HYDROXYCINNAMIC ACID DERIVATIVES AND FLAVONOL PROFILES OF MAQUI (*Aristotelia chilensis*) FRUITS

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ABSTRACT

Maqui (*Aristotelia chilensis*) is an edible berry native to Southern Chile. The anthocyanin profiles and concentrations of this fruit have been widely studied. The high concentration of anthocyanins confers a deep colour to the skin and pulp of maqui berries, and the biological activity of this fruit has been attributed to these compounds. However, other compounds such as flavonols and hydroxycinnamic acid derivatives in this fruit have not been studied to date.

The objective was to determine the hydroxycinnamic acid derivatives and flavonol profiles in maqui berries in order expand the knowledge base for this fruit.

Two extraction methodologies (solid phase extraction using a mixed mode cation exchange cartridge and selective precipitation), followed by HPLC-DAD-ESI-MS/MS were applied to the extraction and identification of the main hydroxycinnamic acids and flavonols in the berries. Quantification was accomplished by using the first extraction procedure.

Low levels of hydroxycinnamic acid derivatives were detected in maqui berries, where 5-galloylquinic acid was the only quantifiable derivative. However, diverse and high levels of flavonols were detected in the fruit, the most relevant of which are myricetin-galactoside, quercetin-rutinoside, and quercetin-3-galactoside. Nineteen other flavonols were identified, with total concentrations between 1.05 and 1.18 µmol/g fresh weight.

The diversity of flavonols makes the profiles and concentrations of these compounds significant, whereas hydroxycinnamic acids in maqui are not significant. The comparatively high levels of flavonols combined with the high concentration of anthocyanins contribute to the consideration of this native fruit from Chile as a "superfruit".

INTRODUCTION

Aristotelia chilensis (Molina) Stuntz (Elaeocarpaceae), also known as Chilean blackberry[1], is a small tree that grows between Coquimbo and Aysén regions of Chile up to 2500 metres above sea level[2]. The fruit of this tree is an edible berry with a diameter of 5 mm, which contains 2 seeds, where the pulp is intensely coloured. Maqui fruits have been traditionally used as a colorant as well as in natural medicine[3] and maqui has thus been the topic of numerous studies[1, 4-12]. Despite the extensive research, the profiles of flavonols and hydroxycinnamic acid derivatives ($HCAD_S$) and their concentrations in maqui berries has not been documented. Prior research has focused primarily on the high anthocyanin content of this fruit[4,8]. The anthocyanin profile of maqui berries has been described, indicating the presence of 3-glucoside, 3,5-diglucoside, 3-sambubioside, and 3-sambubioside-5-glucoside derivatives of delphinidin and cyanidin[4,8], with a total anthocyanin concentration of 137.6 ± 0.4 mg/100 g-fresh weight[4]. The use of maqui berries in functional food preparations, especially juices, based on their rich anthocyanin content and antioxidant activity has also been recently studied [10, 11, 13, 14]. Only quercetin and myricetin were detected during the evaluation of the flavonol profile of maqui berry[6]; however, no derivatives of these compounds or conjugated forms have been described. Evaluation of the hydroxybenzoic acid content by the same authors[6] revealed the presence of gentisic, ferulic, gallic, *p*-coumaric, synapic, and 4-hydroxybenzoic acids; however, as mentioned, there is no available information regarding the profiles and concentrations of HCADs in maqui fruit. However, the phenolic compounds in maqui leaves have been studied, where the presence of flavonoids, indolic alkaloids, coumarins, and triterpenoids has been reported[5].

The antioxidant capacity of maqui fruit was compared with that of other Chilean berries such as calafate (*Berberis microphylla*) and murtilla (*Ugni molinae*) using oxygen radical absorbance capacity methodology (ORAC). The reported antioxidant levels in µmol Trolox equivalents/100 g (fresh weight) were 25662, 19850, 10770, and 8869 for calafate, maqui, murtilla, and blueberries, respectively; these levels are all higher than those detected for other widely consumed fruits such as apples, pears, apricots, peaches, and plums[15].

The biological effects of maqui fruit extracts has been documented based on *in vitro* studies, with claims of anti-inflammatory effects[7,16], antioxidant properties[6,7,15], antiatherogenic activity[17], cardioprotective[1] and hypoglycemic effects[9], as well as antihaemolytic properties[18]. Based on these studies, maqui fruit is considered as a potential source of bioactive compounds; however, to fully understand and exploit the potential of maqui as functional fruit, the content and profiles of flavonols and $HCAD_S$ must be elucidated.

As an analytical challenge, the identification and quantification of other minor phenolic compounds (i.e., flavonols or $HCAD_S$) may be hampered by the extremely high concentrations of anthocyanins in maqui. The absorption spectra of the natural pigments are characterized by an important band in the UV region that interferes with the detection of these phenolic compounds[19]. More selective extraction methodologies are required in order to obtain anthocyanin-free fractions and improve the identification and quantification of non-anthocyanic phenolic compounds in maqui berries.

Based on the aforementioned considerations, the overarching aim of the present study is to expand the knowledge base about maqui berries and their potential as functional fruit. To accomplish this task, we evaluated the profiles and contents of HCADs and flavonols in samples of this fruit. Improved detection of these phenolic compounds was achieved by two complementary extraction procedures, one based on solid phase extraction SPE and the other based on selective precipitation, both followed by HPLC-DAD-ESI-MS/MS analysis.

EXPERIMENTAL

Reagents and standards

Commercial standards of 3-caffeoylquinic (98.87%), 4-caffeoylquinic, and 5-caffeoylquinic acids (98.28%) were obtained from Phytolab (Vestenbergsgreuth, Germany) and standards of myricetin (>85%), quercetin (>98%), kaempferol (>90%), quercetin-3-rutinoside (>95%), quercetin-3-galactoside (>97%), quercetin-3-glucoside (>90%), and quercetin-3-rhamnoside (>85%) were obtained from Sigma-Aldrich (Steinheim, Germany). Formic acid, hydrochloric acid, and ammonia (25%) were obtained from Merck (Darmstadt, Germany). All other solvents (water, acetonitrile, and methanol) were HPLC grade and were purchased from Merck. Mixed phase MCX cartridges were obtained from Waters (Milford, USA).

Sample material

Maqui fruits (*Aristotelia chilensis*) were collected on the campus of the University of Concepción (UDEC) in Concepción (Bío-Bio region), Los Robles (Manzanares, Araucanía region), and Lican Ray (Araucanía region) in Chile during summer 2010 and 2011.

Extraction of hydroxycinnamic acid derivatives and flavonols

Two complementary extraction procedures were carried out in order to obtain a selective extraction for both groups of compounds (flavonols and HCADs). The first procedure was carried out based on a previously described method[19] comprising liquid-solid extraction using methanol acidified with

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formic acid, followed by a clean-up step using Oasis MCX cartridges. Oasis MCX is a mixed-mode cation exchange reversed phase, which allows elimination of sugars and anthocyanins[19, 20].

The second extraction method comprised a fractional precipitation procedure in which 5 g of the dry extract obtained using acidified methanol (with 0.5% HCl) was dissolved in 250 mL of water with 5% formic acid. Using a peristaltic pump, this solution was loaded on Sepabeads SP-850 absorber resin (mean pore size: 38 Å; Supelco, Bellefonte, PA) that was packed in a glass column (40 mm i.d. × 300 mm) pre-conditioned with acidic water. A washing step was performed using 3 L of water. Phenolic compounds were eluted with 1 L of 5% formic acid in methanol and the obtained extract was pre-concentrated and lyophilized. One gram of this extract was re-dissolved in 50 mL of 0.08% trifluoracetic acid (TFA) in ethanol and centrifuged to eliminate the insoluble material. Subsequently, 50 mL of *n*-pentane was added and the precipitate was collected by centrifugation. Precipitation with pentane was repeated two times. Finally, the precipitate was redissolved in 0.08% TFA in a water/acetonitrile 70/30 mixture and loaded on a MCI gel (2.5 × 15 cm) column. Elution was carried out with 0.08% TFA in water/acetonitrile 30/70 (v/v) at a flow rate of 12 mL/min. Under these conditions, anthocyanins were eluted at 10 min, while flavonols and hydroxycinnamic acids were recovered after 45 min.

Evaluation of the flavonol and HCAD profiles of maqui was accomplished by applying both extraction methods in parallel, followed by HPLC-DAD-ESI-MS/MS analysis.

HPLC-DAD-ESI-MS/MS chromatographic conditions

Chromatographic analyses of flavonols and HCADs in maqui fruit extracts was carried out with a Shimadzu HPLC NEXERA system (Kyoto, Japan) equipped with a quaternary LC-30AD pump, a DGU-20A_{5R} degasser unit, a CTO-20AC column oven, a SIL-30AC autosampler, a CBM-20A controller system, and a UV-vis diode array (DAD) SPD-M20A detector, coupled in tandem with a Q-Trap LC/MS/MS 3200 Applied Biosystems MDS Sciex (California, USA) detector. Instrument control and data collection was performed by using a CLASS-VP DAD Shimadzu Chromatography Data System and Analyst Software (Version 1.5.2).

The procedure described by Ruiz[19] was used as the chromatographic technique for identification and quantification of flavonols and HCADs. A C18 column (Kromasil 250 × 4.6 mm, 5 µm) and a C18 pre-column (Nova-Pak Waters, 22 × 3.9 mm, 4 µm) (Milford, MA) were used for separation at 30°C. Gradient elution was performed by varying the mobile phase from 15% to 25% acetonitrile in 14 min, from 25% to 35% for 11 min, from 35% to 100% for 1 min, and from 100% to 15% for 1 min with a final stabilization period of 10 min. MS/MS was performed in negative ionization mode using a collision energy of -5 V, ionization voltage of -4000 V, capillary temperature of 450°C, and the N_2 nebulizer was set to 15 psi. Identification of the analytes was carried out by comparison of their retention time (t_R) and spectral (MS/MS and UV) characteristics with those of the respective commercially available standards. Quantification was performed by using a DAD chromatogram extracted at 320 nm for flavonols and at 360 nm for HCADs. External calibration curves were constructed using 3-caffeoylquinic acid for HCAD determination and using myricetin, quercetin, and kaempferol for quantitative flavonol determination. All results are expressed as µmol/g fresh weight.

RESULTS AND DISCUSSION

HCADs

Only two HCADs were identified in the maqui extracts, based on the UV and MS/MS spectra. These compounds were only detected when the SPE extraction procedure was applied, whereas no HCADs were detected in the extract purified by precipitation. Figure 1A shows the HPLC-DAD chromatogram of the maqui extract at 320 nm obtained by the SPE methodology. The compound with the most abundant signal (peak 1) shows a UV maximum at 331 nm, but this signal was not detected under the MS/MS conditions used for HCADs. A similar situation was observed for the compounds corresponding to signals 3, 5, and 7, which also presented UV absorption at 320 nm; however, these species could not be assigned as HCADs based on the UV and MS/MS spectra.

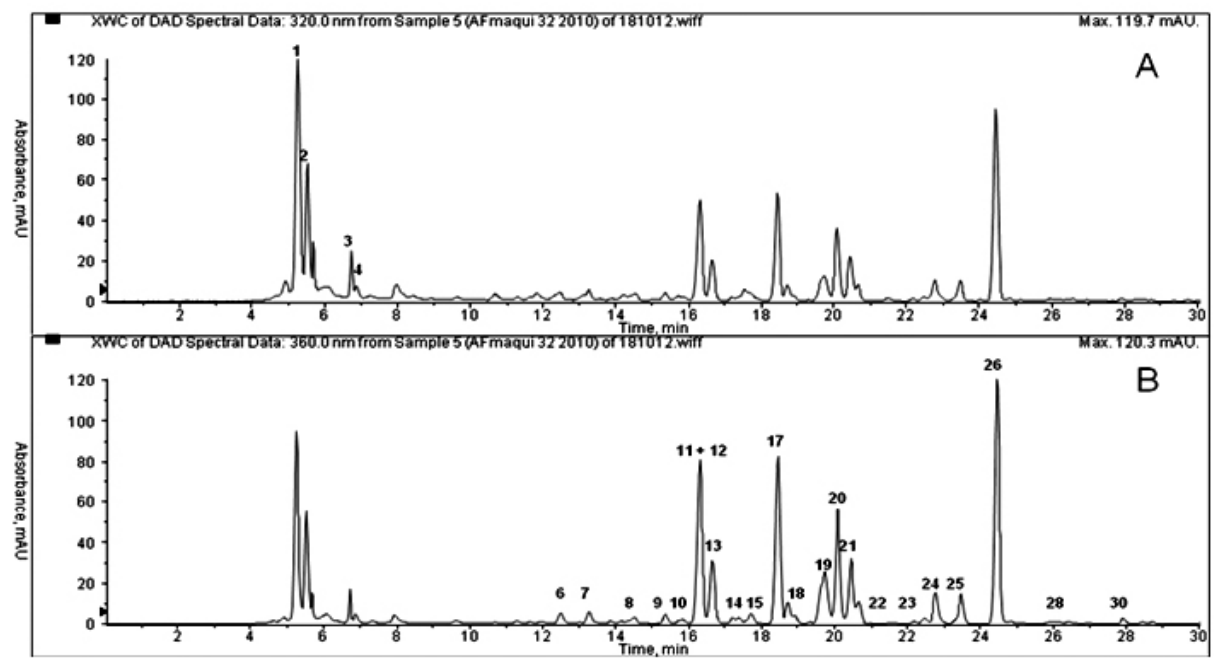

Figure 1: HPLC-DAD chromatogram of flavonols and HCAD in maqui fruits obtained using SPE with mixed phase cartridges. (A) 320 nm, (B) 360 nm. Peaks identities are listed in Table 1.

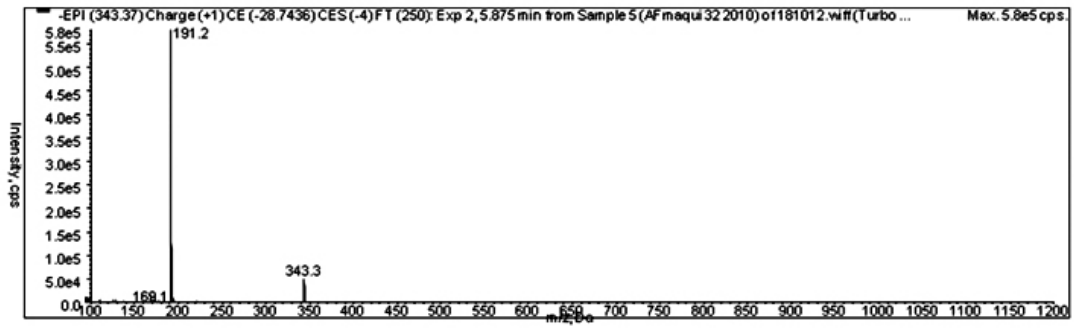

Figure 2: MS/MS spectra of galloylquinic acid in maqui fruits.

Detection of these species was also attempted by using positive ionization in the MS/MS analysis; however MS/MS signals were not detected, making it impossible to identify these compounds. On the other hand, a molecular ion $[M-H]^-$ at 343 and two product ions at *m/z* 191 and 169 (Figure 2) were observed for the most relevant HCAD signal (peak 2) with a maximum UV absorption at 333 nm. On the basis of these characteristics, this compound was tentatively assigned as 5-galloylquinic acid, the spectrum of which is congruent with the data presented by Clifford[21]. Peak 4 was assigned as protocatechuic-4-glucosidic acid based on the MS/MS data in which a pseudomolecular ion peak $[M-H]^-$ was observed at *m/z* 315, which is congruent with the data presented by Rodriguez-Medina[22]. The retention times and spectroscopic characteristics of these compounds are detailed in Table 1.

Table 1: Flavonols and hydroxycinnamic acid derivatives in maqui fruits

Peak	Compound	t_R (min)	λmax (nm)	$[M-H]^-$	Product ions	Kind of Extraction
1	n.i.	5,2	331	-	-	a
2	5-galloylquinic acid	5,5	333	343,3	191,2; 169,1	a
3	n.i.	6,7	317	-	-	a
4	protocatechuic-4-glucosidic acid	7,3	no	315	-	a
5	n.i.	12,3	276	-	-	b
6	quercetin-hexoside	12,7	359	463,3	300,2	a
7	n.i.	13,2	278	-	-	a, b
8	myricetin-galloylhexoside	14,7	no	631,4	479,3; 316,3	a, b
9	myricetin-rutinoside	15,5	no	625,6	316,4	a
10	quercetin-hexoside	16,0	354	463,3	300,3	a
11	myricetin-galactoside	16,3	359	479,3	316,2	a, b
12	myricetin-glucoside	16,6	355	479,3	316,2	a, b
13	quercetin-3-rhamnoside	17,2	no	447,2	300,1	a
14	quercetin-pentoside	17,5	no	433,3	300,1	a
15	quercetin-galloylgalactoside	17,8	359	615	463,3; 300,2	a, b
16	quercetin-galloylglucoside	18,4	no	615,4	463,3; 300,2	b
17	quercetin-3-rutinoside	18,4	355	609,4	300,2	a
18	myricetin-pentoside	19,1	347	449,3	316,2	a, b
19	quercetin	19,9	367	301	-	a, b
20	quercetin-3-galactoside	20,1	355	463,3	300,2	a, b
21	quercetin-3-glucoside	20,5	354	463,3	300,2	a
22	kaempferol-hexoside	20,8	no	447,2	285,3	a
23	myricetin+283	22,5	no	599,3	316,2	a
24	quercetin-pentoside	22,9	355	433,3	300,2	a, b
25	quercetin-pentoside	23,6	352	433,3	300,2	a, b
26	n. i.	24,5	346	329,3	314,2; 271,2	a
27	myricetin-6´´-*O*-hexosil-C-hexoside	24,9	no	599,3	479,4; 316,2	b
28	quercetin-6´´-*O*-hexosil-C-hexoside	26,2	no	583,3	463,3; 300,2	a, b
29	n.i.	27,9	376	-	-	b
30	quercetin	28,1	no	301,3	-	a, b

(a) SPE with MCX cartridges, (b) extraction by precipitation. n.i.: not identified.

Flavonols

A diversity of flavonols was detected in the maqui fruit extracts, where 22 flavonols were identified, corresponding to 14 quercetin derivatives, 7 myricetin derivatives, and 1 kaempferol derivative (Table 1). Both extraction methods (SPE and selective precipitation) functioned complementarily for detection of these compounds. Figure 1B shows the flavonol profile of maqui obtained by the SPE procedure, while Figure 3B shows the flavonol profile obtained by selective precipitation.

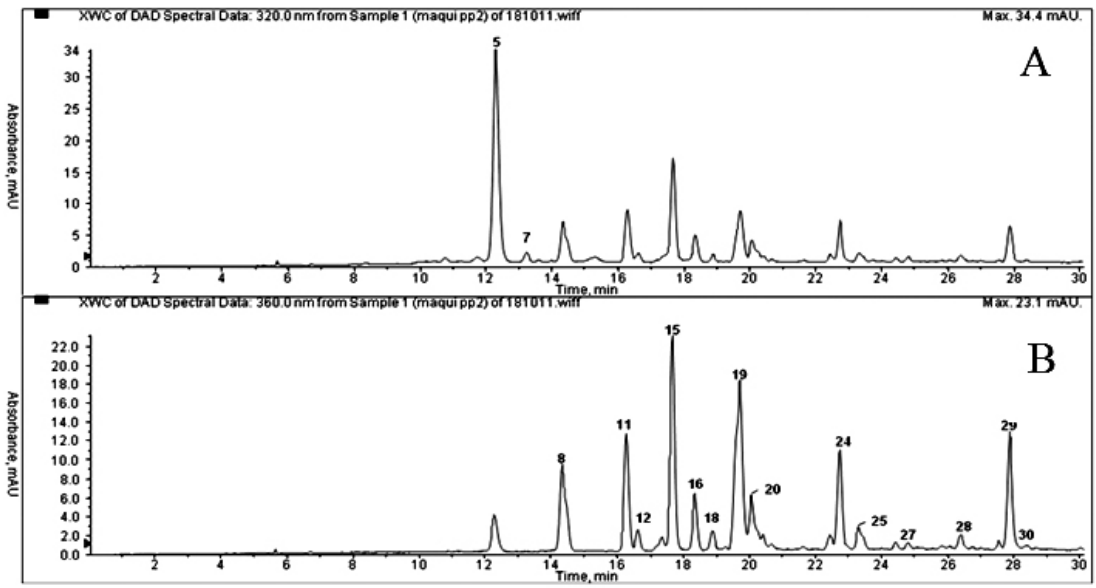

Figure 3: HPLC-DAD chromatogram of flavonols and HCAD in maqui fruits obtained using precipitation extraction. (A) 320 nm, (B) 360 nm. Peaks identities are listed in Table 1.

The quercetin derivatives detected in this fruit included four hexosides, one rhamnoside, one rutinoside, four pentosides, two galloylhexoside derivatives, one unidentified derivative, and a free aglycone. The identities were assigned based on the respective mass spectrometric fragmentation patterns and the UV spectra. A pseudomolecular ion was observed at *m/z* 615 for both galloylhexoside derivatives, with product ions at *m/z* 463 and 300. In this case, the compound identities were assigned by also considering the fragmentation pattern described by Scoparo[23], as well as on the basis of the elution order. The first compound eluted (peak 15) was tentatively assigned as quercetin-galloylgalactoside, while the second peak eluted was assigned as quercetin-galloylglucoside (peak 16); both compounds have previously been detected in blueberries[24]. The myricetin derivatives included one rutinoside, one hexoside, one pentoside, one galloylhexoside, and one unidentified derivative. A pseudomolecular ion $[M-H]^-$ at *m/z* 631 and product ions at *m/z* 479 and 316 were detected for the galloylhexoside derivative, as well as for the other galloylated compounds. The compound was tentatively identified according to the fragmentation pattern described by Scoparo[23].

Myricetin and quercetin derivatives were detected, characterized by respective molecular ions at *m/z* 599.3 and 583.3 and product ions at *m/z* 479 and 316 for the myricetin derivative and at 463 and 300 for the quercetin derivative. These fragmentation patterns have been previously described for 6´´-*O*-hexosil-C-hexosil derivatives of flavones[25], but not for flavonols. Considering that both $[M-H]^-$ ions correspond to myricetin and quercetin derivatives and that the lost fragment in both cases are the same as described for the flavones, we propose that the compounds detected in the maqui extract may correspond to myricetin-6´´-*O*-hexosil-C-hexosil (peak 27) and quercetin-6´´-*O*-hexosil-C-hexosil (peak 28).

A myricetin derivative (peak 23) with a pseudomolecular ion $[M-H]^-$ at *m/z* 599 and a product ion at *m/z* 383 was detected; however, the identity of the conjugate was not unambiguously defined, and the compound was generally assigned as a myricetin-derivative. Only one kaempferol derivative was detected, corresponding to kaempferol-hexoside (peak 22).

The SPE methodology using MCX cartridges facilitated detection of 20 flavonols, one of which was not identified (peak 26 in Figure 1B). The most abundant compounds obtained using this extraction procedure at 359 nm corresponded to myricetin-galactoside (peak 11), quercetin-rutinoside (peak 17), and quercetin-3-galactoside (peak 20).

However, the selective precipitation methodology facilitated detection of only 16 flavonols, coinciding with 13 flavonol signals already identified in the first extraction, whereas the other 3 were not detected in the SPE procedure (peaks 5, 7, and 29). In this case, the most abundant compounds corresponded to quercetin-galloylhexoside (peak 15), quercetin (peak 19), and quercetin-pentoside (peak 24).

These two extraction methodologies evidently gave rise to different flavonol and HCAD profiles for maqui fruits. This approach is an analytical complementary strategy, which allows the extraction of flavonols and HCADs to facilitate identification, and can be used as a tool for isolation of these types of compounds from other fruits rich in anthocyanins.

Concentrations of HCAD and flavonols in maqui fruits

Quantification of the flavonols and HCADs in the maqui fruit extracts was performed with the extract obtained by SPE. The extract obtained via the precipitation methodology was not used for quantification because the extract was prepared only for semi-preparative purposes for identification. Moreover, the complex steps required in this procedure would increase the uncertainty of any quantitative methodology.

The only quantifiable HCAD in maqui fruit extract was 5-galloylquinic acid, with levels between 0.05 and 0.11 mmol/g; this was considered as the total concentration of HCADs in this fruit. This level is lower than those reported for other endemic berries from Chilean Patagonia, such as calafate[19] and other Chilean berries such as *Ribes magellanicum* (3.53 mmol/g) and *Ribes cucullatum* (1.41 mmol/g)[26], as well as other berry crops like black and red currant[27]. However, the HCAD concentration is of the same order of magnitude as reported for other berries such as *Ribes nigrum*[27]. The level of HCADs in maqui fruit is also comparable to that reported for *Ribes rubrum* (0.006–0.3 µmol/g)[27] and lower than the levels reported for, *Vaccinium* spp (0.97–3.21 mmol/g)[28], and *Vaccinium corimbosum L.* (0.73–1.91 mmol/g)[27].

The flavonols detected in maqui extract corresponded to myricetin and quercetin derivatives, of which the most important were quercetin-3-rutinoside (0.22–0.42 µmol/g) and quercetin-3-galactoside (0.17–0.25 µmol/g), with a total flavonol concentration of between 1.05 and 1.14 µmol/g. The total concentrations of $HCAD_S$ and flavonols are summarized in Table 2. The flavonol content of maqui fruit is higher than the $HCAD_S$ content, which is opposite to the trend reported for calafate fruits in which $HCAD_S$ are predominant when anthocyanins, which represent the most abundant group of phenolic compounds in both fruits, are not considered[8].

Table 2: Total concentrations of flavonols and hydroxycinnamic acid derivatives (HCADs) in maqui fruits.

Sample	Total HCADs (µmol/g)	Total flavonols (µmol/g)
Maqui Concepción (Bio-bio region)	0.07	1.14
Maqui Los Robles (Araucania region)	0.05	1.18
Maqui Lican Ray (Araucanía region)	0.11	1.05

The total concentration of flavonols in maqui berries (1.05–1.18 µmol/g) is higher than the levels reported for other berry species, such as blackberry (0.86 µmol/g), rowanberry (0.77 µmol/g), blackcurrant (0.67 µmol/g), strawberry (0.03 µmol/g) [29], *and Vaccinium corymbosum* (0.05–0.11 mmol/g)[27]. This finding is very interesting in terms of the nutraceutical potential of this fruit, especially when the very high anthocyanin concentration of maqui fruit is considered[4,30] in conjunction with the interesting levels of flavonols found in the present study.

CONCLUSIONS

The analytical strategy presented herein, in which selective precipitation and a SPE method were complementarily combined, followed by HPLC-DAD-ESI-MS/MS, facilitated the selective extraction and identification of HCADs and flavonols from maqui fruits. The SPE method facilitated extraction of a larger number of compounds than the precipitation method, and it was also more suitable for quantitative purposes.

Galloylquinic acid was the most abundant HCAD detected in maqui fruits, whereas protocatechuic-4-glucosidic acid was also detected. The other observed signals were not fragmented under the optimal conditions used for HCAD. For this reason, these compounds were not assigned as HCADs. Notably, gallic acid was also detected as a conjugate with flavonols such as quercetin and myricetin.

The bioactive compounds in maqui berries, especially the higher content of flavonols in this fruit relative to the levels reported for other berries, contribute to the consideration of this native fruit from Chile as a "superfruit". The data presented herein represent an expansion of the knowledge-base about phenolic compounds in maqui fruit where the profiles of two new groups (flavonoids and HCADs) of compounds are documented, in addition to that of anthocyanins.

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