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Polyphenols and Antioxidant Activity of Calafate (*Berberis microphylla*) Fruits and Other Native Berries from Southern Chile

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Calafate (*Berberis microphylla*) is a native berry grown in the Patagonian area of Chile and Argentina. In the present study the phenolic composition and antioxidant activity of its fruits were studied and also compared with data obtained for other berry fruits from southern Chile including maqui (*Aristotelia chilensis*) and murtilla (*Ugni molinae*). Polyphenolic compounds in calafate fruit were essentially present in glycosylated form, 3-glucoside conjugates being the most abundant anthocyanins. The anthocyanin content in calafate berries (17.81 ± 0.98 μmol g^{-1}) and flavonol level (0.16 ± 0.01 μmol g^{-1}) are comparable with those found in maqui (17.88 ± 1.15 and 0.12 ± 0.01 μmol g^{-1}, respectively); however, maqui shows lower flavan-3-ol concentration than calafate (0.11 ± 0.01 and 0.24 ± 0.03 μmol g^{-1}, respectively). Maqui and calafate show high antioxidant activity, which correlates highly with total polyphenol content and with anthocyanin concentration.

KEYWORDS: *Berberis microphylla*; calafate; polyphenols; anthocyanins; flavonols; antioxidant activity

INTRODUCTION

Diets rich in fruits and vegetables have been associated with a reduced risk of chronic diseases, such as cancer, cardiovascular disease, and stroke (*1*−*3*). The observed lower mortality rates have, among other things, been attributed to the high concentration of polyphenols, which are important antioxidants (*4*). Polyphenols are ubiquitous secondary plant metabolites that mainly protect plants against biotic and abiotic stresses. Significant amounts of polyphenols are found in highly pigmented blueberries, raspberries, and blackberries as well as in a diverse range of wild berries (*5*).

Wild edible berry species found in central and southern Chile include *Aristotelia chilensis*, which produces an edible black-colored fruit called "maqui"; *Ugni molinae* (murta), an evergreen perennial shrub of the Myrtacea family also called murtilla or mutilla; *Berberis microphylla* (calafate), known also as *Berberis buxifolia*; and *Berberis darwinii* (michay). Calafate and michay are evergreen and semievergreen shrubs or small trees that grow under a wide range of ecological conditions (*6*). Calafate fruits (in English: barberry) are dark purple, black, or bluish berries. They are consumed fresh or prepared as jellies, marmalades, and wines (*7*). Literature data on edible *Berberis* species are scarce. Pomilio (*8*) identified in fruits of *B. buxifolia* the presence of certain anthocyanins by paper chromatography, which included peonidin-3-glucoside, malvidin-3-rutinoside, malvidin-3-glucoside, petunidin-3-rutinoside, petunidin-3-glucoside, peonidin-3-rutinoside-5-glucoside, delphinidin-3-rutinoside, delphinidin-3-glucoside, petunidin-3-rutinoside-5-glucoside, and petunidin-3-gentobioside. More recently, Wallace and Giusti (*9*) studied the composition of a Peruvian berry (*Berberis boliviana* Lechler), which is rich in nonacylated anthocyanins (7−8% dry weight). Addition of *B. boliviana* whole berry powder to yogurt matrices produced an attractive, stable anthocyanin-rich product, thus eliminating the need for industrial colorant extraction.

The main polyphenol classes in berries are flavonoids (anthocyanins, flavonols, flavanols), condensed and hydrolyzable tannins, stilbenoids (resveratrol), and phenolic acids (*10*). Their characterization and quantification in berries is usually carried out by reversed phase high-performance liquid chromatography (RP-HPLC) with diode array detection (DAD) and high-performance liquid chromatography−mass spectrometry (HPLC-MS), after different extraction and hydrolysis procedures (*11*−*14*) or by the colorimetric Folin−Ciocalteu method (*15*, *16*). For determination of the antioxidant capacity, different tests have been developed, such as the Trolox equivalent antioxidant capacity (TEAC), total radical-trapping antioxidant parameter (TRAP), and ferric reducing-antioxidant power (FRAP). Methods available for the measurement of the antioxidant capacity have recently been reviewed by Seeram et al. (*17*).

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Table 1. Proximate Composition, Sugars, and Ascorbic Acid of Calafate Fruits (Fresh Weight Basis)

							sugars		
calafate sample	sampling date	moisture (%)	proteins (%)	ash (%)	ether extract (%)	crude fiber (%)	glucose (%)	fructose (%)	ascorbic acid (mg/100 g)
La Junta	2007	78.6	2.70	0.64	0.18	4.2	0.51	0.33	59.4
Coyhaique	2006	69.9	2.65	1.30	0.37	10.3	0.98	0.42	41.2
Coyhaique	2007	65.6	3.40	1.02	0.13	9.6	1.17	0.73	45.3
San Isidro	March 5, 2008	76.2	2.03	0.89	0.15	4.8	2.64	1.53	90.7
San Isidro	March 10, 2008	76.0	2.20	0.74	0.15	9.3	2.85	0.41	117.5
San Isidro	March 14, 2008	75.4	2.40	0.73	0.14	7.5	4.06	0.79	62.5
San Isidro	March 16, 2008	75.1	2.47	0.76	0.14	5.6	4.66	1.47	59.8
Darwin	2008	72.7	2.57	0.82	0.14	5.4	4.24	0.84	115.7
mean		75	2.6	0.9	0.18	7	3	0.8	74
SD		4	0.4	0.2	0.08	2	2	0.4	30
min		65.6	2.03	0.64	0.13	4.2	0.51	0.33	41.2
max		78.6	3.4	1.30	0.37	10.3	4.66	1.53	117.5

Table 2. Anthocyanins in Calafate and Other Native Berries from Southern Chile

anthocyanin	peak[a]	t_R (min)	molecular ion	product ions	λ_{max} (vis)	detected in[b]
delphinidin-3-glucoside	8	9.5	465	303	524	a, b
cyanidin-3-glucoside	11	11.7	449	287	516	a, b, c
petunidin-3-glucoside	13	13.2	479	317	525	a
peonidin-3-glucoside	15	15.6	463	301	517	a, c
malvidin-3-glucoside	17	16.8	493	331	527	a
delphinidin-3-rutinoside	9	10.3	611	465; 303	525	a
cyanidin-3-rutinoside	12	12.5	595	449; 287	519	a
petunidin-3-rutinoside	14	13.8	625	479; 317	527	a
peonidin-3-rutinoside	16	16.2	609	463; 301	520	a
malvidin-3-rutinoside	18	17.3	639	493; 331	529	a
delphinidin-3,5-dihexoside	1	5.0	627	465; 303	521	a
cyanidin-3,5-dihexoside	3	5.9	611	449; 287	513	a
petunidin-3,5-dihexoside	4	7.0	641	479; 317	521	a
peonidin-3,5-dihexoside	6	8.4	625	463; 301	513	a
malvidin-3,5-dihexoside	7	9.3	655	493; 331	523	a
delphinidin-3-rutinoside-5-glucoside	2	5.5	773	611; 465; 303	524	a
petunidin-3-rutinoside-5-glucoside	5	8.1	787	625; 479; 317	523	a
malvidin-3-rutinoside-5-glucoside	10	10.5	801	639; 493, 331	525	a
delphinidin-3-sambubioside-5-glucoside	30	6.6	759	597; 465; 303	523	b
cyanidin-3-sambubioside-5-glucoside	32	8.8	743	581; 449; 287	515	b
delphinidin-3-sambubioside	34	9.0	597	303	523	b
cyanidin-3-sambubioside	35	11.4	581	287	517	b
delphinidin-3,5-diglucoside	31	7.3	627	465; 303	521	b
cyanidin-3,5-diglucoside	33	8.9	611			b

[a] Peak numbers 1−18 according to **Figure 1A**. [b] Detected in (a) calafate, (b) maqui, (c) murtilla.

The main aim of the present study was to determine the profile and concentration of the main antioxidant compounds in calafate fruit grown in southern Chile. The HPLC profiles of anthocyanins, flavonols, and flavan-3-ols of this fruit were compared with those obtained for maqui and murtilla and for blueberries cultivated in this area. The antioxidant capacity (TEAC assay) was compared with those of other antioxidant-rich barberries, and the correlation between this and polyphenol concentration was also studied and discussed.

MATERIALS AND METHODS

Sample Material. The berries were collected in southern Chile. The specific sampling places were La Junta (XI region, 44° S), Coyhaique (XI region, 45° 34′ S), Darwin and Faro San Isidro (XII region, 54° S) for calafate fruits (*B. microphylla*). Murtilla fruits (*U. molinae*) were obtained from Concepción (VIII region, 33° 46′ S), Loncoche (IX region, 39° 22′ S), and Valdivia (39° 48′ S). Maqui fruits (*A. chilensis*) were obtained from Concepción (VIII region, 33° 46′ S), Temuco (IX region, 38° 45′ S), and Calafquén (XIV region, 39° 29′ S). The samples were collected between February and March of the years 2006, 2007, and 2008 and kept frozen before the analyses.

Instrumentation. An ultrasonic bar homogenizer (Cole Palmer series 4710), a mechanical shaker (Edyman KL2), a desktop centrifuge (Heraeus-Christ GmbH, Osterode, Germany), a Rotavapor (Büchi), and a stirring plate (Corning) were used for sample preparation. Determinations of antioxidant capacity and total polyphenols were made with a Shimadzu UV mini 1240 spectrophotometer. Solid phase extraction was carried out in a Supelco SPE vacuum manifold using a vacuum pump (Millipore).

HPTLC analyses of sugars and ascorbic acid were carried out in a CAMAG Automatic TLC sampler 4, equipped with a CAMAG ADC2 automatic developing chamber, a CAMAG Reprostar 3, and a CAMAG TLC Scanner 3. The control system and data collection were carried out by using Wincats software.

The HPLC-DAD analyses of polyphenols were carried out with a Shimadzu HPLC system equipped with a quaternary LC-10AD*VP* pump, an FCV-10AL*VP* elution unit, a DGU-14A degasser unit, a CTO-10A*VP* oven, and a UV−vis diode array spectrophotometer

model SPD-M10A*VP*. The control system and data collection were carried out by using Shimadzu Chromatography Data System CLASS-*VP* software.

For identity assignment, a HPLC-MS Agilent 1100 series system (Agilent, Waldbronn, Germany), equipped with a photodiode array detector (G1315B), a LC/MSD Trap VL (G244C VL), and an Agilent Chem Station (version B.01.03) with Agilent LC-MS Trap software (version 5.3), was used.

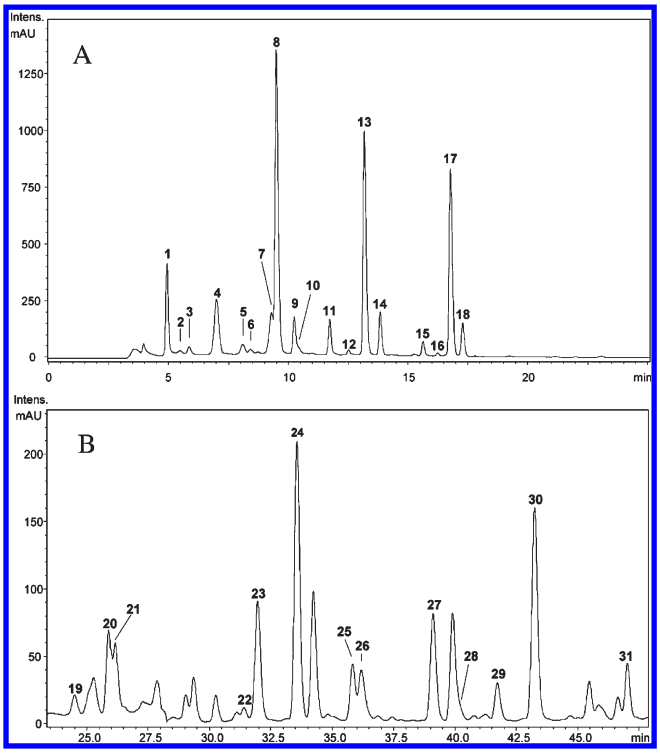

Figure 1. (**A**) HPLC-DAD chromatogram (detection at 520 nm) of anthocyanins in calafate fruits. Peaks: (1) delphinidin-3,5-dihexoside; (2) delphinidin-3-rutinoside-5-glucoside; (3) cyanidin-3,5-dihexoside; (4) petunidin-3,5-dihexoside; (5) petunidin-3-rutinoside-5-glucoside; (6) peonidin-3,5-dihexoside; (7) malvidin-3,5-dihexoside; (8) delphinidin-3-glucoside; (9) delphinidin-3-rutinoside; (10) malvidin-3-rutinoside-5-glucoside; (11) cyanidin-3-glucoside; (12) cyanidin-3-rutinoside; (13) petunidin-3-glucoside; (14) petunidin-3-rutinoside; (15) peonidin-3-glucoside; (16) peonidin-3-rutinoside; (17) malvidin-3-glucoside; (18) malvidin-3-rutinoside. (**B**) DAD chromatogram (detection at 360 nm) of flavonols in calafate fruits. Peaks: (19) myricetin-3-rutinoside-7-glucoside; (20) myricetin-3-glucoside; (21) myricetin-3-rutinoside; (22) quercetin-3-rutinoside-7-glucoside; (23) quercetin-3-galactoside; (24a) quercetin-3-rutinoside + (24b) quercetin-3-glucoside; (25) quercetin-3-(6″-acetyl)-hexoside 1; (26) quercetin-3-(6″-acetyl)-hexoside 2; (27) quercetin-3-rhamnoside; (28) isorhamnetin-3-rutinoside-7-glucoside; (29) isorhamnetin-3-galactoside; (30a) isorhamnetin-3-rutinoside + (30b) isorhamnetin-3-glucoside; (31) isorhamnetin-3-(6″-acetyl)-hexoside.

Reagents and Standards. Commercial standards of malvidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-glucoside, and petunidin-3-glucoside were obtained from Polyphenols (Sandnes, Norway). Commercial standards of flavonol glycosides (3-glucosides of quercetin and isorhamnetin, quercetin-3-rutinoside, and 3-galactoside of quercetin) were from Extrasynthese (Genay, France). A sample of myricetin 3-glucoside was kindly supplied by Prof. Dr. U. Engelhardt (Institute of Food Chemistry, Technische Universität Braunschweig, Germany). Myricetin, quercetin, catechin, epicatechin, potassium persulfate, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were from Sigma-Aldrich (St. Louis, MO). Formic and hydrochloric acid, ammonia, sodium carbonate, Folin−Ciocalteu's phenol reagent, sodium diethyldithiocarbamate trihydrate, ethanol, methanol, and acetonitrile (solvents of HPLC grade) were provided by Merck (Darmstadt, Germany). Stock standard solutions of 100 μg mL^{-1} of each polyphenol were prepared in methanol and stored at 4 °C.

Analytical Methods. *Proximate Composition*. The proximate composition of berries was determined according to usual methods established by AOAC (*18*).

HPTLC Analyses of Sugars and Ascorbic Acid. The procedures were carried out according to the methods described by Morlock and Vega-Herrera (*19*) for determination of sucralose in dietetic products, but starting with 1 g of sample and by CAMAG applications (*20*) for ascorbic acid.

Extraction Procedure. The procedure was based on the method described by Castillo-Muñoz et al. (*22*) with minor modifications. An amount of 10 g of fruit was ground in a stainless steel mixer with 10 mL of methanol/formic acid (97:3% v/v). The sample was submitted to an ultrasonic bar for 30 s followed by 16 h of agitation. A centrifugation step was used to separate the supernatant with two washing steps of the residue with the same solvent. The extract was stored at 4 °C.

Total Phenol Content. Total soluble phenolic compounds in the methanolic extract were determined by measurement of the absorbance at 280 nm (A_{280}) and by the Folin−Ciocalteu method with gallic acid as reference. Results were expressed as absorbance at 280 nm (A_{280}) and Folin−Ciocalteu index (IFC), respectively (*21*).

Solid Phase Extraction and Cleanup of Flavonols and Flavan-3-ols. The method described by Castillo-Muñoz et al. (*22*) was used.

HPLC-DAD and HPLC-MS/MS Analyses. HPLC analyses of polyphenols were carried out by using a C18 Zorbax Eclipse XDB5 μm, 250 × 4.6 mm column (Agilent, Palo Alto, CA) with a C18, Nova-Pak Waters, 22 × 3.9 mm, 4 μm precolumn (Milford, MA) at 40 °C. The analysis of anthocyanins and flavan-3-ols was carried out using a mobile phase gradient constituted by water/acetonitrile/formic acid (87:3:10% v/v/v) (solvent A) and water/acetonitrile/formic acid (40:50:10% v/v/v) (solvent B). The flow rate was 0.8 mL min^{-1}, and the gradient program was from 94 to 70% of solvent A in 15 min, from 70 to 50% in 15 min, from 50 to 40% by 5 min, and from 40 to 94% in 6 min followed by 9 min of stabilization at 94% A. The DAD wavelengths were 518 and 280 nm for anthocyanins and flavan-3-ols, respectively. For flavan-3-ol determination, the cited SPE procedure was applied prior to the chromatographic separation. For anthocyanin determination the methanolic extract was filtered using a 0.22 μm membrane (PDVC) and directly injected into the HPLC

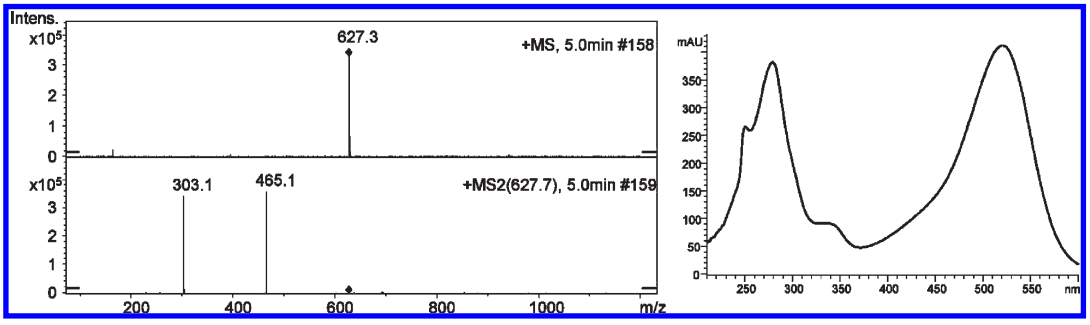

Figure 2. MS and MS/MS spectra and DAD-UV−vis spectrum of delphinidin-3,5-dihexoside.

Table 3. Flavonols Detected in Calafate Fruit

flavonol	peak[a]	t_R (min)	pseudomolecular ions (+M and −M modes)	product ions	λ_{max} (vis)
myricetin-3-rutinoside-7-glucoside	19	24.5	789 787	nd 625; 317	356
myricetin-3-glucoside	20	25.9	481 479	319 317	355
myricetin-3-rutinoside	21	26.1	627 625	481; 319 317	356
quercetin-3-rutinoside-7-glucoside	22	31.4	773 771	611; 303 609; 301	355
quercetin-3-galactoside	23	31.9	465 463	303 301	353
quercetin-3-rutinoside	24a	33.5	611 609	465; 303 301	354
quercetin-3-glucoside	24b	33.7	465 463	nd nd	nd
quercetin-3-(6″-acetyl)-hexoside 1	25	35.8	nd 505	nd 463; 301	354
quercetin-3-(6″-acetyl)-hexoside 2	26	36.1	nd 505	nd 463; 301	354
quercetin-3-rhamnoside	27	39.1	449 447	303 301	348
isorhamnetin-3-rutinoside-7-glucoside	28	40.1	787 785	317 315	nd
isorhamnetin-3-galactoside	29	41.7	479 477	317 315	353
isorhamnetin-3-glucoside	30b	43.1	479 477	nd nd	nd
isorhamnetin-3-rutinoside	30a	43.2	625 623	479; 317 315	354
isorhamnetin-3-(6″-acetyl)-hexoside	31	47.0	nd 519	nd 315	352

[a] Peak numbers according to **Figure 1B**.

equipment; the injection volume was 50 μL and the temperature was 40 °C. For identification of anthocyanins, ESI-MS/MS was used in positive ionization mode (*23*).

HPLC analyses of flavonols were performed on the same chromatographic system as formerly described for anthocyanins using chromatographic conditions previously reported and specifically developed for grape and wine flavonol analysis (*24*) and by injection of the anthocyanin-free flavonol fractions isolated by SPE (*22*, *24*). Identity assignment of flavonols was by ESI-MS/MS in both positive and negative ionization modes (*24*) and quantification by DAD chromatograms extracted at 360 nm.

Total Anthocyanins. The total concentration of monomeric anthocyanins was obtained as the sum of individual monomeric anthocyanins obtained by the HPLC method.

Determination of the Antioxidant Capacity (*TEAC Assay*). The TEAC assay consists of the formation of the $ABTS^{\bullet+}$ radical by reaction with potassium persulfate. The decrease of $ABTS^{\bullet+}$ radical by antioxidants was measured at 734 nm, using Trolox (a water-soluble analogue of vitamin E) for method calibration (*25*).

RESULTS AND DISCUSSION

Characteristic Parameters of Analytical Methods. Before determination of the polyphenol profiles, total polyphenols, antioxidant activity, and fructose, glucose, and ascorbic acid concentrations in calafate and the other berries under investigation, various methods adapted from wine analysis were optimized and validated for berries. Precision, limits of quantification, and linear ranges satisfied statistical requirements.

Composition of Calafate Fruits. The proximate composition of whole calafate fruit is shown in **Table 1**. To the best of our knowledge, no previous data about these parameters have been published for calafate before. Comparison of these results with those obtained for fruits of other *Berberis* species shows that the protein levels of calafate are 3−4 times lower than those described for *Berberis vulgaris* and *Berberis pimana* (fresh weight) (*26*, *27*). It should be noted that in our case, the quantification was a direct determination of glucose and fructose by HPTLC, whereas in the cited references, results were expressed as total reducing sugars. The glucose and fructose concentration in calafate varied between 0.3 and 5% of fresh weight. This can be explained in part by the different degrees of maturity of fruit, as shown by the samples of San Isidro; however, the area of collection also seems to have an impact on sugar levels, too, being lower in samples collected in the XI region (La Junta, Coyhaique) compared to the XII region (San Isidro, Darwin).

Ascorbic Acid in Calafate Fruits. Vitamin C levels in calafate vary between 41.2 and 117.5 mg/100 g of fruits (**Table 1**), whereas for *B. vulgaris* the concentration is 6.52 mg/100 g of fruit (*26*) and for *B. aristata*, 11.97 mg/100 g of fruit (*28*). It is important to consider that this compound also contributes to the antioxidant activity of the berry.

HPLC-DAD-MS/MS Analyses of Flavonoids. **Table 2** presents the spectral data and retention time of each anthocyanin detected in calafate and in the other berries as obtained by HPLC-DAD-MS/MS analysis. For this fruit, 18 anthocyanins were detected, that is, the 3-glucosides, 3,5-dihexosides (glucoside and/or galactoside conjugates), and 3-rutinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin. Also, the 3-rutinoside-5-glucosides of delphinidin, petunidin, and malvidin were detected in the extract. **Figure 1A** shows the HPLC-DAD profile of the anthocyanins from calafate fruit extract. The identities of peaks 8, 11, 13, 15, and 17 were confirmed by comparison of their retention time and their UV−vis spectra with commercial standards of each anthocyanidin-3-glucoside as well as by their MS^n spectra. The latter compounds were characterized by MS spectra showing their expected molecular ions (M^+) and by MS/MS spectra showing a unique fragment ion ($[M - 162]^+$) that is in agreement with the loss of a hexose moiety, which could be glucose or galactose. Taking into account the coincidence in retention times with authentic standards of anthocyanidin-3-glucosides and that cyanidin-3-glucoside and cyanidin-3-galactoside show different retention times under the chromatographic conditions used (*29*), we assigned the aforementioned peaks as anthocyanidin-3-glucosides. The identity assignment for the other anthocyanins was carried out using their UV−vis and MS^n spectra.

In the case of 3,5-dihexosides conjugates, a loss of two m/z 162 fragments was observed, which could correspond to both glucoside and galactoside groups. **Figure 2** shows the fragmentation patterns of the delphinidin-3,5-dihexoside with its corresponding fragments (molecular ion of m/z 627 and fragments at m/z of 303 and 465). A diglucoside conjugation was unlikely because standard solutions of 3,5-diglucosides have different retention times than the dihexosides observed in the calafate extract. For a standard solution of peonidin-3,5-diglucoside the retention time was 11.9 min, and for malvidin-3,5-diglucoside it was 13.8 min, whereas for peonidin- and malvidin-dihexosides observed in the calafate extract, their retention times were 8.4 and

Table 4. Flavonoid Concentration, Total Phenols, and Antioxidant Activity in Calafate, Maqui, and Murtilla Berries (Fresh Weight)[a]

		anthocyanins (μmol/g of fresh wt)	flavonols (μmol/g of fresh wt)	flavan-3-ols (μmol/g of fresh wt)	total phenols		antioxidant activity (Trolox equivalent, μmol/g of fresh wt)
					IFC[b]	A_{280}[c]	
calafate	La Junta 2007	19.63 ± 1.26	0.14 ± 0.01	0.43 ± 0.02	70 ± 7	188 ± 11	50.8 ± 12.4
	Coyhaique 2006	16.73 ± 1.08	0.13 ± 0.01	0.85 ± 0.04	110 ± 12	227 ± 13	94.7 ± 23.1
	Coyhaique 2007	26.13 ± 1.68	0.13 ± 0.01	0.61 ± 0.03	123 ± 13	278 ± 16	99.5 ± 24.3
	Faro San Isidro a 2008	14.21 ± 0.91	0.19 ± 0.01	traces[d]	70 ± 7	154 ± 9	78.0 ± 19.0
	Faro San Isidro b 2008	14.51 ± 0.93	0.21 ± 0.01	traces	79 ± 8	158 ± 9	51.3 ± 12.5
	Faro San Isidro c 2008	15.44 ± 0.99	0.14 ± 0.01	traces	75 ± 8	192 ± 11	81.2 ± 19.8
	Faro San Isidro d 2008	19.07 ± 1.23	0.12 ± 0.01	traces	84 ± 9	194 ± 11	75.9 ± 18.5
	Darwin 2008	16.76 ± 1.08	0.20 ± 0.01	traces	84 ± 9	195 ± 11	64.3 ± 15.7
	mean	17.81 ± 0.98 a	0.16 ± 0.01 a	0.24 ± 0.03 a	87 ± 9 a	198 ± 11 a	74.5 ± 15.9 a
maqui	Concepción	20.22 ± 1.30	0.11 ± 0.01	0.09 ± 0.01	113 ± 12	213 ± 10	100.5 ± 24.5
	Temuco	17.40 ± 1.12	0.11 ± 0.01	0.11 ± 0.01	75 ± 8	164 ± 8	69.9 ± 17.1
	Calafquén	16.01 ± 1.03	0.15 ± 0.01	0.12 ± 0.01	103 ± 11	276 ± 14	93.9 ± 22.9
	mean	17.88 ± 1.15 a	0.12 ± 0.01 a	0.11 ± 0.01 b	97 ± 10 a	218 ± 11 a	88.1 ± 21.5 a
murtilla	Concepción	0.20 ± 0.01	0.25 ± 0.01	0.16 ± 0.01	35 ± 4	60 ± 2	19.3 ± 4.7
	Loncoche	0.22 ± 0.01	0.32 ± 0.01	0.35 ± 0.02	27 ± 3	69 ± 3	8.6 ± 2.1
	Valdivia a	0.24 ± 0.02	0.29 ± 0.01	0.36 ± 0.02	37 ± 4	71 ± 3	10.4 ± 2.5
	Valdivia b	0.19 ± 0.01	0.28 ± 0.01	0.21 ± 0.01	27 ± 3	73 ± 3	8.4 ± 2.0
	mean	0.21 ± 1.08 b	0.29 ± 0.01 b	0.27 ± 0.01 a	32 ± 4 b	68 ± 3 b	11.7 ± 2.3 b
blueberries	Temuco	2.53 ± 0.16	0.12 ± 0.01	0.07 ± 0.01	17 ± 1	65 ± 3	14.5 ± 0.59

[a] In each column values with different letters are significantly different ($\alpha < 0.05$) obtained by ANOVA. [b] Folin−Ciocalteu method. [c] Absorbance 280 nm method. [d] Traces: detected but not quantified.

9.3 min, respectively. It was not possible to ascertain if this compound corresponds to a 3,5-digalactoside or to a mixed 3,5-galactoside/glucoside conjugates. For this reason, identification is given as dihexosides. The isolation and structural characterization of the dihexosides are in progress and will be communicated separately.

The 3-rutinoside conjugates of anthocyanin show a loss of a m/z 146 fragment, corresponding to a rhamnose group and the loss of a glucose group (m/z 162 fragment), constituting the rutinoside conjugate. For the 3-rutinoside-5-glucoside conjugates, the observed loss of an additional m/z 162 fragment corresponded to the 5-glucoside conjugation.

Considering these results, the predominant anthocyanins found in calafate fruit were glycosylated derivatives of delphinidin (m/z 303), petunidin (m/z 317), malvidin (m/z 331), peonidin (m/z 301), and cyanidin (m/z 287), with the most abundant being delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin 3-glucoside. In addition, the UV−vis spectra were in agreement with the aforementioned peak assignations. The most significant features were the presence of a characteristic shoulder at around 440 nm (*30*) for the 3-monosubstituted anthocyanidins and the absence of this shoulder, together with a relative enhancement of the absorbance at around 280 nm, for the 3,5-disubstituted anthocyanidins.

Table 3 shows the spectral data and retention times of flavonols found in calafate extract, and **Figure 1B** presents their HPLC-DAD profile using the specific chromatographic method developed for flavonols (detection at 360 nm). A total of 13 flavonols could be assigned on the basis of, first, their characteristic UV−vis spectra and, second, their MS and MS/MS spectra (both positive and negative ionization modes). In addition, some compounds could be tested against true standards. Only glycosylated flavonols derived from the flavonoid aglycones quercetin, isorhamnetin, and myricetin were found in the calafate berry extract. In the case of the B-ring trisubstituted flavonol myricetin, the main derivatives were the 3-glucoside (peak 20) and the 3-rutinoside (peak 21). In addition, its 3-rutinoside-7-glucoside was found as a minor constituent (peak 19). Peak 20 eluted at the same retention time of a true standard of myricetin-3-glucoside, and its mass spectral data were in agreement with the suggested assignation. Similarly, the MS and MS/MS data for peaks 19 and 21 supported their identity. It is remarkable that only myricetin-3-rutinoside showed the expected sequential losses of the rhamnosyl and glucosyl moieties of the rutinoside sugar under positive ionization mode (**Table 3**), the product ions at m/z values of 481 and 319, generated from the fragmentation of the pseudomolecular ion, $[M + H]^+$, with m/z 627, whereas the rutinoside residue was cleaved in a unique step under negative ionization conditions (pseudomolecular ion, $[M - H]^-$, at m/z 625 and only one product ion at m/z 317). Peak 19 was intense enough to yield product ions under negative ionization mode, and the losses of two fragments of 162 and 308 amu were compatible with glucose and rutinose, respectively. The assignation as a 3-rutinoside-7-glucoside derivative was suggested on the basis of the structures of the naturally occurring flavonol glycosides described in the literature (*31*).

In the case of B-ring disubstituted flavanols (quercetin and isorhamnetin), the main derivatives were also the 3-rutinosides (peaks 24a and 30a), but now followed by the 3-galactosides (peaks 23 and 29). The assignation of the 3-rutinoside derivatives was based on the m/z values obtained for the expected pseudomolecular ions (peak 24a, $[M + H]^+$ at 611 and $[M - H]^-$ at 609; peak 30a, $[M + H]^+$ at 625 and $[M - H]^-$ at 623) and the subsequent losses of the rutinosyl moiety in the ion trap. Peaks 23 and 29 showed the expected pseudomolecular and product ions for 3-hexoside derivatives of quercetin and isorhamnetin (**Table 3**), but they showed the same retention times as true standards of the 3-galactoside derivatives of quercetin and isorhamnetin, respectively (*24*). However, the 3-glucosides of quercetin and isorhamnetin were also detected (peaks 24b and 30b, respectively), but they partially coeluted with their respective 3-rutinoside derivatives, as has been described for quercetin-3-glucoside and quercetin-3-rutinoside (*24*, *23*). The 3-glucoside and 3-rutinoside derivatives of myricetin give stable pseudomolecular ions in negative ionization mode that further suffered

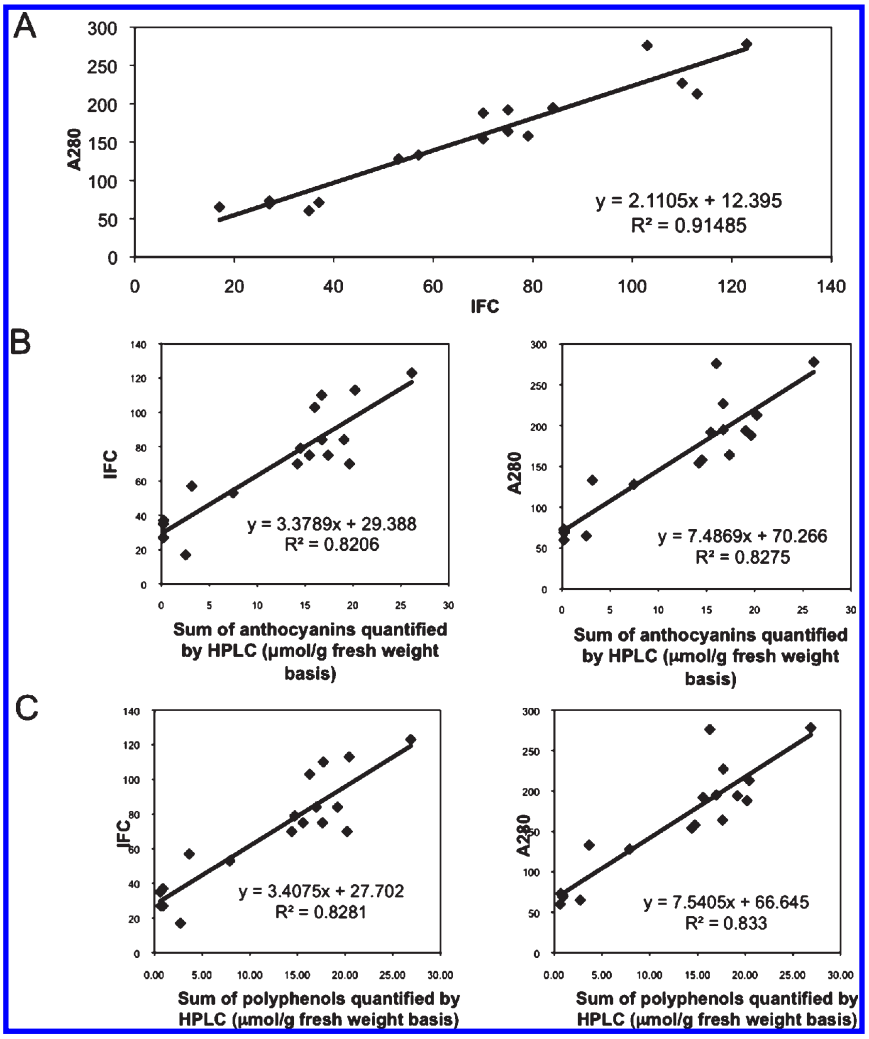

Figure 3. Correlation between the results of total polyphenols obtained by different estimation methods: (**A**) IFC (Folin−Ciocalteu) versus A_{280} (absorbance at 280 nm); (**B**) IFC and A_{280} versus sum of anthocyanins by HPLC; (**C**) IFC and A_{280} versus TF by HPLC (sum of all anthocyanins, flavonols, and flavan-3-ols obtained by HPLC).

fragmentation in the ion trap. The same was found for authentic standards of quercetin-3-glucoside and quercetin-3-rutinoside (data not shown). On the basis of this behavior, we extracted selected ion chromatograms at the expected negative pseudomolecular ion m/z values for the couples of 3-glucoside and 3-rutinoside derivatives of myricetin (479 and 625, respectively), quercetin (463 and 609, respectively), and isorhamnetin (477 and 623, respectively). This was the only way to detect the presence of such 3-glucoside derivatives, because they cannot be distinguished from their respective 3-rutinoside derivatives by their UV−vis spectra.

In addition to the above-mentioned quercetin and isorhamnetin derivatives, the corresponding 3-rutinoside-7-glucoside derivatives (peaks 22 and 28, respectively) were also tentatively identified on the basis of the UV−vis and mass spectral data (**Table 3**). Isorhamnetin-3-rutinoside-7-glucoside partially coeluted with a non-flavonol peak, and only MS data could be obtained. Peaks 25 and 26 showed identical UV−vis and MS data (only under negative ionization mode; **Table 3**); the loss of mass fragments of 42 (the corresponding signal at m/z 463 was of low intensity) and 162 amu suggested that both compounds were 3-(6″-acetyl)-hexoside derivatives of quercetin (the lighter product ion had a m/z value of 301), but we did not get any supporting data for the assignment of the sugar moieties or whether they were position isomers of the same sugar having the acetyl group linked to different positions. Only one similar 3-(6″-acetyl)-hexoside derivative was also found for isorhamnetin (peak 31), although it showed the signal to the loss of the complete acetylhexoside group only in its MS/MS spectrum (**Table 3**). Finally, peak 27 was assigned as quercetin-3-rhamnoside because it exhibited a loss of 146 amu resulting in a product ion matching the aglycone quercetin (**Table 3**). In the context of flavan-3-ol profiles, only catechin and epicatechin were detected in the calafate extracts at trace levels.

Quantitative Determination of Flavonoids in Calafate Fruits and Comparison with Other Berries. By HPLC-DAD analysis, the polyphenolic compounds of calafate fruits were quantified, and higher levels of anthocyanins than flavonols and flavan-3-ols could be determined. Their total concentrations are presented in **Table 4**. The most abundant anthocyanins were the group of 3-glucoside conjugates, with a high concentration of delphinidin-3-glucoside (between 1.38 and 10.14 μmol/g) followed by petunidin-3-glucoside and malvidin-3-glucoside, with concentrations between 0.83 and 4.99 μmol/g and between 0.63 and 6.02 μmol/g, respectively. The total concentration of anthocyanins in calafate was between 14.21 and 26.13 μmol/g of fresh weight, the highest concentrations being detected in fruits from Coyhaique. In parallel, levels and profiles of these compounds were compared with other fruits from southern Chile as maqui and murtilla. In maqui, the sum total of detected anthocyanins varied between 16 and 20 μmol/g. For this fruit, the most abundant anthocyanin

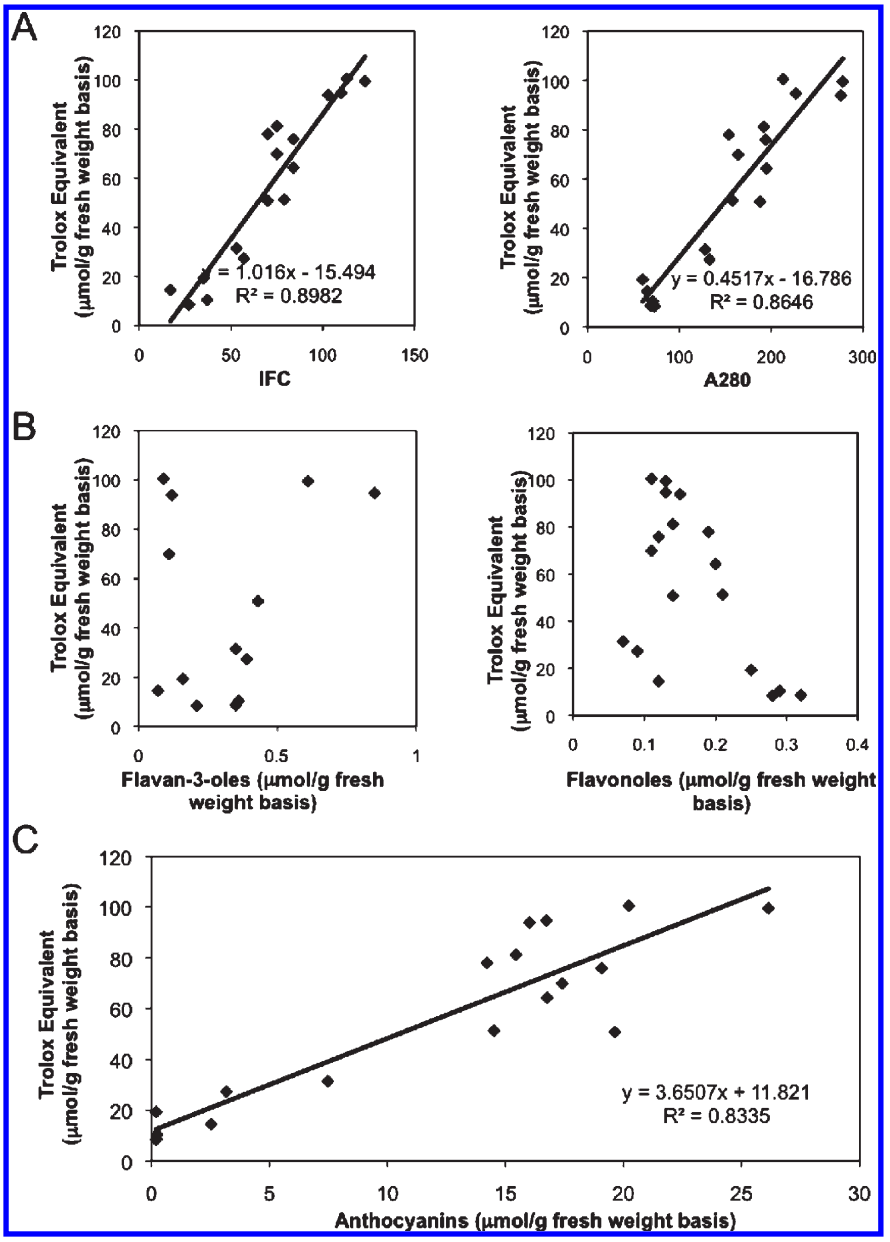

Figure 4. Correlation between antioxidant activity and total polyphenols (**A**), flavonols and flavan-3-ols (**B**), and anthocyanin concentration (**C**). IFC, Folin−Ciocalteu method; A_{280}, absorbance 280 nm method.

was delphinidin-3-sambubioside-5-glucoside with a mean concentration of 5.14 μmol/g. The anthocyanin profiles for these species were in agreement with those described by Escribano-Bailón et al. (*30*) and very recently by Céspedes et al. (*32*). In murtilla fruits only cyanidin-3-glucoside, with a concentration range of 0.05−0.09 μmol/g of fresh weight, and peonidin-3-glucoside, with 0.13−0.15 μmol/g, were detected by HPLC-DAD. The level of total anthocyanins in murtilla fruit was much lower than those found in calafate and maqui fruits, which explains the weaker coloration of this fruit (rose), in comparison with the black and purple colors of maqui and calafate fruits, respectively.

For all berries under investigation, total flavonol concentrations were lower than total anthocyanins, with the exception of murtilla, with 0.25−0.32 μmol/g of flavonols and 0.19−0.24 μmol/g of anthocyanins. For this berry, the concentration of flavonols was 2 times higher than those observed for maqui and calafate, which varied between 0.11 and 0.21 μmol/g of fresh weight, with the most abundant being the quercetin derivatives (**Table 4**). For maqui fruits, the flavonol concentrations were comparable to calafate fruits (mean of 0.12 μmol/g of fresh weight).

The flavan-3-ols identified in calafate and murtilla were catechin and epicatechin; however, in calafate samples from the year 2008, only traces of epicatechin were detected at concentrations below the limit of quantitation of the method. In maqui only epicatechin was detected. The total concentrations of the identified flavan-3-ols were comparable for the studied berries. For murtilla, this concentration was also higher than its anthocyanin concentration.

Total Polyphenol Content and Antioxidant Capacity. Total phenol content was determined by using both the Folin−Ciocalteu (IFC) as well as the absorbance at 280 nm (A_{280}) methods. Furthermore, the concentrations of total anthocyanins, flavonols, and flavan-3-ols as obtained by HPLC analyses (TF by HPLC) are presented. A good correlation between these three total polyphenolic estimation methods was observed for all studied berries (**Figure 3**). The three methods showed higher total polyphenol content for maqui, followed by calafate and murtilla.

In the case of a blueberry sample, its level was closer to that of murtilla than to that of maqui or calafate. For the latter species the high polyphenol concentration can be explained by their high level of anthocyanins. Highest total polyphenol concentration in calafate was observed in samples from Coyhaique, whereas samples from San Isidro harvested at different dates during 2008 showed rather constant concentrations at the two first harvest dates and an increase in the following weeks, which can be attributed to fruit ripening. This last observation indicates a low influence of maturation process produced between the 11 sampling days on the levels of these compounds. In this context, it is important to consider that a difference in sugar concentration was not observed for these fruits, but neither is a correlation between polyphenol content and sugar concentration observed.

The antioxidant activities obtained by TEAC method for calafate, maqui, murtilla, and blueberry are also presented in **Table 4**. In calafate fruits, TEAC values varied over a wide range, from 50.8 to 99.5 μmol/g of Trolox equivalents. The highest antioxidant activity in calafate samples was comparable with those observed in maqui. Murtilla and blueberry samples showed antioxidant activity much lower than that of these fruits. Antioxidant activity in these berries showed certain correlation with total polyphenols and also with their anthocyanins levels (**Figure 4**), which was attributed to highest concentrations of these compounds compared with the other polyphenolic compounds present in the fruits.

These results suggest that calafate fruits contain mainly anthocyanins and minor concentrations of other polyphenols as flavonols and flavan-3-ols. The predominant anthocyanin family was that formed by the glucose derivatives of delphinidin, petunidin, and cyanidin. The concentration of total anthocyanins in calafate was comparable with those found in maqui; however, one calafate sample showed an anthocyanin concentration 30% higher than the highest concentration observed in maqui samples. Blueberries and murtilla showed low concentrations of these compounds, but in murtilla fruits the total flavonol level was twice the concentration observed in the other berries under investigation. For all berries, total anthocyanin concentrations were highly correlated with antioxidant activity, which could be attributable to the anthocyanins present in these fruits, which represent approximately 98% of the studied polyphenolic compounds in calafate fruits. For the first time these results point out the high polyphenol content of calafate berries and confirm that previously reported for maqui berries, offering for both "superfruits" potential to be included in functional foods and nutraceuticals.

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Supporting Information Available: Additional table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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