberry

			LC, t_R (min)				content[a,b] (mg)	
number	constituent	linkage[c]	ODS column	diol column	LC/MS ($M^{-/+/-2}$)	LC/MS/MS or [TOF−MS] (m/z)	in Bs (100 g)	in Bj (100 mL)
1	catechin		18.8	6.4	289^-	125	5.43	0.88
2	epicatechin		30.0	6.8	289^-	125	7.41	1.06
	total flavanol monomers (as EC equivalent)						12.8	1.94
3	procyanidin B3	CA → CA	14.3	15.6	577^-	289, 287	3.44	0.35
4	procyanidin B4	CA → EC	19.3	15.6	577^-	289, 287	13.4	0.62
5	procyanidin C2	CA → CA → CA	16.5	24.2	865^-	577, 575	7.41	0.09
6	procyanidin trimer	(E)CA → (E)CA → (E)CA	21.8	25.2	865^-	577, 575	11.9	–
7	propelargonidin dimer	(E)AF → (E)CA	20.3	12.7	561^-	289, 271	2.53	–
8	propelargonidin dimer	(E)AF → (E)CA	24.8	12.7	561^-	289, 271	7.96	0.09
9	propelargonidin trimer	(E)AF → (E)AF → (E)CA	25.6	19.2	833^-	543, 561	3.80	–
10	propelargonidin trimer	(E)AF → (E)AF → (E)CA	30.3	20.5	833^-	543, 561	4.70	–
11	propelargonidin trimer	(E)AF → (E)AF → (E)CA	20.2	21.8	849^-	559, 561	4.34	–
12	propelargonidin trimer	(E)CA → (E)AF → (E)CA	21.9	21.8	849^-	559, 577	7.96	–
13	propelargonidin trimer	(E)AF → (E)CA → (E)CA	24.6	22.9	849^-	559, 561	3.07	–
14	propelargonidin trimer	(E)AF → (E)CA → (E)CA	27.5	22.9	849^-	559, 577	5.61	–
	total proanthocyanins (as B2 and C2 equivalent)						76.3	1.14
15	peduculagin or its isomer		7.0		783^-	301	70.5	3.43
16	peduculagin or its isomer		12.0		783^-	301	42.5	4.40
17	sanguiin H-10 or its isomer		25.7		$[783]^{-2}$	$[1590.7, +Na]^+$	66.6	8.10
18	sanguiin H-10 or its isomer		30.7		$[783]^{-2}$	$[1590.7, +Na]^+$	41.1	4.31
19	bis-HHDP galloylglucose		30.8		935^-	$[974.9, +K]^+$	70.7	–
20	sanguiin H-6 and/or lambertianin A		32.5		$[935]^{-2}$	$[1892.7, +Na]^+$	371	51.3
21	sanguiin H-6 and/or lambertianin A		34.2		$[935]^{-2}$	$[1892.7, +Na]^+$	170	–
22	bis-HHDP galloylglucose		36.8		935^-	$[974.9, +K]^+$	52.6	–
23	sanguiin H-2 or its isomer		35.0		1103^-	$[1126.9, +Na]^+$	10.9	6.60
24	lambertianin C		31.7		$[1401]^{-2}$	$[2826.7, +Na]^+$	16.3	38.0
	total ellagitannins (as punicalagins A&B equivalent)						912	116
25	cyanidin-3-[2-(glucosyl)glucoside]		27.7		611^+	284	10.5	50.5
26	cyanidin-3-glucoside		29.3		449^+	284	20.8	28.8
27	cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside]		31.9		757^+	284	2.17	22.4
28	cyanidin-3-[6-(rhamnosyl)glucoside]		33.8		595^+	284	–	2.90
	total anthocyanins (as C3Glc equivalent)						33.5	105
29	ellagic acid		40.6		301^-	284, 273	38.2	6.51
	total ellagic acids (as ellagic acid equivalent)						38.2	6.51
30	quercetin-3-arabinoside		40.1		433^-	300	15.4	1.85
31	quercetin-3-glucuronide		40.8		477^-	301	4.70	1.41
32	kaempferol-3-*O*-(6″-*O*-*p*-coumaroyl)-glucoside		43.8		593	285	5.97	–
	total flavonols (as K3Glc equivalent)						25.9	3.26

[a] Contents of individual and total components are expressed as the mean of three replicates. The standard deviations (SDs) of the replicate values were < ±15%. [b] –, less than 0.2 and 0.09 mg in Bs and Bj, respectively. [c] Abbreviations: EC, epicatechin; CA, catechin; (E)CA, catechin or epicatechin; (E)AF, afzelechin or epiafzelechin.

at 520 nm were assigned to anthocyanins. The MS data of the molecular and product ions (Table 1) of compounds **25**, **26**, **27**, and **28** were consistent with four constituents of boysenberry anthocyanins previously reported:[12] cyanidin-3-[2-(glucosyl)glucoside], cyanidin-3-glucoside, cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside], and cyanidin-3-[6-(rhamnosyl)glucoside], respectively.

Identification of ellagitannins was based on HPLC−MS/MS and MALDI−TOF−MS data. In RP HPLC−DAD−MS/MS chromatograms of Bs-XAD and Bj-XAD, the presence of ellagitannins was suggested by absorption traces at 280 nm (panels A and G of Figure 5) and product ions of m/z 301 [hexahydroxy-diphenoyl (HHDP)]$^-$ (data for compounds **15** and **16** alone are presented in Table 1). Analysis by extracting ions at m/z ratios of 783.2−783.7 (panels B and H of Figure 5), 934.3−934.8 (panels C and I of Figure 5), 935.2−935.7 (panels D and J of Figure 5), 1103−1104 (panels E and K of Figure 5), and 1401−1402 (panels F and L of Figure 5) gave corresponding peaks of pedunculagin or its isomer $[783]^-$ (**15** and **16**), sanguiin H-10 or its isomer $[783]^{-2}$ (**17** and **18**), bis-HHDP galloylglucose $[935]^-$ (**19**), sanguiin H-6 and/or lambertianin A $[935]^{-2}$ (**20** and **21**), and lambertianin C

Table 2. Structural Composition and mDP of PAs from Boysenberry Seed and Juice Extracts[a]

		terminal unit (mol %)			extension unit (mol %)			
boysenberry	extract	CA	EC	(E)AF	CA	EC	(E)AF	mDP
seeds	Bs-XAD	14.4 ± 0.4	27.7 ± 1.7	nd	43.3 ± 1.2	1.9 ± 0.6	12.7 ± 0.3	2.8 ± 0.3
	Bs-LH	11.7 ± 0.2	26.1 ± 0.2	nd	46.2 ± 0.4	1.8 ± 0.1	14.2 ± 0.1	2.7 ± 0.0
juice	Bj-XAD	21.9 ± 0.3	40.0 ± 1.2	nd	27.7 ± 1.0	8.9 ± 0.1	1.5 ± 0.0	1.9 ± 0.1
	Bj-LH	19.2 ± 0.8	31.5 ± 0.3	nd	37.3 ± 0.2	10.3 ± 0.6	1.8 ± 0.1	2.1 ± 0.0

[a] Abbreviations: CA, catechin; EC, epicatechin; (E)AF, afzelechin or epiafzelechin; Bs, boysenberry seeds; XAD, Amberlite XAD-7HP; LH, Sephadex LH-20; Bj, boysenberry juice; nd, not detected; mDP, mean degree of polymerization. Molar ratios (mol %) of terminal units and extension units and mDP are expressed as the mean of three replicates.

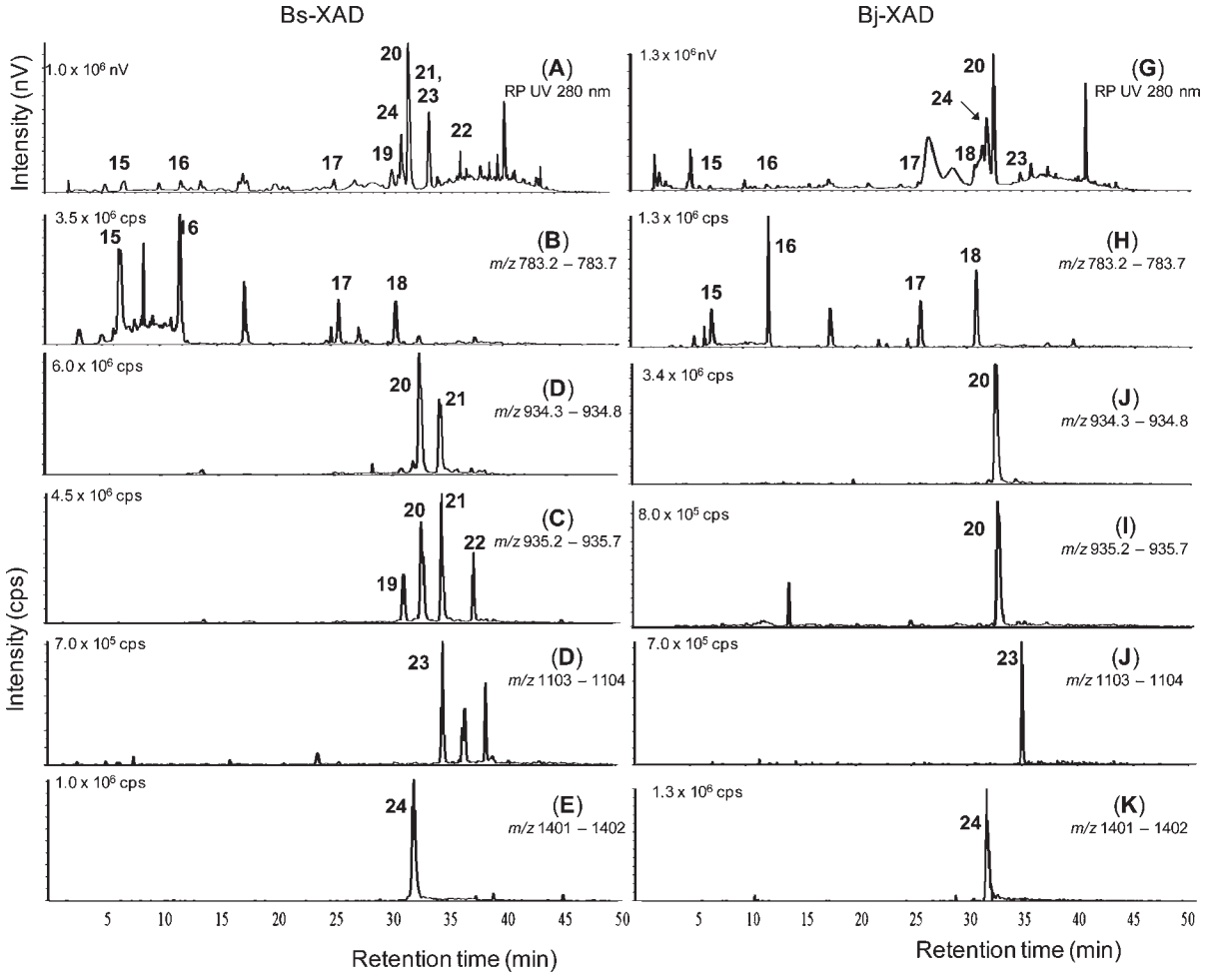

Figure 5. (A and G) RP HPLC and (B−F and H−K) HPLC−MS chromatograms of major ellagitannins in Amberlite XAD-7HP extracts from boysenberry seeds (Bs-XAD) and juice (Bj-XAD). Components characterized are numbered and referenced to Table 1.

$[1401]^{-2}$ (**24**). Ellagitannins with a large molecular mass (>750 Da) were verified by MALDI−TOF−MS, as shown in Figure 6. Mass peaks of *m/z* 974.9, 1126.9, 1590.7, 1892.7, and 2826.7 corresponded to alkali metal adducts of bis-HHDP galloylglucoses $[936.6 + K]^+$ (**22**), sanguiin H-2 or its isomer $[1104.7 + Na]^+$ (**23**), sanguiin H-10 or its isomers $[1569.1 + Na]^+$, sanguiin H-6 and/or lambertianin A $[1871.3 + Na]^+$, and lambertianin C $[2804.0 + Na]^+$, respectively. The major ellagitannins in boysenberry seeds consisted of 10 components (**15**−**24**), in contrast to those in boysenberry juice, which consisted of just 7 components (**15**−**18**, **20**, **23**, and **24**), lacking compounds **19**, **21**, and **22** (Figure 5).

Quantification of Polyphenols in Boysenberry Seeds and Juice. The individual polyphenolic components were classified into six polyphenolic classes (flavanol monomers, PAs, ellagitannins, anthocyanins, ellagic acid, and flavonol glycosides) corresponding to their molecular structures. Quantification of these compounds was performed against authentic standards using RP HPLC, except for PAs, which were measured by HPLC−MS. The contents of polyphenols in the waste seeds

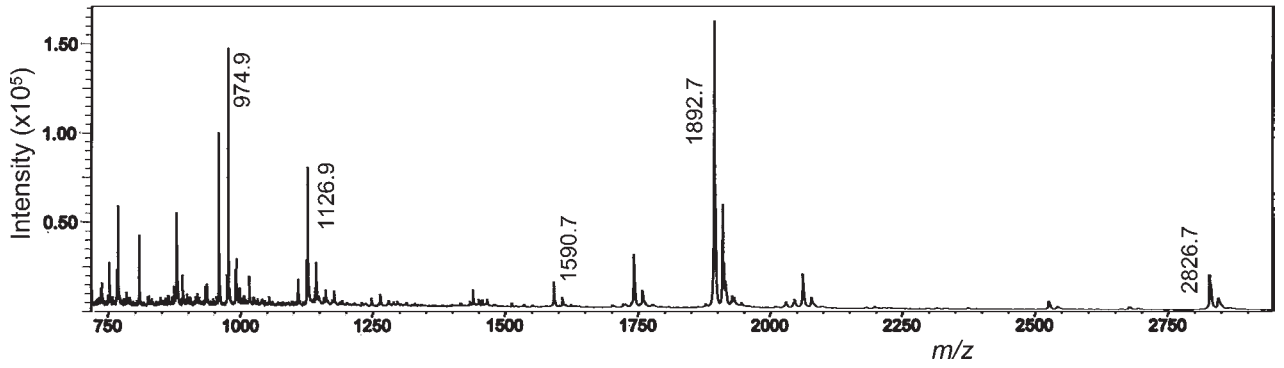

Figure 6. MALDI−TOF−MS spectrum (>750 Da) of Na^+ and K^+ adducts of ellagitannins in a Sephadex LH-20 extract (Bs-LH) from boysenberry seeds.

and commercial juice of boysenberry were calculated from those in Bs-XAD and Bj-XAD, respectively. The results are summarized in Table 1. A comparison of the contents in the seeds and juice reveals that PAs and ellagitannins were found at levels 72- and 7.9-fold higher, respectively, in the seeds (76.3 and 912 mg/100 g) compared to juice (1.14 and 116 mg/100 mL). In contrast, the anthocyanin content in the seeds was 0.33-fold the anthocyanin content in juice. The most abundant PA component in the seeds was PC B4. There was a remarkable difference in the number of PA components between the seeds and juice, with 12 identified in the seeds and only 4 in juice.

DISCUSSION

Polyphenols in boysenberry seeds and juice were extracted and partially purified through Amberlite XAD-7HP columns to give Bs-XAD and Bj-XAD extracts, respectively. HPLC−DAD−MS/MS and MALDI−TOF−MS were invaluable tools for revealing the polyphenol profiles of the extracts, which both consisted of flavanol monomers, PAs, anthocyanins, ellagitannins, ellagic acid, and flavonol glycosides. Consistent with previous studies,[12] anthocyanins were found to be one of the major polyphenolic components of boysenberry juice; however, this study identified numerous other polyphenols in both seed and juice extracts.

Research into PAs has proven to be a challenging field. The structural complexity and diversity of PAs makes identification and characterization difficult, and their physiological activities and bioavailability are similarly complex.[7,8] There have been several papers reporting the presence of PAs in berry fruits,[11,15,19,20] but these studies have not extended to the structural identification of individual PA components. Furthermore, no study to date has examined the PA content of the seeds. In this study, the PAs present in boysenberry seeds and juice were analyzed and were found to have similar characteristics (DP, flavanol units, and contents) compared to PAs in other dietary plants.[11,15] The boysenberry PAs in both seeds and juice consisted of CA, EC, and (E)AF units, with the DP higher in seed PAs than juice PAs. The DP for both seed and juice PAs indicates that boysenberry PAs are characterized by short oligomers: dimers and trimers. In contrast, PAs from cacao, blueberry, blackberry, blackcurrant, grape, and apple have been reported to range from monomers to polymers, and PAs in banana, dates, and beer contain up to pentamers.[15] Short oligomeric PAs are considered to have an important advantage in terms of their health-promoting effects, because only PAs with DPs of 1−2 and 1−4 are confirmed to be orally absorbed in humans[21] and rats,[22] respectively. PAs in boysenberry seeds and juice were also found to contain characteristic PP oligomers with AF units, which were interlinked as extension units. The presence of PPs has been reported in a limited number of berries, such as strawberry (*Fragaria* × *ananassa*),[11,23] raspberry (*Rubus idaeus*),[11] and cloudberry (*Rubus chamaemorus*).[11] The structural characteristics of these PPs are similar to those in boysenberry, and their presence in boysenberry is not surprising, given that boysenberry is thought to be a hybrid of *Rubus* species. The overall PA content of the seed extract was found to be 72-fold higher than that of juice.

Interesting data also emerged on the ellagitannins occurring in boysenberry seeds and juice. Ellagitannins (6 components) and anthocyanins were the major polyphenols in juice; however, ellagitannins were more abundant (10 components) in the seeds (Table 1). Recently, Kool et al. isolated and identified 4 main ellagitannins from a boysenberry seed extract,[13] 3 of which agreed with the structures discovered in our study (**17** or **18**, **20** or **21**, and **23**). We propose that the number of components identified in our study was more abundant because we analyzed crude polyphenolic extracts. Studies on blackberry, considered to be the parent species of the hybrid boysenberry, have reported information regarding the anatomical location of ellagitannins within the *Rubus* fruit. Ellagitannin contents were more than 50% lower in fruits without seeds than in fruits with seeds.[24] The number of ellagitannin structures was greater in the seeds (14 components) than in the flesh (3 components), which contained structural isomers, such as two components with the same molecular mass as sanguiin H-6 or lambertianin A.[25] These reports on blackberry support the possibility that boysenberry seeds contain a larger number of ellagitannins, including their structural isomers.

In conclusion, the polyphenols in the waste seeds and commercial juice of boysenberry were found to span six classes: flavanol monomers, PA, ellagitannins, anthocyanins, ellagic acid, and flavonol glycosides. The most abundant class in both seeds and juice was the ellagitannins, and the second most abundant in seeds was the PAs. The PAs in both materials were found to consist of short oligomeric PCs and PPs, ranging from dimers to trimers, and the seeds contained a 72-fold higher amount of PAs than the juice. This study shows that boysenberry seeds are a very good source of short oligomeric PAs, and further studies could confirm any potential health-promoting properties, including effective bioavailability. These findings highlight the possibility of producing value-added products from the seeds left over from the processing of boysenberry fruits for juice and puree.

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ABBREVIATIONS USED

Bj, boysenberry juice; Bs, boysenberry seeds; PA, proanthocyanidin; PC, procyanidin; PP, propelargonidin; CA, (+)-catechin; EC, (−)-epicatechin; (E)CA, catechin or epicatechin; (E)AF, afzelechin or epiafzelechin; Q3Glc, quercetin-3-*O*-glucoside; C3Glc, cyanidin-3-*O*-glucoside; DAD, diode array detector; NP, normal diol phase; RP, reversed phase; DP, degree of polymerization

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