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Underutilized Native Biobío Berries: Opportunities for Foods and Trade

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The Biobío Region in Chile presents special edaphoclimatic characteristics and biodiversity that make it suitable for the introduction and development of some berries that are understudied in terms of their health-promoting composition and characteristics. Chilean strawberry (*Fragaria chiloensis* ssp. *chiloensis,* form *chiloensis*) and calafate (*Berberis microphylla* G. Forst.) have been underused despite having interesting nutritive values and health-promoting capacities. The polyphenolic compositions of Chilean strawberry and calafate were characterized by HPLC-DAD-ESI-MSn analyses, as well as their antioxidant potential (in ORAC and DPPH assays). The major secondary metabolites present in Chilean strawberry and calafate were ellagic acid derivatives and anthocyanins, respectively, being the anthocyanins more closely related to the higher antioxidant capacity found in the berries. Therefore, the Biobío Region berries analyzed are rich in bioactive phytochemicals with health-promoting characteristics. This represents an opportunity for the food industry and for international trade with Chilean berries, as well as a potential way of promoting rural development in the region.

Keywords: Chilean strawberry, Calafate, Antioxidant potential, BioBío Region, Anthocyanins, Ellagitannins.

The Biobío Region (VIII Region) in central Chile, with a predominant Mediterranean climate, marks the transition between the dry temperate and temperate rainy climates with a constant humidity located in the southern part of the country [1]. These characteristics confer on the Region a great potential for agricultural development and make it suitable for the introduction of new crops.

Edible berries, such as blackberries and raspberries, are rich sources of anthocyanins, which are water-soluble compounds responsible for the red, purple, and blue colors of fruits, contributing also to their taste and to the main bioactivities reported for these fruits [2,3]. These berries also show a wide range of other (poly)phenolic compounds, such as flavonols and phenolic acids. All these phytochemicals, which have shown antioxidant and anti-inflammatory activities, have been related to health-promoting effects, including protection of DNA and cardioprotective, neuroprotective, and anticarcinogenic activities [4-6]. Ellagitannins have been found to exhibit significant anti-atherogenic, anti-thrombotic, anti-inflammatory, and anti-angiogenic effects, involved directly in vascular health benefits [7].

Chilean strawberry (*Fragaria chiloensis* ssp. *chiloensis* f. *chiloensis*) is a native wild species of southern Chile and one of the ancestors of the well-known commercial strawberry (*Fragaria* x *ananassa* Duch.), with pale red or white fruits [8]. Calafate (*Berberis microphylla* G. Forst., family Berberidaceae) is a wild shrub bearing black berries, and is native to the Aysen and Magallanes Region in Patagonia. Its use has been limited to local juice and jams [9]. Both fruits, Chilean strawberry and calafate berry, possess attractive nutritional value for the local people in the Andean Region of Chile, and have been used for decades as food and remedies by indigenous groups [10].

What is lacking is the characterization of the composition of these underutilized native berries of the Biobío Region, as nutritious and healthy fresh fruit options for Chile and elsewhere, to stimulate their production and international trade. The present study addresses that gap in our knowledge.

The polyphenols found in Chilean strawberry by HPLC-DAD-ESI/MS^n analyses were mainly ellagitannin derivatives (Table 1).

Table 1: Phenolic compounds identified by HPLC-DAD-ESI/MS^n in Chilean strawberry.

Peak	RT (min)	λmax (nm)	M^+ or M^-	Ion	MSn	Compound
1	5.3	320	785	-	481, 301, 275	Ellagitannin
2	8.0	288	785	-	481, 301, 275	Ellagitannin
3	12.2	278	634	-	301	Pedunculagin I (Galloyl-hexahydroxyphenoyl (HHDP))-hexose)
4	22.4	272	935	-	633, 451, 301	Ellagitannin
5	23.7	252, 360	935	-	633, 451, 301	Ellagitannin
6	24.2	278	935	-	633, 451, 301	Ellagitannin
7	24.9	278, 516	449	+	287	Cyanidin-3-*O*-glucoside
8	26.9	278, 502	433	+	271	Pelargodinin-3-*O*-glucoside
9	32.1	276, 518	535	+	287, 449	Cyanidin-malonyl-glucoside
10	32.9	250, 362	935	-	633, 451, 301	Ellagitannin
11	34.2	256, 368	935	-	633, 451, 301	Ellagitannin
12	36.2	254, 350	955	-	477, 301	Isorhamnetin-3-*O*-glucoside
13	42.2	258, 350	433	-	301	Quercetin-3-*O*-ribose
14	44.4	264, 346	461	-	285	Kaempferol derivative

These compounds represented a large part of the total (poly)phenols present in the berries (peaks 1, 2, 3, 4, 5, 6, 10, and 11), and were identified as either ellagic acid derivatives or hexahydroxydiphenolic acid (HHDP) derivatives, all producing characteristic fragments at *m/z* 301 [11-12]. Also, anthocyanin derivatives - specifically cyanidin-3-*O*-glucoside (peak 7), pelargonidin-3-*O*-glucoside (peak 8), and cyanidin-malonyl-glucoside (peak 9) - were detected. Cyanidin-malonyl-glucoside (*m/z* 535) showed characteristic fragmentation ions of cyanidin with hexoside (*m/z* 449, 287) and a malonyl acid attached (*m/z* 86). It has been suggested that these anthocyanins contribute greatly to the high antioxidant capacity of the Chilean strawberry [8]. Flavonols

were also detected in the Chilean strawberry (peaks 12, 13, and 14), which were tentatively identified as isorhamnetin-3-*O*-glucoside, quercetin-3-*O*-ribose, and a kaempferol derivative, respectively.

The HPLC-DAD-ESI/MS^n analyses identified 20 compounds in calafate berries, namely anthocyanins and flavonol 3-*O*-glycoside conjugates, and their derivatives (Table 2).

Table 2: Phenolic compounds identified in calafate (*Berberis microphylla*) by HPLC-DAD-ESI/M.

Peak	RT (min)	λmax (nm)	M^+ or M^-	Ion	MSn	Phenolic Compounds
1	14.1	278, 524	640	+	317	Petunidin-3-*O*-dihexoside
2	16.9	280, 522	654	+	331	Malvidin-3-*O*-dihexoside
3	22.3	276, 524	464	+	303	Delphinidin-3-*O*-hexoside
4	24.7	280, 516	448	+	287	Cyanidin-3-*O*-hexoside
5	26.3	276, 526	478	+	317	Petunidin-3-*O*-hexoside
6	27.6	276, 524	624	+	317,146,162	Petunidin-3-*O*-coumaroyl-hexoside
7	28.7	278, 514	462	+	301	Peonidin-3-*O*-hexoside
8	28.9	344	510	-	346,193	Feruloyl derivative
9	29.9	274, 526	492	+	331	Malvidin-3-hexoside
10	31.1	280, 530	638	+	331,146, 162	Malvidin-3-coumaroyl-hexoside
11	34.9	350	609/610	-	301	Quercetin-3-rutinoside
12	36.1	352	463/464	-	301	Quercetin-3-glucoside
13	39.8	324	515	-	353,191	3,5- Di-caffeoylquinic acid(CQA)
14	40.5	352	506	-	463,301	Quercetin-hexoside derivative
15	43.4	352	447	-	301	Quercetin-3-*O*-rhamnoside
16	44.4	350	477	-	315	Isorhamnetin-3-*O*-hexoside
17	44.9	354	623	-	315	Isorhamnetin-3-*O*-hexoside-derivative
18	46.1	350	477	-	315	Isorhamnetin-3-*O*-hexoside
19	50.6	352	519/520	-	477,315	Isorhamnetin-3-*O*-hexoside derivative
20	53.1	352	519/520	-	477,315	Isorhamnetin-3-*O*-hexoside derivative

Two di-hexosides were identified, petunidin-3-*O*-di-hexoside and malvidin-3-*O*-dihexoside, but it was not possible to determine if the compounds corresponded to either a 3,5-digalactoside or to mixed 3,5-galactoside/glucoside conjugates [13]. In addition, delphinidin-3-*O*-hexoside, cyanidin-3-*O*-hexoside, petunidin-3-*O*-hexoside, and peonidin-3-*O*-hexoside were also detected, as had been predicted for calafate [14]. Peaks 3 and 9 were identified as the mono-glycosides delphinidin-3-*O*-glucoside and malvidin-3-*O*-galactoside, with characteristic MS ions at *m/z* 464 and 492, respectively, as had been anticipated [15]. Also, other anthocyanins, peaks 6 and 10, were determined to be petunidin-3-*O*-coumaryl-hexoside (MS ions at *m/z* 317, 146, and 162) and malvidin-3-*O*-coumaryl-hexoside (MS ions at *m/z* 331, 146, and 162), respectively. Flavonols (quercetin and isorhamnetin derivatives; peaks 11, 12, 14, 15, 16, 17, 18, 19, and 20) were identified, as well as two hydroxycinnamic acid derivatives, a feruloyl derivative, and a caffeoylquinic acid (MS ions at *m/z* 510 and 515, respectively). Similarly, other authors found 20 hydroxycinnamic acids in calafate berries, although their MS/MS spectra were not conclusive enough to allow a full description or identification [15-16].

The total contents of polyphenolic compounds (mg/g DW) in Chilean strawberries and calafate berries are shown in Table 3, divided into three main groups: anthocyanins, flavonols, and hydroxycinnamic acid derivatives.

The calafate berries are notable for their total anthocyanin content (9.4 mg/g DW), which is far higher than that of Chilean strawberries (0.4 mg/g DW). In Chilean strawberry only three anthocyanins were quantified, cyanidin-3-*O*-glucoside (0.2 mg/g), pelargonidin-3-*O*-glucoside (0.1 mg/g), and cyanidin-malonyl-glucoside (0.1 mg/g); these have been reported as helpful to distinguish between native Chilean and other strawberry genotypes by different authors [8, 17]. The concentration of anthocyanins in Chilean strawberry was higher than that found by other authors

Table 3: Total concentration (mg/g DW) of different phenolic compounds present in Chilean strawberry and calafate by HPLC-DAD.

	Chilean Strawberry	Calafate Berry
Anthocyanins		
Delphinidin-3-*O*-hexoside		2.5 ± 0.02
Cyanidin-3-*O*-hexoside		0.8 ± 0.04
Petunidin-3-hexoside		2.9 ± 0.05
Petunidin-3-O-coumaroyl-hexoside		0.4 ± 0.02
Peonidin-3-*O*-hexoside		0.5 ± 0.02
Malvidin-3-*O*-hexoside		2.3 ± 0.07
Cyanidin-3-*O*-glucoside	0.2 ± 0.001	
Pelargonidin-3-*O*-glucoside	0.1 ± 0.001	
Cyanidin-malonyl-glucoside	0.1 ± 0.0	
Total anthocyanins	0.4	9.4
Flavonols		
Quercetin-3-rutinoside		0.6 ± 0.1
Quercetin-3-glucoside		0.3 ± 0.01
Quercetin-hexoside-derivative		0.5 ± 0.01
Quercetin-3-*O*-rhamnoside		0.4 ± 0.001
Isorhamnetin-3-*O*-hexoside		0.5 ± 0.03
Isorhamnetin-3-O-hexoside-derivative		0.3 ± 0.002
Isorhamnetin-3-*O*-hexoside		0.3 ± 0.02
Isorhamnetin-3-*O*-hexoside-derivative		0.3 ± 0.004
Isorhamnetin-3-*O*-hexoside-derivative		0.1 ± 0.01
Isorhamnetin-3-*O*-glucoside	0.2 ± 0.005	
Kaempferol	0.01 ± 0.001	
Total Flavonols	0.2	3.2
Hydroxycinnamic acid derivatives		
Feruloyl-derivative		0.4 ± 0.01
Caffeoylquinic acid		0.3± 0.01
Ellagitannin	0.5 ± 0.001	
Ellagitannin	0.5 ± 0.008	
Ellagitannin	0.1 ± 0.002	
Ellagitannin	0.3 ± 0.001	
Ellagitannin	0.2 ± 0.002	
Ellagitannin	0.5 ± 0.003	
Total hydroxycinnamic acid derivatives	2.1	0.6

(0.02 mg/g FW) [8]. In calafate berries, six anthocyanins were quantified, delphinidin-3-*O*-hexoside (2.5 mg/g DW), petunidin-3-*O*-hexoside (2.9 mg/g DW), and malvidin-3-*O*-hexoside (2.3 mg/g DW), representing 80 % of the total amount. The flavonols found in calafate berries presented an interesting profile, being mainly quercetin and isorhamnetin derivatives and reaching 3.2 mg/g DW, much higher than in Chilean strawberry (0.2 mg/g DW). In the case of Chilean strawberry, two flavonols were quantified, isorhamnetin-3-*O*-glucoside (0.2 mg/g DW) and kaempferol (0.01 mg/g DW), in agreement with previous work [12]. Concerning the phenolic acids present in the samples, the content of ellagitannin derivatives in Chilean strawberry (2.1 mg/g DW) was quite interesting, being higher than that reported by other authors (1.5 mg/g FW after acid hydrolysis) [8]. Additionally, two phenolic acid derivatives - a feruloyl derivative and caffeoylquinic acid - were also quantified in calafate berries (0.6 mg/g DW).

Differences between these samples and berries of the same species growing in the wild can be clearly observed since the production of natural antioxidants in berries is determined by several factors - such as location of the crop, genetic factors, state of maturation, distribution of antioxidants (since it varies within the different tissues of the fruit), and agronomic conditions, among others [18-21]. But, as remarked by other authors [13], these species have often been investigated less in terms of crop feasibility and the potential benefits of their agricultural use.

The antioxidant capacity evaluated by the ORAC and DPPH$^{\cdot}$ assays shows relevant data for these species grown in the Biobío Region, since the rationale of this study was based on obtaining berries with high functional and antioxidant value for their potential use in this location. These different methods to evaluate the antioxidant capacity are complementary, as at least two of these assays are needed to obtain reliable information to confirm the potential beneficial antioxidant effects. The values obtained in the ORAC assay ranged from 467.5 ± 25.5 (Calafate berry) to 313.1 ± 29.9 (Chilean strawberry) μmol Trolox/g DW. These Biobío berries

exhibited a great radical scavenging activity, similar to those found for other Chilean berries, such as maqui (grown in the IV-XI Regions) and American berries such as açai, which are widely studied because of their strong antioxidant capacity [22]. Therefore, these novel Chilean fruits represent promising sources of natural antioxidants. In the DPPH$^{\cdot}$ assay, calafate berries (51.5 ± 5.6 µmol Trolox/g DW) exhibited a much higher antioxidant capacity than Chilean strawberries (37.4 ± 5.1 µmol Trolox/g DW). These high values for calafate berries are attributable to their notable anthocyanin contents [23-24]. The outcomes of the antioxidant capacity tests are positive for the agricultural potential of these underutilized berries and the development of the Biobío Region.

To sum up, two species of wild growing berries collected in the Biobío Region, the Chilean strawberry (*Fragaria chiloensis*) and the calafate berry (*Berberis microphylla*), showed potential for fresh fruit consumption and national and international trade, due to their rich polyphenolic profiles and strong antioxidant capacities.

According to Hoffmann [25], calafate could have a geographical distribution in Chile that extends from Maule Region (VII) to Tierra del Fuego (XII) (at the southern end of Patagonia), and it grows at diverse altitudes. Thus, calafate berries might potentially be produced in a variety of areas to stimulate food production and trade. Chilean strawberry turned out to be a highly interesting berry too, with a high content of ellagitannins - which promote human health (e.g. due to their anti-atherogenic, anti-thrombotic, anti-inflammatory, and anti-angiogenic effects).

For the future, these berries clearly represent fresh and healthy fruits, and their cultivation would boost the rural and native economies in the Biobío region of Chile.

Experimental

Chemicals: The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$), fluorescein (free acid), 2,2-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), monobasic sodium phosphate, and dibasic sodium phosphate were obtained from Sigma–Aldrich (Steinheim, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system.

Fruit origin and extractions: Lyophilized Chilean strawberries and calafate berries were obtained from different places in the Biobío Region in Chile (Figure 1). Chilean strawberries came from Contulmo (38.01º S, 73.20º W) whereas Calafate berries were collected from San Ignacio in the Andean foothills (36.85º S, 71.95º W). For individual phenolic compounds, the protocol described by Gironés-Vilaplana *et al*. [26] was followed. Briefly, freeze-dried samples (100 mg) were mixed with 5 vol. of methanol/formic acid/water (25:1:24). Then, the samples were vortexed and sonicated for 60 min. The samples were kept at 4ºC overnight and sonicated again for 60 min. Centrifugation was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.22-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4ºC before analysis.

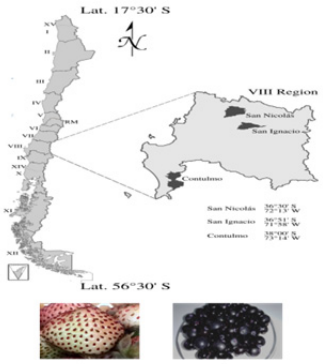


Figure 1: Locations of Biobio Region where Chilean strawberry (A) and Calafate berry (B) were obtained.

Identification of phenolic compounds by HPLC–DAD–ESI/MS^n and quantification by HPLC–DAD: The chromatographic analyses for the identification and quantification of (poly)phenols were carried out in an Agilent HPLC 1200 system (Agilent Technologies, Waldbronn, Germany) coupled to a mass detector series, following a published protocol [26]. The full-scan mass covered the range of *m/z* from 100 to 1200. The mass spectrometry data were acquired in the positive ionization mode for anthocyanins and in the negative ionization mode for other flavonoids and phenolic acids. Chromatograms were recorded at 280, 320, 360, and 520 nm. For the quantification, an Agilent HPLC-DAD 1100 system (Agilent Technologies, Waldbronn, Germany), using the analysis procedure described for identification, was employed and quantification was based on calibration curves using external standards: anthocyanins were quantified using cyanidin 3-*O*-glucoside at 520 nm, flavonols using quercetin 3-*O*-rutinoside at 360 nm, ellagic acid derivatives using ellagic acid at 320 nm, and cinnamic acid derivatives as 3-*O*-caffeoylquinic acid at 320 nm. All the compounds were obtained from Sigma Aldrich (Steinheim, Germany). The samples were analyzed in triplicate.

Antioxidant capacity assays: The free radical scavenging activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) method [27], as well as the *oxygen radical absorbance capacity* ($ORAC_{FL}$) assay [28], both adapted to a microscale. Briefly, the antioxidant capacity for DPPH$^{\cdot}$ was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction with the radical. For $ORAC_{FL}$, this parameter was evaluated by measuring the variation in fluorescence after 120 min of reaction with the radical. The assays were performed using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite-M200 micro plate reader (Tecan, Grödig, Austria). The results were expressed as µmol Trolox/100 g dry weight. Six replications were carried out.

Statistical analyses: The results are presented as descriptive statistics according to the available data. The data are expressed as the mean and standard deviation. All analyses were performed with the GraphPad Prism 6.0 statistical package (GraphPad Software Inc., San Diego, CA, USA).

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