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Analytical Methods

Anthocyanins and antioxidant capacities of six Chilean berries by HPLC–HR-ESI-ToF-MS

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ABSTRACT

The HPLC profiles of six fruits endemic of the VIII region of Chile were investigated using high resolution mass analysis (HR-ToF-ESI-MS). The anthocyanin fingerprints generated for the fruits were compared and the antioxidant capacities measured by the scavenging of the DPPH radical, the ferric reducing antioxidant power (FRAP), the superoxide anion scavenging activity assay (SA), and correlated with the inhibition of lipid peroxidation in human erythrocytes (LP) and total content of phenolics, flavonoids and anthocyanins measured by spectroscopic methods. Several anthocyanins were identified, including 3-*O*-glycosides derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin. Three phenolic acids (feruloyl-quinic acid, chlorogenic acid, and neochlorogenic acid) and five flavonols (hyperoside, isoquercitrin, quercetin, rutin, myricetin and isorhamnetin) were also identified. Calafate fruits showed the highest antioxidant activity. However, the highest LP activity was found for Chilean blueberries (>95%) followed by calafate fruits (91.27%) and luma (83.4%).

1. Introduction

Several epidemiological studies have showed the association between the consumption o foods with high content of phenolics mainly flavonoids and anthocyanins and the prevention of some diseases such as heart attack, cancer and other degenerative disorders (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004; Zamora-Ros et al., 2013). Fruits and vegetables are considered protective for human health due to their content of beneficial compounds particularly against various oxidative-stress related diseases (Pennington & Fisher, 2010). Anthocyanins are a group of water soluble polyphenolic pigments widely found in berry fruits which can act as free radical scavengers mitigating oxidative stress (Pojer, Mattivi, Johnson, & Stockley, 2013). The term berry fruit generally refers to small fruits without big seeds that can be eaten whole. Berry fruits are often the richest source of antioxidant phenolics among fruits and vegetables (Lachman, Orsák, & Pivec, 2000), thus the study of native berry fruits is of great economic significance since it can support the consumption and commercial activities. Chilean fruits such as arrayán, chequen, calafate, meli, maqui and murta (Fig. 1) are small pigmented native berries which were used as a food source since ancient times. At present, there is some regional use of berries from trees and shrubs belonging to Myrtaceae (Chilean myrtle, murta, arrayán, chequén, luma and meli), Berberidaceae (michay and calafate) as well as Eleaocarpaceae (maqui) occuring in south America. In Chile, "murtilla" (*Myrtus ugni* Molina or *Ugni molinae* Turczaninov), is the best-known of the native Myrtaceae plants, where the people appreciate its red edible berries for its unique aroma. Infusions of the leaves of this species are anti-inflammatory and analgesic (Delporte et al., 2007) and the fruits contain several volatile compounds responsible for the aroma (Scheuermann et al., 2008). Arrayán (*Luma apiculata* (DC.) Burret is an evergreen Myrtaceae tree of about 10 m in height with orange-red trunk and edible purple black berries, 1–1.5 cm in diameter, ripe in early autumn and are half the size, more intense color but similar aspect and consistence than the worldwide commercialized blueberries (*Vaccinium corymbosum*) occurring in southern Chile and Argentina. Murillo (Murillo, 1889) describes the medicinal properties of *Eugenia apiculata* D.C., (a synonym for *L. apiculata,* (Hoffmann, 1995) as anti-inflammatory and balsamic. The fruits were used to prepare an aromatic liquor. The fruits of *Luma chequén* (Molina) A. Gray, syn: *Myrceugenella chequen* (Mol.) Kaus are edible small berries with similar size than those of arrayán and murta. de Mösbach (de Mösbach, 1991) refer to *L. chequen* with uses in infusion and syrup as an astringent. Both *L. apiculata* and *L. chequen* fruits were used to prepare a South American native fermented beverage (Hoffmann, 1995). Calafate or Magellan barberry (*Berberis microphylla* G. Forst) is another

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Fig. 1. Pictures of (a) Highbush blueberries, (*Vaccinium corymbosum*), (b) murta, (*Ugni molinae*) (c) calafate, (*Berberis microphylla*), (d) arrayán, (*Luma apiculata*), (e) chequén, (*Luma chequen*), and (f) meli, (*Amomyrtus meli*). Picture (e) was obtained from the following web site: http://www.borago.cl.

Patagonian shrub with edible dark small berries that can grow in a great variety of areas (Mariangel et al., 2013). The production of calafate is concentrated in small gardens in the Regions of Aysén and Magallanes for local production of jams and juices (Mariangel et al., 2013). Calafate, maqui and murta are antioxidant berries considered superfruits due to their high content of phenolic compounds including several anthocyanins (Delporte et al., 2007; Ruiz et al., 2010, 2013). Indeed, several worldwide known edible Myrtaceae fruits presented free radical scavenging constituents including anthocyanins (Reynertson, Yang, Jiang, Basile, & Kennelly, 2008), while Chilean Myrtaceae with high content of anthocyanins have been assessed for antioxidant activity and showed good antioxidant capacities (Simirgiotis, Borquez, & Schmeda-Hirschmann, 2013; Theoduloz, Franco, Ferro, & Schmeda Hirschmann, 1988; Theoduloz, Pacheco, & Schmeda Hirschmann, 1991). Several antioxidant phenolics in edible plants (Bórquez, Kennelly & Simirgiotis, 2013); fruits (Simirgiotis, Ramirez, Schmeda Hirschmann, & Kennelly, 2013; Wu, Dastmalchi, Long, & Kennelly, 2012; Wu et al., 2013); nuts (Verardo et al., 2010) and food byproducts (Qiu et al., 2012) were analyzed using HPLC coupled to PDA and accurate high resolution time of flight analyzers (HPLC-PDA–ToF-MS). However, the chemical analysis regarding anthocyanins or metabolomics present in wild Chilean berries including arrayán, chequén, murta, and calafate was generally performed using low resolution methods (ESI-MS) (Ruiz et al., 2010, 2013; Simirgiotis, Borquez, et al., 2013).

In the present work the anthocyanin profiles and polyphenolic content of six small Chilean berries (arrayán, chequén, murta, calafate, meli and Chilean blueberry var. Brigitta) from the VIII region of Chile were compared and correlated with the antioxidant capacities measured by the bleaching of the DPPH radical, the ferric reducing antioxidant power (FRAP), the superoxide anion scavenging activity assay (SA) and the inhibition of lipid peroxidation in human erythrocytes (LP). The anthocyanins in berries were accurately identified with the help of PDA analysis and high resolution time of flight mass spectrometry (HPLC–ESI-ToF-MS) plus comparison with authentic standards.

2. Materials and methods

2.1. Chemicals and plant material

Folin–Ciocalteu phenol reagent (2 N), reagent grade Na_2CO_3, $AlCl_3$, HCl, $FeCl_3$, $NaNO_2$, NaOH, quercetin, trichloroacetic acid, sodium acetate, HPLC-grade water, HPLC-grade acetonitrile, reagent grade MeOH and formic acid were obtained from Merck (Darmstadt, Germany) Cyanidin, delphinidin 3-*O*-galactoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, petunidin-3-*O*-galactoside, peonidin-3-*O*-galactoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside (all standards with purity higher than 95% by HPLC) were purchased either from ChromaDex (Santa Ana, CA, USA), Extrasynthèse (Genay, France) or

Wuxi apptec co. Ltd. (Shangai, China). Gallic acid, TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine), Trolox, thiobarbituric acid, tert-butylhydroperoxide, dimethylsulfoxide, nitro blue tetrazolium, xanthine oxidase and DPPH (1,1-diphenyl-2-picrylhydrazyl radical) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All ripe fruits for this study (aprox. 500 g each) were collected at Región del Bio-Bio, Chile. Sampling was performed using sterile disposable gloves and rigid plastic sample containers and each sample was submitted individually by overnight courier to our laboratory in Antofagasta to prevent deterioration. Random healthy ripe fruits, representative of the lot, were collected from various specimens (at least 10 fruits per specimen) and different locations (at least 3) in each growing area. Ripe fruits of arrayán *(L. apiculata* (DC.) Burret, chequén (*L. chequén* (Molina) A. Gray), and Murta (*U. molinae* Turcz) were collected in Re-Re, Chile in May 2011. Meli (*A. meli* (Phil.) D. Legrand & Kausel and calafate (*B. microphylla* G. Forst) were collected in the Andean woods of Santa Bárbara, in May 2011. Blueberries (*V. corymbosum*) variety highbush Brigitta were collected in April 2011 in the area of Chillán. Voucher herbarium specimens including samples of fruits were deposited at the Laboratorio de Productos Naturales, Universidad de Antofagasta, Antofagasta, Chile, with the numbers La-111505-1, Lc-111505-2, Um-111505-1, Am-111805-1, Bm-111805-1 and Vc-110704-1, respectively.

2.2. Sample preparation

Fresh fruits were carefully washed, separately homogenized in a blender and freeze-dried (Labconco Freezone 4.5 l, Kansas, MO, USA). Ten grams of each lyophilized fruit was finally pulverized in a mortar, defatted thrice with 100 ml of n-hexane and then extracted with 100 ml of 0.1% HCl in MeOH in the dark in an ultrasonic bath for one hour each time. The extracts were combined, filtered and evaporated *in vacuo* in the dark (40 °C). The extracts were suspended in 20 ml ultrapure water and loaded onto an XAD-7 (100 g) column. The column was rinsed with water (100 ml) and phenolic compounds were eluted with 100 ml of MeOH acidified with 0.1% HCl. The solutions were combined and evaporated to dryness under reduced pressure (40 °C) to give 634.20, 739.20, 499.93, 672.24, 489.93 and 521.38 mg of *L. apiculata*, *L. chequén*, *B. microphylla*, *V. corymbosum*, *A. meli* and *U. molinae* fruits, respectively.

2.3. Liquid chromatography analysis

A portion of each extract (approximately 2 mg) obtained as explained above was dissolved in 2 ml 0.1% HCl in MeOH, filtered through a 0.45 μm micropore membrane (PTFE, Waters) before use and was injected into the HPLC-PDA and ESI-ToF-MS equipment. Qualitative HPLC-PDA analysis of the extracts was performed using a Waters (Milford, MA) Alliance 2695 system equipped with 2695 separation module unit and 2996 PDA detector and a 250 × 4.6 mm, 5 μm, 100 Å, Luna C-18 column (Phenomenex, Torrance, CA), with a linear gradient solvent system of 0.1% aqueous formic acid (solvent A) and acetonitrile 0.1% formic acid (solvent B) as follows: 90% solvent A until 4 min, followed by 90–75% solvent A over 25 min, then 75–10% A over 35 min, then going back to 90% solvent A until 45 min. and finally reconditioning the column with 90% solvent A isocratic for 15 min. The flow rate and the injection volume were 0.5 ml/min and 20 μl, respectively. The compounds were monitored using a wavelength range of 210–800 nm.

2.4. Mass spectrometric conditions

Hyphenated PDA with high-resolution electrospray ionization-time of flight-mass spectrometry (HR-ESI-ToF-MS) analysis was performed using a LCT premier XE ToF mass spectrometer (Waters, Milford, MA) equipped with an ESI interface and controlled by MassLynx V4.1 software, using the chromatographic conditions as stated above. The compounds were monitored using PDA with a wavelength range of 210–800 nm, while mass spectra were acquired with electrospray ionization and the ToF mass analyzer in both positive and negative modes over the range *m/z*: 100–1000. The capillary voltages were set at 3000 V (positive mode) and 2800 V (negative mode), respectively, and the cone voltage was 20 V. Nitrogen was used as the nebulizer and desolvation gas. The desolvation and cone gas flow rates were 300 and 20 l/h, respectively. The desolvation temperature was 400 °C, and the source temperature was 120 °C. For the dynamic range enhancement (DRE) lockmass, a solution of leucine enkephalin (Sigma–Aldrich, Steinheim, Germany) was infused by a secondary reference probe at 200 pg/ml in CH_3CN/water (1:1) containing 0.1% formic acid with the help of a second LC pump (Waters 515 HPLC

Table 1
Scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP), superoxide anion scavenging activity (SA), inhibition of lipid peroxidation in human erythrocytes (LP), total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and extraction yields of six edible berry fruits from the VIII region of chile.

Species	DPPH[A]	FRAP[B]	SA[G]	LP[H]	TPC[C]	TFC[D]	TAC[E]	Extraction yields (%)[I]
Vaccinium corymbosum	3.32 ± 0.18*a*	96.15 ± 5.39*d,f*	72.61 ± 1.91*r*	96.49 ± 0.84*u,v*	45.86 ± 3.46	18.50 ± 3.75*p*	21.41 ± 1.65	6.72
Berberis microphylla	2.33 ± 0.21*a,b*	124.46 ± 6.54	81.31 ± 2.95*s*	91.27 ± 1.82*u,x*	65.53 ± 1.35	45.72 ± 2.68	51.62 ± 1.78	4.99
Luma chequén	12.92 ± 0.30	76.22 ± 3.45*e*	43.79 ± 2.91*t*	62.95 ± 2.3	5.11 ± 0.18*k*	2.57 ± 0.11*m*	1.54 ± 0.05	7.39
Luma apiculata	5.88 ± 0.21	93.4 ± 4.68*d,g*	64.22 ± 3.46	83.40 ± 1.90*y*	27.61 ± 1.61	12.80 ± 2.43*n,p*	15.24 ± 1.49*l*	6.34
Ugni molinae	10.94 ± 0.32*c*	81.10 ± 4.58*e,h,j*	52.22 ± 1.81*t*	53.55 ± 2.92	9.24 ± 0.28*k*	5.54 ± 0.91*m,o*	6.85 ± 0.10	5.21
Amomyrtus meli	7.46 ± 0.10*b*	88.29 ± 6.34*f,g,h,i*	56.44 ± 2.32	75.29 ± 2.13	17.52 ± 0.66	11.76 ± 2.04*n,o*	13.33 ± 2.69*l*	4.89
Gallic acid[F]	1.36 ± 0.22 (7.99 ± 1.29 μM)	148.1 ± 8.35	94.39 ± 1.98	91.24 ± 0.91*v,x*	–	–	–	–
Cyanidin 3-O-glucoside[F]	8.47 ± 1.23*c* (17.47 ± 2.53 μM)	95.48 ± 6.72*i,j*	76.85 ± 1.71*r,s*	72.88 ± 1.90*y*	–	–	–	–

[a] Antiradical DPPH activities are expressed as IC_{50} in μg/ml for extracts and compounds.
[b] Expressed as μM trolox equivalents/g dry weight.
[c] Total anthocyanin content (TAC) expressed as mg cyanidin 3-O-glucoside/g dry weight.
[d] Total flavonoid content (TFC) expressed as mg quercetin/g dry weight.
[e] Total phenolic content (TPC) expressed as mg gallic acid/g dry weight.
[f] Used as standard antioxidants. Values in the same column marked with the same letter are not significantly different (at $p < 0.05$).
[g] Expressed in percentage scavenging of superoxide anion at 100 μg/ml.
[h] Expressed in percentage inhibition of lipid peroxidation in human erythrocytes at 100 μg/ml.
[i] Extraction yields expressed in percent W/W extraction on the basis of freeze dried material.

pump). The reference mass was scanned once every five scans for each positive and negative data collection. Both positive and negative ESI data were collected using a scan time of 0.2 s, with an interscan time of 0.01 s, and a polarity switch time of 0.3 s. The full chromatograms were recorded at two different aperture voltages. The most intense fragmental ions and molecular ions could be obtained, when the aperture voltage were set at 60 V and 0 V, respectively. V-optics mode was used for increased intensity.

2.5. Antioxidant assays

2.5.1. Free radical scavenging capacity

The free radical scavenging capacity of the extracts was determined by the DPPH assay as previously described (Simirgiotis & Schmeda-Hirschmann, 2010), with some modifications. Briefly, 50 μl of processed SPE MeOH extract or pure compound prepared at different concentrations was added to 2 ml of fresh 0.1 mM solution of DPPH in methanol and allowed to react at 37 °C in the dark. After thirty minutes the absorbance was measured at 517 nm. The DPPH scavenging ability as percentage was calculated as: DPPH scavenging ability = $(A_{control} - A_{sample}/A_{control}) \times 100$. Afterwards, a curve of % DPPH bleaching activity versus concentration was plotted and IC_{50} values were calculated. IC_{50} denotes the concentration of sample required to scavenge 50% of DPPH free radicals. The lower the IC_{50} value the more powerful the antioxidant capacity. Gallic acid (from 1.0 to 125.0 μg/ml, $R^2 = 0.991$) and cyanidin 3-O-glucoside (from 1.0 to 125.0 μg/ml, $R^2 = 0.997$) were used as standard antioxidant compounds.

2.5.2. Ferric reducing antioxidant power

The determination of ferric reducing antioxidant power or ferric reducing ability (FRAP assay) of the extracts was performed as described by (Benzie & Strain, 1996) with some modifications. The stock solutions prepared were 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. Plant extracts or standard methanolic Trolox solutions (150 μl) were incubated at 37 °C with 2 ml of the FRAP solution (prepared by mixing 25 ml acetate buffer, 5 ml TPTZ solution, and 10 ml $FeCl_3{\cdot}6H_2O$ solution) for 30 min in the dark. Absorbance of the blue ferrous tripyridyltriazine complex formed was then read at 593 nm. Quantification was performed using a standard calibration curve of antioxidant Trolox (from 0.2 to 2.5 μmol/ml, R^2: 0.995). Samples were analyzed in triplicate and results are expressed in μmol TE/gram dry mass.

2.5.3. Superoxide anion scavenging activity

The enzyme xanthine oxidase is able to generate superoxide anion radical ($O_2^{\cdot-}$) "in vivo" by oxidation of reduced products from intracellular ATP metabolism. The superoxide anion generated in this reaction sequence reduces the nitro blue tetrazolium dye (NBT), leading to a chromophore with a maximum of absorption at 560 nm. Superoxide anion scavengers reduce the speed of generation of the chromophore. The Superoxide anion scavenging activities of isolated compounds and fractions were measured spectrophotometrically in a microplate reader as reported previously (Simirgiotis, Ramirez, et al., 2013). All compounds, and berry extracts were evaluated at 100 μg/ml. Values are presented as mean ± standard deviation of three determinations.

2.5.4. Inhibition of lipid peroxidation in human erythrocytes

Studies on erythrocyte lipid peroxidation were carried out as described by (Luna et al., 2013) with minor modifications. Human red blood cells obtained from healthy non smoking donors were washed three times in cold phosphate buffered saline (PBS) by centrifugation at 3500 rpm. Afterwards, cells were suspended in PBS and its density adjusted to 1 mM hemoglobin in each reaction

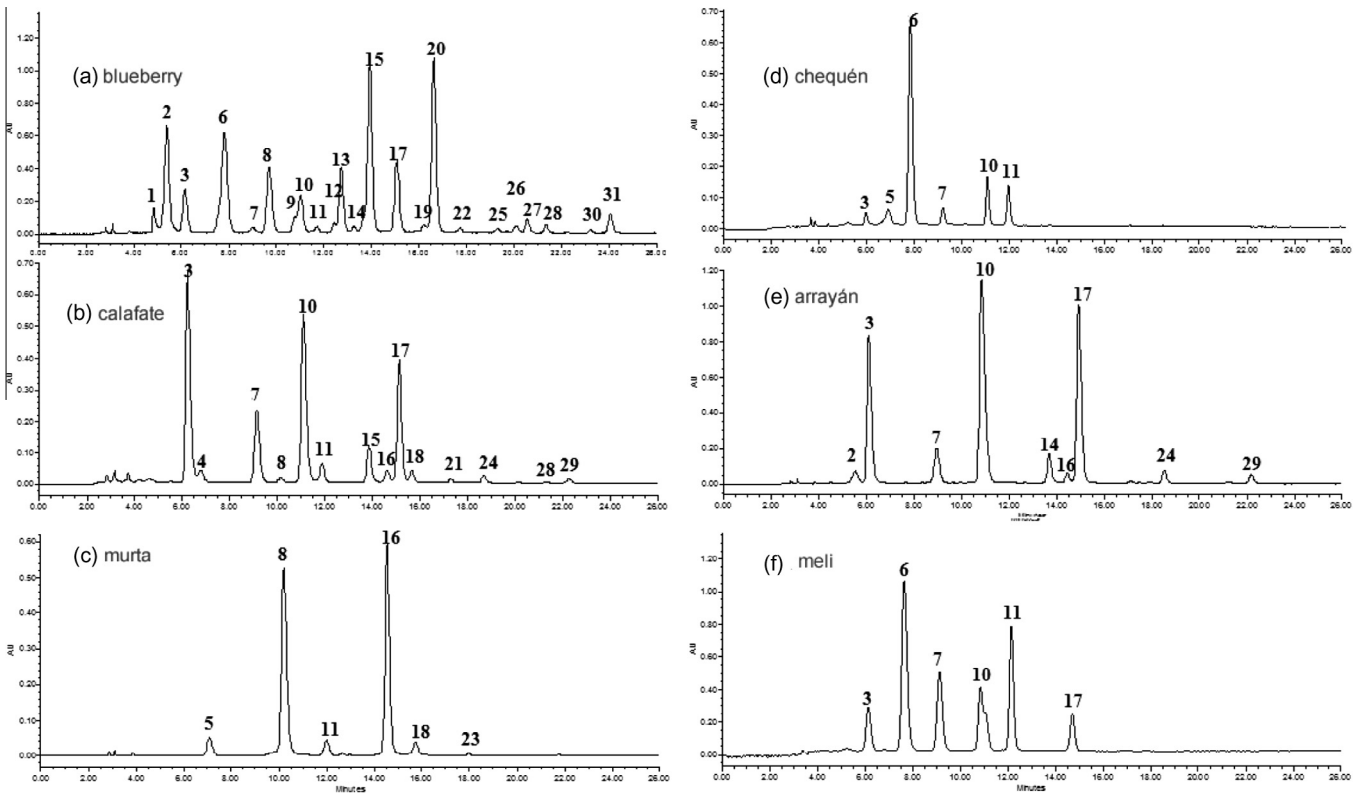


Fig. 2. HPLC–PDA chromatograms of six berries from the VIII region of Chile. (a) *Vaccinium corymbosum*, (b) *Berberis microphylla*, (c) *Ugni molinae*, (d) *Luma chequén*, (e) *Luma apiculata*, and (f) *Amomyrtus meli* monitored at 520 nm. Peaks numbers refer to those indicated in Table 2.

tube. The final concentration of samples and controls in DMSO was 1%. The final cell suspensions were incubated with different concentrations of the test extracts and compounds dissolved in DMSO and PBS during 10 min at 37 °C. After incubation cells were exposed to tert-butylhydroperoxide (1 mM) during 15 min at 37 °C under vigorous shaking. After treatment lipid peroxidation was determined indirectly by the TBARs formation (De Azevedo et al., 2000). Results (in triplicate) are expressed as percentage of inhibition compared to controls.

2.5.5. Polyphenol, flavonoids and anthocyanin contents

The total polyphenolic contents (TPC) of *Luma* fruits and leaves were determined by the Folin–Ciocalteau method (Simirgiotis, 2013; Simirgiotis, Borquez, et al., 2013; Simirgiotis, Caligari, &

Table 2
Identification of phenolic compounds in Chilean berries by LC-PDA–HR-ToF-ESI-MS data.

Peak number	Retention time (min.)	Uv max	HR-M+ ion (ppm)	Other ions (Aglycon moiety)	Formula	Identification	Fruit
1	4.8	276–523	641.1687 (−4.8)	317.0618 (Petunidin)	$C_{28}H_{33}O_{17}$	Petunidin-3-O-di-hexoside	Blue
2	5.9	280–517	611.1614 (0.3)	449.1709 (Cyanidin-3-O-hexoside)	$C_{27}H_{31}O_{16}$	Cyanidin-3-O-di-hexoside	Blue, arr
3	6.3	276–523	465.1040 (1.8)	303.0500 (Delphinidin)	$C_{21}H_{21}O_{12}$	Delphinidin 3-O-galactoside[a]	Blue, cal, che, arr, lu
4	6.8	276–525	465.1038 (1.1)	303.0495 (Delphinidin)	$C_{21}H_{21}O_{12}$	Delphinidin 3-O-glucoside[a]	Cal
5	7.1	280–517	595.1478 (−31.0)	449.1089 (Cyanidin-3-O-glucoside)	$C_{27}H_{31}O_{15}$	Cyanidin 3-O-rutinose	Mu
6	7.8	280–511	449.1052 (−7.1)	287.0675 (Cyanidin)	$C_{21}H_{21}O_{11}$	Cyanidin-3-O-galactoside[a]	Blue, che, lu
7	9.1	280–517	449.1099 (3.3)	287. 0507 (Cyanidin)	$C_{21}H_{21}O_{11}$	Cyanidin-3-O-glucoside[a]	Blue, che, lu
8	9.8	276–526	625.1789 (3.2)	479.1198 (Petunidin-3-O-glucoside)	$C_{28}H_{33}O_{16}$	Petunidin-3-O-rutinoside	Blue, cal, mu
9	10.7	276–527	639.1911 (−2.2)	493.1136 (Malvidin-3-O-glucoside)	$C_{29}H_{35}O_{16}$	Malvidin-3-O-rutinose	Blue
10	11.2	276–526	479.1233 (9.0)	317.0672 (Petunidin)	$C_{22}H_{23}O_{12}$	Petunidin-3-O-glucoside[a]	Blue, che, arr, lu
11	11.9	276–523	479.1224 (7.1)	317.0646 (Petunidin)	$C_{22}H_{23}O_{12}$	Petunidin-3-O-galactoside[a]	Blue, cal, mu, che, lu
12	12.5	276–525	609.1825 (0.8)	301.0829 (Peonidin)	$C_{28}H_{33}O_{15}$	Peonidin 3-O-rutinose	Blue
13	12.7	279–520	463.1234 (−1.3)	301.0689 (Peonidin)	$C_{22}H_{23}O_{11}$	Peonidin-3-O-galactoside[a]	Blue
14	13.4	276–527	493.1361 (3.0)	331.0832 (Malvidin)	$C_{23}H_{25}O_{12}$	Malvidin-3-O-galactoside[a]	Blue, arr
15	14.0	276–523	435.0936 (2.1)	303.0472 (Delphinidin)	$C_{20}H_{19}O_{11}$	Delphinidin-3-O-arabinoside	Blue, cal
16	14.7	276–527	463.1258 (3.9)	301.1257 (Peonidin)	$C_{22}H_{23}O_{11}$	Peonidin-3-O-glucoside	Cal, mu, arr
17	15.3	276–527	493.1252 (−19.0)	331.0789 (Malvidin)	$C_{23}H_{25}O_{12}$	Malvidin-3-O-glucoside[a]	Blue, cal, arr, lu
18	15.6	279–527	433.1131 (−0.92)	301.0709 (Peonidin)	$C_{21}H_{21}O_{10}$	Peonidin-3-O-arabinoside	Cal, mu
19	16.2	276–526	449.1066 (−4.0)	317.1969 (Petunidin)	$C_{21}H_{21}O_{11}$	Petunidin-3-O-arabinoside	Blue
20	16.7	280–517	419.0978 (−1.9)	287. 0696 (Cyanidin)	$C_{20}H_{19}O_{10}$	Cyanidin-3-O-arabinoside[a]	Blue
21	17.3	276–311–527	639.1933 (34.2)	493.1382 (Malvidin-3-O-glucoside)	$C_{32}H_{31}O_{14}$	Malvidin 3-O-(6″ coumaroyl) glucoside	Cal
22	17.8	276–527	463.1284 (9.5)	330.1706 (Malvidin)	$C_{22}H_{23}O_{11}$	Malvidin-3-O-arabinose[a]	Blue
23	18.0	280–517	549.1639 (7.1)	449.1082 (Cyanidin-3-O-glucose)	$C_{25}H_{25}O_{14}$	Cyanidin-3-O-(6″ succinoyl)-glucose	Mu
24	18.6	279–523	625.1820 (8.2)	463.0905 (Peonidin-3-O-hexoside)	$C_{28}H_{33}O_{16}$	Peonidin 3-O-di hexoside	Cal
25	19.4	276–311-523	919.4460 (2.1)	303.0504 (Delphinidin)	$C_{42}H_{47}O_{23}$	Delphinidin-3-O-rutinose (6″-p-coumaroyl)-2″-O-glucose	Blue
26	20.0	276–523	507.1135 (−0.4)	303.0495 (Delphinidin)	$C_{23}H_{23}O_{13}$	Delphinidin 3-O-(6″ acetyl) glucoside	Blue
27	20.6	280–517	491.1206 (3.6)	287.1232 (Cyanidin)	$C_{23}H_{23}O_{12}$	Cyanidin 3-O-(6″ acetyl) glucoside	Blue
28	21.4	276–526	521.1293 (−0.4)	317.0676 (Petunidin)	$C_{24}H_{25}O_{13}$	Petunidin 3-O-(6″ acetyl) glucoside	Blue, cal
29	22.3	276–527	535.1451 (−0.2)	331.0789 (Malvidin)	$C_{25}H_{27}O_{13}$	Malvidin 3-O-(6″ acetyl) galactoside	Cal, arr
30	23.2	276–321-523	627.1393 (−6.8)	287.0743 (Cyanidin)	$C_{30}H_{27}O_{15}$	Delphinidin-3-O-(6″caffeoyl)-glucose	Blue
31	24.0	276–527	535.1463 (1.5)	331.0673 (Malvidin)	$C_{25}H_{27}O_{13}$	Malvidin 3-O-(6″ acetyl) glucoside	Blue

Abbreviations: blue, blueberry; cal, calafate; mu, murta; che, chequén; arr, arrayán; me, meli.
[a] Identified by spiking experiments with authentic compounds.

Schmeda-Hirschmann, 2009) with some modifications. An aliquot of each processed SPE extract (200 μl, approx. 2 mg/ml) was added to the Folin–Ciocalteau reagent (2 ml, 1:10 v/v in purified water) and after 5 min of reaction at room temperature (25 °C), 2 ml of a 100 g/l solution of Na_2CO_3 was added. Sixty minutes later the absorbance was measured at 710 nm. The calibration curve was performed with gallic acid (concentrations ranging from 16 to 500 μg/ml, $R^2 = 0.999$) and the results were expressed as mg gallic acid equivalents/g dry mass. Determination of total flavonoid content (TFC) of the methanolic extracts was performed as reported previously (Simirgiotis et al., 2008) using the $AlCl_3$ colorimetric method. Quantification was expressed by reporting the absorbance in the calibration graph of quercetin, which was used as a standard (from 0.1 to 65.0 μg/ml, $R^2 = 0.994$). Results are expressed as mg quercetin equivalents/g dry weight. The assessment of total anthocyanin content (TAC) was carried out by the pH differential method according to AOAC as described by (Lee, Durst, & Wrolstad, 2005; Simirgiotis & Schmeda-Hirschmann, 2010). Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. Pigment concentration is expressed as mg cyanidin 3-glucoside equivalents/g dry mass and calculated using the formula:

$$\mathrm{TA(mg/g)} = \frac{A \times \mathrm{MW} \times \mathrm{DF} \times 10^3}{\varepsilon \times 1}$$

where $A = (A510\ \mathrm{nm} - A700\ \mathrm{nm})\ \mathrm{pH}\ 1.0 - (A510\ \mathrm{nm} - A700\ \mathrm{nm})$ pH 4.5; MW (molecular weight) = 449.2 g/mol; DF = dilution factor; 1 = cuvette pathlength in cm; ε = 26,900 L/mol cm, molar extinction coefficient for cyanidin 3-*O*-β-D-glucoside. 10^3: factor to convert g to mg. All spectrometric measurements were performed using a Unico 2800 UV–vis spectrophotometer (Shangai, Unico instruments, Co, Ltd).

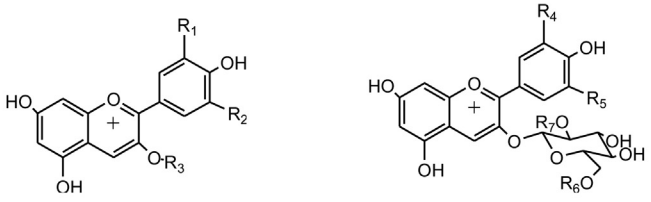
2.5.6. Statistical analysis

The statistical analysis was carried out using the originPro 9.0 software packages (Originlab Corporation, Northampton, MA, USA). The determination was repeated at least three times for each sample solution. Analysis of variance was performed using ANOVA. Significant differences between means were determined by Tukey comparison test (p values < 0.05 were regarded as significant).

3. Results and discussion

3.1. Antioxidant capacity and total phenolics, flavonoids and anthocyanin contents

Calafate showed the highest antioxidant activity (2.33 ± 0.21 μg/ml and 124.46 ± 6.54 μM TE/g dry weight in the DPPH and FRAP assays, respectively, Table 1 and Fig. S1, supplementary material), followed by blueberry (3.32 ± 0.18 μg/ml and 96.15 ± 5.39 μM TE/g DW), and arrayán (5.88 ± 0.21 and 93.4 ± 4.68 μM TE/g DW, Table 1). However, Blueberries showed the highest inhibition of lipoperoxidation in human erythrocytes (96.49%). The bleaching of the radical DPPH for calafate was close to that showed by the standards gallic acid and cyanidin-3-glucoside (1.36 ± 0.22 And 8.47 ± 1.23 μg/ml, respectively). The antioxidant activities showed positive correlation with polyphenolic content assays ($0.67 \geqslant R^2 \geqslant 0.9856$). The total phenolic content (TPC) varied from 5.11 ± 0.18 for chequén to 65.53 ± 1.35 μM trolox equivalents/g DW for calafate fruits, and showed linear correlation with the antioxidant assays ($R^2 = 0.8755$ and $R^2 = 0.9143$ for TPC/DPPH and TPC/FRAP assays, respectively, Table 1) the TPC of our sample of calafate showed values two times higher than a Chilean sample from mañihuales (Mariangel et al., 2013) but was close to that reported for a Chilean sample from Faro San Isidro (Ruiz et al., 2010). The total anthocyanin content (TAC) ranged from 1.54 ± 0.05 for chequén to 51.62 ± 1.78 mg cyanidin-3-glucoside/g DW for calafate and showed strong linear correlation with the antioxidant assays ($R^2 = 0.7044$ and $R^2 = 0.9914$ for TAC/DPPH and TAC/FRAP assays, respectively, Table 1). The total flavonoid content (TFC) showed similar trend, varying from 2.57 ± 0.11 for *L. chequén* to 45.72 ± 2.68 mg quercetin/g DW for *B. microphylla*. The TFC showed linear correlation with the antioxidant assays ($R^2 = 0.678$ for TFC/DPPH and $R^2 = 0.9856$ for TFC/FRAP assays, respectively. The total anthocyanin content for our sample of calafate was close to the values reported for Chilean samples collected



Peak	R_1	R_2	R_3	Peak	R_4	R_5	R_6	R_7
3*	OH	OH	Gal	**1**	OH	OCH_3	Glu	H
4	OH	OH	Glu	**2**	OH	OCH_3	Glu	H
6*	OH	H	Gal	**5**	OH	H	Rha	H
7*	OH	H	Glu	**8**	OH	OCH_3	Rha	H
9	OCH_3	OCH_3	Rha	**9**	OCH_3	OCH_3	Rha	H
10*	OH	OCH_3	Glu	**12**	H	OCH_3	Rha	H
11*	OH	OCH_3	Gal	**21**	OCH_3	OCH_3	Cou	H
13*	H	OCH_3	Gal	**23**	OH	H	Succ	H
14	OCH_3	OCH_3	Gal	**24**	H	OCH_3	Glu	H
15	OH	OH	Ara	**25**	OH	OH	Cou-rha	Glu
16*	H	OCH_3	Glu	**26**	OH	OH	Ac	H
17*	OCH_3	OCH_3	Glu	**27**	OH	H	Ac	H
18	H	OCH_3	Ara	**28**	OH	OCH_3	Ac	H
				29	OCH_3	OCH_3	Ac	H
				30	OH	OH	Caff	H
				31	OCH_3	OCH_3	Ac	H

*Identified using standard compounds. Gal: galactose; Glu: Glucose; Ara: Arabinose; Rha: Rhamnose; Cou: Coumaric acid; Succ: Succinic acid; Ac: Acetyl group; Caff:Caffeic acid.

Fig. 3. Structures of the anthocyanins identified in six berries from the VIII region of Chile.

in La Junta and Darwin (16.76 mmol/g fresh weigh) and Faro San Isidro (15.44 mmol/g fresh weigh) taking into account conversion factors and 85% water loss (approximately 50.11 and 46.21 mg/g dry weight, respectively) (Ruiz et al., 2010). The levels of anthocyanins in the fruits can explain the different intensity in the color especially for murta, which is red-rose, in comparison with calafate which is purple and blueberry and arrayán which are black (Fig. 1). It is reported that fruits antioxidant activities and composition of phenolics are dependent of genetic differences among different species and environmental conditions and harvest and/or ripeness within the same species (Carbone, Giannini, Picchi, Lo Scalzo, & Cecchini, 2011; Mariangel et al., 2013) which can explain the differences in phenolic composition and antioxidant capacities found between the species under study and among other reports of antioxidant activities and phenolic composition of the same species from other zones of Chile (Mariangel et al., 2013; Ruiz et al., 2010, 2013). Human erythrocytes were used as a cell-based lipid peroxidation assay to further explore the antioxidant properties of Chilean berries. As shown in Table 1 and Supplementary material (Fig. S1) the highest activity was found for Chilean blueberries (>95%) followed by calafate fruits (91.27%) and luma (83.4%). The inhibitory activity presented by calafate was close to that of gallic acid (91.24%) and higher than that showed by the anthocyanin cyanidin 3-*O*-glucoside (72.88%). However, all alcoholic fruit extracts prevented the hemolysis caused by the rupture of cell membranes induced by lipid peroxidation (Table 1). Since lipid peroxidation inhibition by antioxidant compounds present in fruits is crucial to mitigate the propagation of oxidative stress related diseases (Mendes, De Freitas, Baptista, & Carvalho, 2011) the inhibitory activity on lipid peroxidation showed by all berries investigated can further support their health benefits and therapeutic potential.

3.2. MS-PDA identification of anthocyanins in six small berry fruits from southern Chile

Anthocyanins in berry fruits were identified using HPLC with UV–visible data (PDA, Fig. 2 and Table 2) and high resolution time of flight mass spectrometry (HR-ToF-MS, Table 2). The 31 anthocyanins identified in the six berries (Fig. 3) were mainly 3-O glycoside conjugates and their derivatives. Twenty three compounds were detected in blueberry (peaks **1**–**3**, **6**–**15**, **17**, **19**, **20**, **22**, **25**–**28**, **30** and **31**, Table 2) fourteen in calafate (peaks **3**, **4**, **7**, **8**, **10**, **11**, **15**, **16**–**18**, **21**, **24**, **28** and **29**), nine in arrayán (peaks **2**, **3**, **7**, **10**, **14**, **16**, **17**, **24** and **29**), and six in meli (peaks **3**, **6**, **7**, **10**, **11** and **17**), chequén (peaks **3**, **5**, **6**, **7**, **10** and **11**) and murta (peaks

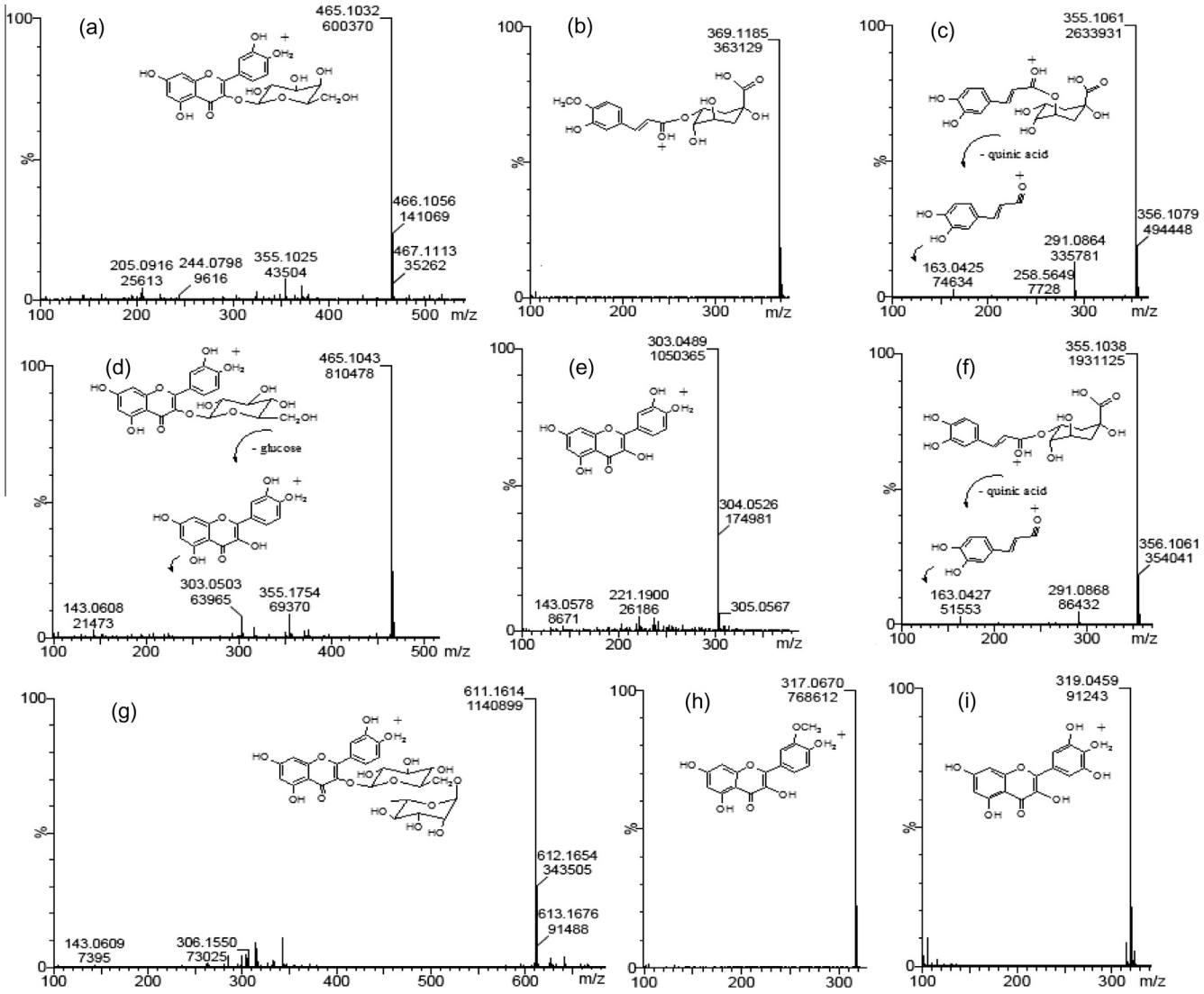


Fig. 4. Full scan ToF MS spectra and structures of minor phenolic compounds detected in six berries from the VIII region of Chile. (a) Hyperoside, (b) feruloyl-quinic acid, (c) chlorogenic acid (d) isoquercitrin (e) quercetin, (f) neochlorogenic acid (g) rutin (h) myricetin and (i) isorhamnetin.

5, **8**, **11**,**16**, **18** and **23**). Figs. S2 and S3 (Supplementary material) showed as examples full scan ToF-MS spectra of peaks **3**, **8**, **9**, **10**, **16**, **17**, **21**, **22** and **28**). Peaks **3**, **6**, **7**, **10**, **11**, **13**, **16** and **17** were identified by spiking experiments with authentic standards as delphinidin 3-*O*-galactoside (HR-MS ion at *m*/*z* 465.1043, λ_{Max}: 276–523), cyanidin-3-*O*-galactoside (HR-MS ion at *m*/*z* 449.1052, λ_{Max}: 280–511), cyanidin-3-*O*-glucoside (HR-MS ion at *m*/*z* 449.1099, λ_{Max}: 280–517), petunidin-3-*O*-glucoside (HR-MS ion at *m*/*z* 479.1233, λ_{Max}: 276–526), petunidin-3-*O*-galactoside (HR-MS ion at *m*/*z* 479.1233, λ_{Max}: 276–523), peonidin-3-*O*-galactoside (HR-MS ion at *m*/*z* 463.1234, λ_{Max}: 279–520), peonidin-3-*O*-glucoside (HR-MS ion at *m*/*z* 463.1258, λ_{Max}: 279–523), and malvidin-3-*O*-glucoside (HR-MS ion at *m*/*z* 493.1252, λ_{Max}: 276–527), (Table 2) respectively. Peaks **4** and **14** were identified as the monoglucosides delphinidin 3-*O*-glucoside and malvidin-3-*O*-galactoside (HR-MS ions at *m*/*z* 493.1361 and 465.1038, respectively (Li et al., 2012; Lätti, Riihinen, & Jaakola, 2011; Simirgiotis, Borquez, et al., 2013). Peaks **1**, **2** and **24** showing HR-MS molecular ions at *m*/*z* 611.1614, 641.1687 and 625.1820 coincident with the formulas $C_{27}H_{31}O_{16}$ (0.3), $C_{28}H_{33}O_{17}$ (−4.8) and $C_{28}H_{33}O_{16}$ (8.2) were identified as petunidin (λ_{Max}: 276–523), cyanidin (λ_{Max}: 280–517), and peonidin (λ_{Max}: 279–523), dihexosides (Aguirre et al., 2011; Ruiz et al., 2010). In a similar manner, peaks **5** (HR-MS at *m*/*z* 595.1478, $C_{27}H_{31}O_{15}$, −31.0), **8** (HR-MS at *m*/*z* 625.1789, $C_{28}H_{33}O_{16}$, 3.2), **9** (HR-MS at *m*/*z* 639.1911, $C_{29}H_{35}O_{16}$, −2.2) and **12** (HR-MS at *m*/*z* 609.1825, $C_{28}H_{33}O_{15}$, 0.8) were assigned as cyanidin, petunidin, malvidin and peonidin rutinosides (Abdel-Aal, Young, & Rabalski, 2006; Lätti et al., 2011; Ruiz et al., 2010; Wu & Prior, 2005). Peaks **15**, **18**–**20** and **22** (Fig. 2) with HR-MS molecular ions at *m*/*z* 435.0936 ($C_{20}H_{19}O_{11}$, 2.1), 433.1131 ($C_{21}H_{21}O_{10}$, −0.92), 449.1066 ($C_{21}H_{21}O_{11}$, −4.0), 419.0978 ($C_{20}H_{19}O_{10}$, −1.9) and 463.1284 ($C_{22}H_{23}O_{11}$) were identified as delphinidin (λ_{Max}: 276–523), peonidin (λ_{Max}: 276–527), petunidin (λ_{Max}: 276–523), cyanidin (λ_{Max}: 280–517) and malvidin (λ_{Max}: 276–527) arabinosides, respectively (Lätti et al., 2011; Yousef et al., 2013), While peaks **21** (HR-MS at *m*/*z* 639.1933, $C_{32}H_{31}O_{14}$) and **23** (HR-MS at *m*/*z* 549.1639, $C_{25}H_{25}O_{14}$) were identified as malvidin 3-*O*-(6″ coumaroyl) glucoside and cyanidin-3-*O*-(6″ succinoyl)-glucose (Abdel-Aal et al., 2006; Aguirre et al., 2011). Peak **25** with a molecular ion at *m*/*z* 919.4460 ($C_{42}H_{47}O_{23}$) present in blueberries was identified as the complex anthocyanin: delphinidin-3-*O*-rutinose (4‴-*O*-p-coumaroyl)-2″-*O*-glucose (Li et al., 2012; Zheng et al., 2011). Peaks **26**–**28** and **31** with HR-MS peaks at *m*/*z* 507.1135 ($C_{23}H_{23}O_{13}$), 491.1206 ($C_{23}H_{23}O_{12}$), 521.1293 ($C_{24}H_{25}O_{13}$), and 535.1463 ($C_{24}H_{25}O_{13}$), were identified as delphinidin, cyanidin, petunidin, and malvidin 3-*O*-(6″ acetyl) glucosides as reported (Li et al., 2012; Yousef et al., 2013), while peak **30** (HR molecular ion at *m*/*z* 627.1393 coincident with a formula of $C_{30}H_{27}O_{15}$ (−6.8) was identified as delphinidin-3-*O*-(6″caffeoyl)-glucose (Wu & Prior, 2005). An Isomer of peak **31** (peak **29**, HR-MS ion at *m*/*z* 535.1451 ($C_{25}H_{27}O_{13}$, −0.2), was identified as malvidin 3-O-(6″ acetyl) galactoside (Li et al., 2012; Yousef et al., 2013).

3.3. Identification of phenolic acids and flavonols

Some minor phenolic compounds (Ruiz et al., 2010, 2013; Simirgiotis, 2013) were present in all six blueberries analyzed which were identified (Fig. 4). The phenolic acids: feruloyl-quinic acid (HR-ToF-MS: 369.1105, MF: $C_{17}H_{21}O_9$, −0.3), chlorogenic acid (HR-ToF-MS: 355.1061, MF: $C_{16}H_{19}O_9$, 9.0) and neochlorogenic acid (HR-ToF-MS: 355.1038, molecular formula: $C_{16}H_{19}O_9$, 2.5), the flavonols quercetin (HR-ToF-MS: 303.0489, MF: $C_{15}H_{11}O_7$, error −5.3), myricetin (HR-ToF-MS: 319.0459, molecular formula: $C_{15}H_{11}O_8$, −1.6) rutin (HR-ToF-MS: 611.1614, MF: $C_{27}H_{31}O_{16}$, 0.3) hyperoside (HR-ToF-MS: 465.1043, MF: $C_{21}H_{21}O_{12}$, 2.2) isoquercitrin (HR-ToF-MS: 465.1032, MF: $C_{21}H_{21}O_{12}$, −0.2) and isorhamnetin (HR-ToF-MS: 317.0670, MF: $C_{16}H_{13}O_7$, 2.8, this last flavonoid was only present in chequén fruits).

4. Conclusions

Several anthocyanins and other phenolics were identified by HPLC-MS in six Chilean berries which showed high antioxidant capacities including the inhibition of lipoperoxidation in human erythrocytes and superoxide anion scavenging activity. Among the anthocyanins identified in the six berries, twenty three compounds were detected in blueberry, fourteen in calafate, nine in arrayán and six were present in meli, chequén and murta. The major anthocyanins detected were 3-*O*-glycoconjugates of malvidin, delphinidin, peonidin, petunidin and cyanidin. However, significant differences in the amount of anthocyanins were found for the six berries, which presented also different antioxidant capacities. Blueberry fruits showed the most complex anthocyanin profile, and the highest Inhibition of lipid peroxidation in human erythrocytes (96.49%) while the fruits of chequen and murta showed a simpler pattern with only six anthocyanins, whereas arrayán and chequén showed a more complex pattern. However, the fruits of calafate (*B. microphylla*) presented the highest antioxidant capacity and poliphenolic content followed by the fruits of Chilean blueberries (*V. corymbosum*), arrayán (*L. apiculata*) and meli (*A. meli*). These Chilean berries can be functional food and their alcoholic extracts candidates for their use as functional ingredients and naturally healthy products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.12.039.

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