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Direct identification of phenolic constituents in Boldo Folium (*Peumus boldus* Mol.) infusions by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry

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ABSTRACT

A very simple and direct method was developed for the qualitative analysis of polyphenols in boldo (*Peumus boldus* Mol., Monimiaceae) leaves infusions by high-performance liquid chromatography with diode array detection (HPLC-DAD) and electrospray ionization tandem mass spectrometry (HPLC-MS^n). The phenolic constituents identified in infusions of the crude drug Boldo Folium were mainly proanthocyanidins and flavonol glycosides. In the infusions, 41 compounds were detected in male and 43 compounds in female leaf samples, respectively. Nine quercetin glycosides, eight kaempferol derivatives, nine isorhamnetin glycosides, three phenolic acids, one caffeoylquinic acid glycoside and twenty one proanthocyanidins were identified by HPLC-DAD and ESI-MS for the first time in the crude drug. Isorhamnetin glucosyl-di-rhamnoside was the most abundant flavonol glycoside in the male boldo sample, whereas isorhamnetin di-glucosyl-di-rhamnoside was the main phenolic compound in female boldo leaves infusion. The results suggest that the medicinal properties reported for this popular infusion should be attributed not only to the presence of catechin and boldine but also to several phenolic compounds with known antioxidant activity. The HPLC fingerprint obtained can be useful in the authentication of the crude drug Boldo Folium as well as for qualitative analysis and differentiation of plant populations in the tree distribution range.

1. Introduction

Boldo (*Peumus boldus* Mol., Monimiaceae), an endemic dioic tree 3–8 m in height is the source of the worldwide known crude drug Boldo Folium. Infusions of boldo leaves are recommended for the treatment of gastrointestinal spasms, dyspeptic disorders and hepatobiliary ailments. The reported bioactive components from the crude drug are the flavonoid catechin and the alkaloid boldine but little is known on the composition of infusions. Most studies on Boldo Folium constituents focus on the alkaloids and particularly on boldine, the main aporphine present in the tree bark. Boldine isolated from *P. boldus* present antioxidant and chemopreventive effect [1–4] and the biological effects of the alkaloid was recently revised [4]. The essential oil obtained by hydrodistillation was analyzed using GC–MS techniques [5,6]. However, very little of the volatile essential oil is present in the infusions. A clear relation between the plant phenolics and antioxidant effect was disclosed for aqueous extracts (infusions and decoctions) [7,8] and a recent study [9] with a boldo leaf infusion showed the protective effect of the aqueous extract as well as pure boldine and mainly catechin on cisplatin-induced lipoperoxidation in mice liver.

Boldo hydroalcoholic extract present a relevant hepatoprotective effect and boldine was at least partially responsible for the reported liver protecting but not for the anti-inflammatory effect [10]. The in vitro antioxidant effect of herbal teas consumed in Chile, including *P. boldus* leaf infusion was reported [9,11,12]. In a previous communication [7], we showed that catechin was one of the antioxidant compounds of boldo leaves, and that the relative concentration of alkaloids and phenolics in boldo extracts suggested that free-radical scavenging effect is mainly due to catechin and flavonoids rather than to the aporphine alkaloids content. Boldo is a dioecious tree, with female and male individuals [13]. A question not previously addressed is the possible differentiation of both genders by phytochemical constituents.

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Recently, an improvement in detection and characterization of natural polar and ionic species in solution has been achieved by the introduction of atmospheric pressure ionization (API) methods, especially electrospray ionization mass spectrometry (ESI-MS), coupled to high-performance liquid chromatography. After HPLC separation of the mixture of compounds, the ESI process transfers small and medium size molecules present in the liquid phase into the gas phase, in order to obtain after the transition protonated (positive mode) or deprotonated (negative mode) molecules, and in some cases (some highly polymerized compounds such as tannins, for instance) multiply charged ions. These ions can be isolated and analyzed by mass spectrometry for structural analysis and identification purposes.

Phenolic compounds such as phenolic acids, tannins and flavonoids are broadly distributed in plants, constitute their most abundant secondary metabolites with many biological activities [14] and can be used as chemotaxonomic markers [15,16]. Liquid chromatography with diode array detection (DAD) hyphenated with tandem mass spectrometry (LC–MS/MS) has been successfully applied to provide tentative structures of phenolic compounds in extracts from natural sources. Indeed, metabolome analysis based on HPLC-DAD–ESI-MS fingerprinting technique is a powerful tool in phytochemistry [17], plant taxonomy and fast characterization of phenolic compounds in medicinal herbs [18,19] vegetables [20] and edible fruits [21,22].

The aim of the present study was to develop a direct method to detect and identify phenolic compounds in boldo leaf infusions by LC-DAD and LC–MS and to provide an HPLC fingerprint as a tool for qualitative analysis of healthy phytochemicals, which could be useful as a reference for nutraceutical polyphenolic non-alkaloid constituents of boldo infusions.

2. Materials and methods

2.1. Chemicals and plant material

Authentic standards of isorhamnetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, quercetin 3-*O*-glucoside (quercitrin), quercetin 3-*O*-rutinoside (rutin), caffeic and ferulic acids were purchased from Sigma–Aldrich (St. Louis, MO, USA) or ChromaDex (Santa Ana, CA, USA). The purity of the reference standards was determined to be more than 95% for flavonoid glycosides and more than 98% for caffeic and ferulic acids by HPLC. Methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA) and formic acid from Merck (Darmstadt, Germany). All solvents used were of HPLC grade.

Leaves from mature male and female *P. boldus* Mol. trees were collected nearby Armerillo, VII Region, Chile, on October 2007. Both trees belong to the same population and were collected the same day, at the same time and the distance of both specimens was about 4 m. Voucher herbarium specimens are kept at the Herbario de la Universidad de Talca and were identified by Patricio Peñailillo.

2.2. Preparation of samples for the HPLC-DAD–MS analysis

The leaves were air-dried and ground in a mill. Infusions were immediately prepared by adding 5 g of powdered leaves (corresponding to about 2 tea bags) to 250 mL (1 cup) of hot (90 °C) deionized water (Milli-Q) and left to stand for 5 min, filtered through a 0.45 μm PTFE filter (Waters) and directly injected (20 μl) for HPLC-DAD and HPLC–MS–MS analysis. The infusions were lyophilized to obtain the w/w extraction yields which were 16.9% for female and 17.7% for male boldo leaves, respectively.

2.3. Instrumentation

A Merck-Hitachi (LaChrom, Tokio, Japan) system equipped with an L-7100 pump, an L-7455 UV diode array detector and a D-7000 chromatointegrator was used for qualitative HPLC-DAD analysis. A 250 mm × 4.60 mm i.d., 5 μm C18-RP Luna column (Phenomenex, Torrance, CA, USA) maintained at 25 °C was used. LC–MS–MS was conducted using the same column with an Agilent 1100 HPLC system connected through a split to an Esquire 4000 Ion Trap LC/MS System (Bruker Daltoniks, Germany).

2.4. LC-UV and LC–MS analysis

The filtered aqueous infusions and extracts obtained from both male and female boldo leaves were immediately submitted to HPLC-DAD and HPLC-MS analysis, which were performed using a linear gradient solvent system of 1% formic acid (A) and methanol (B) as follows: 90% A over 10 min, followed by 90–80% A over 45 min; and 80–25% A from 45 to 50 min at a flow rate of 1 mL/min. The volume injected was 20 μl. The compounds were monitored at 254 nm, and UV spectra from 200 to 600 nm were recorded for peak characterization.

2.5. ESI mass spectrometric conditions

Full scan ESI mass spectra were measured between *m/z* 150 and 2000 u in negative ion mode for all compounds. Nitrogen was used as nebulizer gas at 172.36 kPa, 350 °C and at a flow rate of 8 L/min. The mass spectrometric conditions were as follows: electrospray needle, 4000 V; end plate offset, −500 V; skimmer 1, −56.0 V; skimmer 2, −6.0 V capillary exit offset, −84.6 V; collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (ms/ms) using helium as the collision gas.

3. Results and discussion

3.1. Profile and phenolic characterization

The phenolics present in aqueous infusions of male and female boldo leaves were immediately separated by HPLC and the UV and MS spectra of the different peaks were recorded. We have slightly modified our previous method [21] changing the HPLC run time and substituting acetonitrile by methanol. The modifications allowed a good separation of most of the peaks in the chromatogram. However, we have not obtained a better separation between peaks 47 and 48 using several different HPLC conditions without co-elution of some of the other main peaks. The HPLC-DAD chromatograms of male and female boldo infusions recorded at 254 nm are presented in Fig. 1. Diode array detection (UV spectra recorded from 200 to 600 nm) allowed characterization of phenolics mainly as procyanidins and flavonol 3-*O*-glycosides. Definitive identification of some of the phenolic derivatives was performed by spiking experiments with authentic compounds.

In this study, flavonols showed absorption maxima at 260 and 346 nm for kaempferol and 254 and 354 nm for quercetin and isorhamnetin derivatives [15,23], and were tentatively identified as *O*-conjugates of sugars, taking into consideration MS data and literature reports [21,24,25]. The solvent system employed using negative ion mode proved to be a very sensitive method for ionization of phenolics. Positive ion mode with the same solvent system was almost unable to ionize all compounds, especially phenolic acids and flavonoid glycosides. ESI-MS analysis (full scan, product ion scan and precursor ion scan) showed cleavage of the glycosidic bond of the flavonol 3-*O*-glycoside leading to elimination of the sugar residue after proton rearrangement. Precursor ion scans of the 301 (deprotonated quercetin daughter MS ions at *m/z* 179 and 151 [21], 285 (deprotonated kaempferol, daughter MS ions at *m/z* 151 and 133 [26] and 315 (deprotonated isorhamnetin, daughter MS ions at *m/z* 300, 285, 179 and 151)

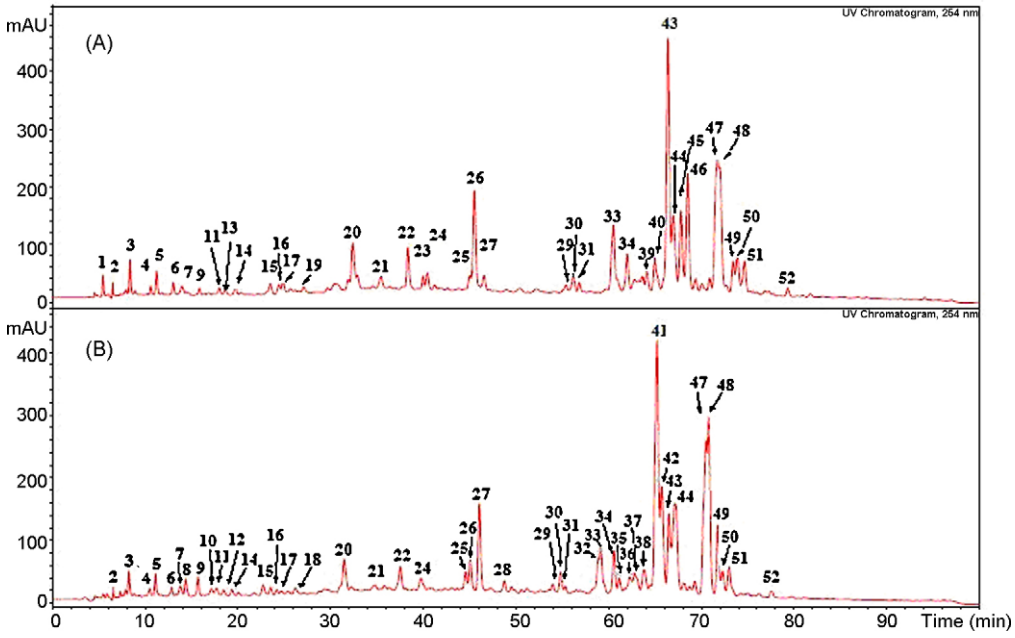


Fig. 1. HPLC-DAD chromatograms at 254 nm of *Peumus boldus* leaf infusion. (A) Male and (B) female plant.

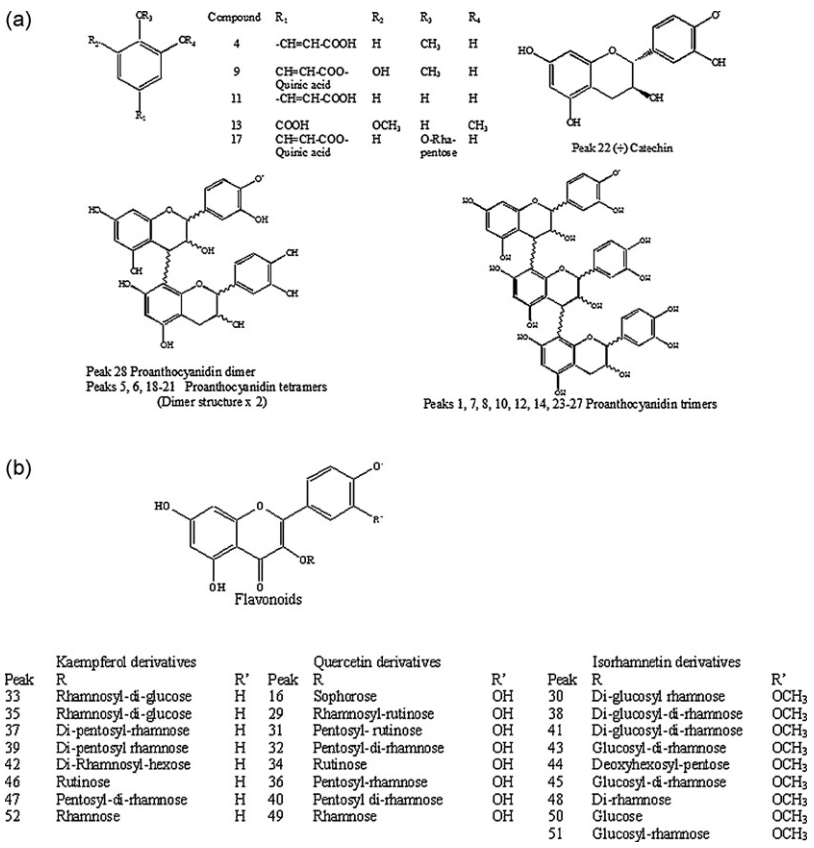
ions allowed the detection of several quercetin, kaempferol and isorhamnetin glycoside derivatives [23]. The detected pseudomolecular ions were further fragmented to obtain the tentative sugar moieties. In flavonol glycosides detected in plants and fruits, glucose is the most common hexose attached at the C-3 position, but galactose and glucuronic acid have also been reported [21,27,28]. In this work, neutral loss scan experiments with losses of 132, 162, 146, 308 and 324 mass units allowed identification of pentosides (xylose or arabinose), hexosides (glucose or galactose), deoxyhexosides (rhamnose), and rutinosides (rhamnose–glucose) and di-hexosides (glucosides), respectively [26].

Some authors have used relative abundances of the ions obtained by MS/MS experiments to differentiate flavonoid rutinosides (α-rhamnose 1 → 6 glucose) from flavonoid neohesperidosides (α-rhamnose 1 → 2 glucose) both showing a neutral loss of 308 u [26,29]. However, confirmation of the sugar moiety of the flavonoid glycoside and exact structure of these compounds cannot be achieved only by HPLC-UV–MS. It requires isolation of the unknown compound and NMR spectral data analysis or UV spectral shifts to confirm the position of attachment of the sugar moiety to the aglycon and hydrolysis followed by co-chromatography with standards sugars. Thus, tentative designation of sugar moieties in this work was based on MS analysis and literature data. The assignment of the glycoside substitutions to position 3 of the flavonoid ring was based on the literature data and in some cases, confirmed by comparison with a standard compound. Fig. 2 shows tentative structures of some of the identified compounds, Fig. 3 shows Full ESI mass spectra of peaks 21, 41–44, 47, 48 and 51 and Fig. 4 shows proposed fragmentations, Full ESI-MS, MS^2 and MS^3 mass spectra of peaks 41 and 43. The 52 compounds detected and identified in boldo infusions are listed in Table 1 and explained below.

3.2. Identification of phenolic compounds

Proanthocyanidins absorbed at 275–280 nm [24]. ESI-MS analysis (Table 1) showed the characteristic pattern of catechin/epicatechin dimers, trimers and tetramers, resulting from differences in the stereochemistry and/or point of attachment of the monomeric units (Fig. 2) [30].

In this work, 20 proanthocyanidins showing different degrees of polymerization (peaks 1–3, 5–8, 10, 12, 14, 18–21, 23–28) as well as 3 phenolic acids (peaks 9, 11 and 13), and one proanthocyanidin monomer (peak 22) were detected and identified on the basis of mass spectral data (Fig. 2). Furthermore, 26 compounds were tentatively identified as flavonol glycoside derivatives in the infusions. Nine compounds were identified as quercetin glycosides (λ max 254, 354 nm, peaks 15, 16, 29, 31, 32, 34, 36, 40 and 49), eight as kaempferol derivatives (λ max 262, 346 nm, peaks 33, 35, 37, 39, 42, 46, 47 and 52) and nine as isorhamnetin glycosides (λ max 254, 264 sh, 354 nm, peaks 30, 38, 41, 43, 44, 45, 48, 50, 51, Fig. 2). Peak 22 was identified as the procyanidin monomer (+) catechin [21], while peak 28 was identified as a proanthocyanidin dimer with a pseudomolecular $[M-H]^-$ ion at *m/z* 577 and MS^2 fragments at *m/z* 531 and 205. Peaks 2 and 3 could be also identified as proanthocyanidins according to their UV spectra (λ max 237, 278 nm) but the tentative structure remains unknown. Peaks 1, 7, 8, 10, 12, 14 and 23–27 were identified as proanthocyanidin trimer isomers with a MW of 866 u due to a deprotonated molecule at *m/z* 865 and characteristic MS^2 ions at *m/z* 577, 407 and 289 [21]. Similarly, peaks 5, 18–21 were identified as isomers of a proanthocyanidin tetramer with MS^n fragments at *m/z* 865, 577, 407 and 289 and a deprotonated molecule at *m/z* 1153 [24,30,31]. Peak 6 was identified also as a proanthocyanidin tetramer with an $[M-H]^-$ ion at *m/z* 1167 according to the literature [32]. Peak 4, 9, 11 and 13 were identified as ferulic, hydroxychlorogenic, caffeic and syringic acid as reported in the literature [33–36] and comparison with authentic compounds for ferulic and caffeic acids. Peak 15 is tentatively a quercetin derivative which showed in full scan mode an $[M-H]^-$ ion at *m/z* 603, an adduct $[2M-H]^-$ ion at *m/z* 1207, MS^2 ions at *m/z* 423 and 379 and a ion at *m/z* 301, characteristic of the flavonol quercetin (MS^3 179 and 151 u) but the tentative structure is unknown. Peak 16 was identified as a quercetin sophoroside with a MW of 626, it showed a neutral loss of 324 corresponding to sophoroside (glucose 1 → 2 glucose) [37], a MS^2 fragment at *m/z* 463 ($[M-H-glucose]^-$), and a MS^3 ion at 301 ($[M-H-diglucose]^-$) [23], while peak 17 was identified as a caffeoylquinic acid glucoside ($[M-H]^-$ ion at *m/z* 631) according to previously published MS and UV spectral data [38]. Full scan mass spectrum of peak 22 showed the deprotonated molecule $[M-H]^-$ at *m/z* 289 and an



	Kaempferol derivatives			Quercetin derivatives			Isorhamnetin derivatives	
Peak	R	R'	Peak	R	R'	Peak	R	R'
33	Rhamnosyl-di-glucose	H	16	Sophorose	OH	30	Di-glucosyl rhamnose	OCH_3
35	Rhamnosyl-di-glucose	H	29	Rhamnosyl-rutinose	OH	38	Di-glucosyl-di-rhamnose	OCH_3
37	Di-pentosyl-rhamnose	H	31	Pentosyl- rutinose	OH	41	Di-glucosyl-di-rhamnose	OCH_3
39	Di-pentosyl rhamnose	H	32	Pentosyl-di-rhamnose	OH	43	Glucosyl-di-rhamnose	OCH_3
42	Di-Rhamnosyl-hexose	H	34	Rutinose	OH	44	Deoxyhexosyl-pentose	OCH_3
46	Rutinose	H	36	Pentosyl-rhamnose	OH	45	Glucosyl-di-rhamnose	OCH_3
47	Pentosyl-di-rhamnose	H	40	Pentosyl di-rhamnose	OH	48	Di-rhamnose	OCH_3
52	Rhamnose	H	49	Rhamnose	OH	50	Glucose	OCH_3
						51	Glucosyl-rhamnose	OCH_3

Fig. 2. Proposed structures of phenolics identified in *Peumus boldus* infusions.

adduct $[2M-H]^-$ ion at *m/z* 579 while MS–MS experiments showed MS^2 ions at *m/z* 245, 221 characteristic of catechin, identity confirmed by comparison with a reference compound [21]. Peak 29 showed an $[M-H]^-$ ion at *m/z* 755 and was identified as an *O*-glycoside of quercetin according to the UV spectrum (λ max 254, 354 nm) and MS/MS analysis of the $[M-H]^-$ ion, which yielded a base peak at *m/z* 301 (M−448) and MS fragments at *m/z* 179 and 151 [21]. Peak 30 showed a deprotonated molecule at *m/z* 785, which, after loss of 146 u (deoxyhexose) and 324 u (dihexose moiety) gave 3-methyl-quercetin (isorhamnetin) MS^2 fragment at *m/z* 315 (MS^3

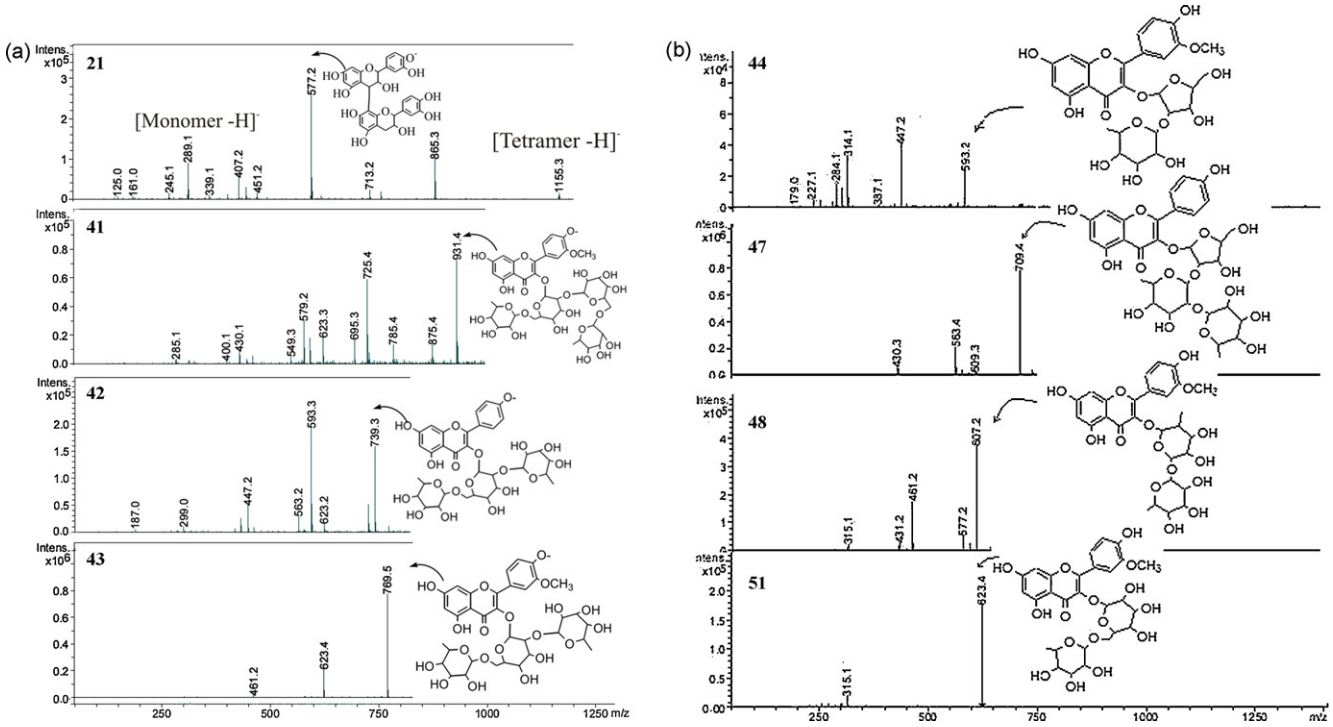


Fig. 3. Full ESI mass spectra of peaks 21, 41–44, 47, 48 and 51.

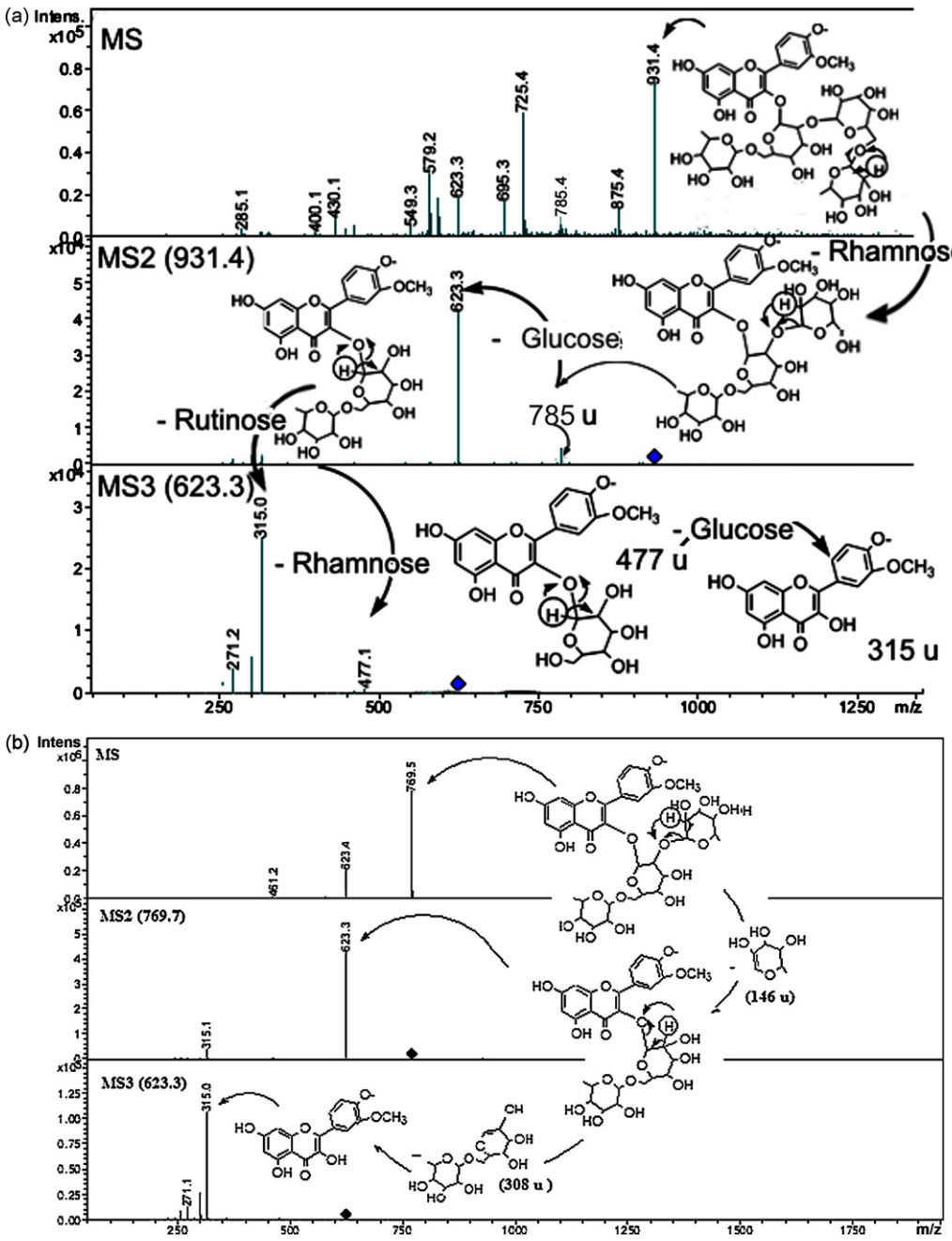


Fig. 4. Proposed fragmentations, Full ESI-MS, MS^2 and MS^3 mass spectra of peaks 41 and 43.

ions at *m/z* 300, 179 and 151) [39] and thus was identified as an isorhamnetin rhamnosyl-di-glucoside.

Peak 31 (λ max 254, 351) was identified as a quercetin pentosyl-rutinoside. It had an $[M-H]^-$ ion at *m/z* 741, MS^2 ions at *m/z* 609 ($[M-H-pentose]^-$), 595 (loss of 146 u, a rhamnose moiety from the parent ion at 741) and MS^3 ions at 445 and the quercetin ion at *m/z* 301 (loss of 294, pentose and glucose moieties). Similarly, peaks 32 and 40 were identified as isomers of a quercetin pentosyl-di-rhamnoside. They both showed an $[M-H]^-$ ion at *m/z* 725, MS^2 ions at *m/z* 579 (loss of 146 u, a rhamnose moiety) 447 (loss of pentose unit) and the quercetin ion at 301 (loss of 146 u, a second rhamnose moiety). MS Analysis of peak 47 showed the neutral losses corresponding to the same triglycoside unit (424 u) and monoglycoside (146, 132 and 146 u) moieties than peaks 32 and 40, but the shape of its UV spectrum was different (λ max 262, 341) and assigned to the kaempferol aglycon (MS^3 at *m/z* 285), and was identified as a kaempferol pentosyl-di-rhamnoside. Tandem mass spectra of peaks 33 and 35 (Table 1) also showed the pattern typical for a kaempferol glycoside (λ max 262, 341) with an $[M-H]^-$ ion at *m/z* 755, which, after losing 146 u (rhamnose) produced an MS^2 fragment at *m/z* 609 and MS^3 fragments at *m/z* 447 (loss of 162 u, a hexose from the ion at *m/z* 609, or loss of 308, rutinoside, from the deprotonated molecule at *m/z* 755) and *m/z* 285 (loss of hexose from the ion at *m/z* 447). They were identified as isomers of a kaempferol rhamnosyl-di-glucoside, as reported previously [40]. Peak 34 showed a $[M-H]^-$ ion at *m/z* 609, and MS^n ions at *m/z* 447, 301, 179 and 151, and was identified as the quercetin 3-*O* rutinoside rutin by comparison with an authentic sample [22]. Peak 36 ($[M-H]^-$ ion at *m/z* 579) was identified as a quercetin

Table 1
Tentative identification of phenolic compounds in *Peumus boldus* leaf infusion by LC-DAD, LC–MS and MS/MS data.

Peak #	Rt (min)	λ max (nm)	MW	$[M-H]^-$	$[2M-H]^-$	MS/MS ions	Tentative assignment	Gender
1	5.4	234, 279	866	865		577, 407, 289	Proanthocyanidin trimer	M
2	6.4	237, 278	504	503	1007	341, 179, 143	Unknown proanthocyanidin	M/F
3	8.4	233, 279	541	540		328, 272, 210, 158	Unknown proanthocyanidin	M/F
4	10.6	240, 323	192	191		157, 147, 110	Ferulic acid	M/F
5	11.1	237, 278	1154	1153		889, 763, 721, 575	Proanthocyanidin tetramer	M/F
6	12.8	237, 278	1168	1167		1041, 1015, 995, 862, 683	Proanthocyanidin tetramer	M/F
7	13.8	237, 278	866	865		577, 407, 287	Proanthocyanidin trimer	M/F
8	14.6	234, 280	866	865		739, 407, 287	Proanthocyanidin trimer	F
9	15.8	235, 315	384	383		225, 192, 177	Hydroxy-chlorogenic acid	M/F
10	17.5	234, 280	866	865		739, 575, 449	Proanthocyanidin trimer	F
11	17.7	241, 323	180	179		175, 171, 165, 135, 107	Caffeic acid	M/F
12	18.5	236, 279	866	865		793, 575, 407, 289	Proanthocyanidin trimer	F
13	18.7	240, 275	198	197		179, 164, 153, 121	Syringic acid	M
14	19.8	234, 279	866	865		577, 407, 289	Proanthocyanidin trimer	M/F
15	23.5	254, 351	604	603	1207	423, 379, 301, 299, 257, 231	Unknown quercetin glycoside derivative	M/F
16	24.4	253, 351	626	625		463, 423, 379, 299, 257	Quercetin sophoroside	M/F
17	24.7	240, 330	632	631		353, 315, 191, 135	Caffeoylquinic acid glycoside	M/F
18	26.2	237, 278	1154	1153		865, 575, 407, 289	Proanthocyanidin tetramer	F
19	27.2	237, 278	1154	1153		865, 561, 289	Proanthocyanidin tetramer	M
20	32.4	234, 278	1154	1153		865, 695, 577, 407, 289, 287	Proanthocyanidin tetramer	M/F
21	35.8	234, 278	1154	1153		865, 695, 577, 407, 289	Proanthocyanidin tetramer	M/F
22	38.0	243, 278	290	289	579	123, 149, 221, 245	(+)Catechin	M/F
23	39.9	234, 278	866	865		695, 577, 407, 289, 287	Proanthocyanidin trimer	M
24	40.5	234, 278	866	865		695, 577, 407, 289, 287	Proanthocyanidin trimer	M/F
25	44.5	246, 278	866	865		695, 577, 407, 289, 287	Proanthocyanidin trimer	M/F
26	45.0	246, 278	866	865		695, 577, 407, 289, 287	Proanthocyanidin trimer	M/F
27	46.0	246, 278	866	865		695, 577, 407, 289, 287	Proanthocyanidin trimer	M/F
28	48.7	251	578	577		531, 205	Proanthocyanidin dimer	M
29	61.2	254, 351	756	755		609, 489, 429, 327, 301, 179, 151	Quercetin rhamnosyl-rutinoside	M/F
30	56.2	254, 346	786	785		639, 459, 315, 300, 271, 151	Isorhamnetin di-glucosyl-rhamnoside	M/F
31	56.8	254, 351	742	741		609, 595, 445, 300, 255, 179, 151	Quercetin pentosyl-rutinoside	M/F
32	58.7	254, 351	726	725		579, 447, 429, 327, 301	Quercetin pentosyl-di-rhamnoside	F
33	59.2	266, 343	756	755		609, 447, 429, 327, 285, 255, 151, 133	Kaempferol rhamnosyl-diglucoside	M/F
34	60.8	254, 346	610	609		447, 301, 279, 151	Quercetin rhamnosyl-glucoside (rutin)	M/F
35	55.4	266, 346	756	755		609, 447, 429, 327, 285, 151, 133	Kaempferol rhamnosyl-diglucoside	F
36	62.3	256, 349	580	579		447, 301, 179, 151	Quercetin pentosyl-rhamnoside	F
37	62.7	268, 344	696	695		549, 285, 255, 151, 133	Kaempferol dipentosyl-rhamnoside	F
38	63.8	264, 346	932	931		623, 315, 300, 271	Isorhamnetin di-glucosyl-di-rhamnoside (isorhamnetin di-rutinoside)	F
39	64.1	264, 346	696	695		549, 285, 255, 151, 133	Kaempferol dipentosyl-rhamnoside	M
40	64.8	254, 351	726	725		579, 447, 429, 327, 301	Quercetin pentosil di-rhamnoside	M
41	65.2	262, 346	932	931		785,725, 623, 477, 315, 300	Isorhamnetin di-glucosyl-di-rhamnoside (isorhamnetin-rutinoside neohesperidoside)	F
42	65.8	264, 346	740	739		593, 447, 413, 284, 255, 151, 133	Kaempferol di-rhamnosyl-hexoside	F
43	66.6	254, 352	770	769		623, 315, 300, 271, 285, 179, 151	Isorhamnetin glucosyl-di-rhamnoside	M/F
44	67.0	254, 351	594	593		447, 315, 300, 285, 179, 151	Isorhamnetin deoxyhexosyl-pentoside	M/F
45	67.9	254, 352	770	769		623, 315, 300, 271, 179, 151	Isorhamnetin glucosyl-di-rhamnoside	M
46	68.5	262, 346	594	593		447, 285, 151, 133	Kaempferol rutinoside	M
47	71.8	262, 341	710	709		563, 431, 413, 285, 163	Kaempferol pentosyl-di-rhamnoside	M/F
48	72.1	254, 346	608	607		461, 315, 300, 285	Isorhamnetin di-rhamnoside	M/F
49	73.9	254, 346	448	447		301, 271, 179, 151	Quercetin rhamnoside (quercitrin)	M/F
50	74.0	254, 343	478	477		314, 300, 285, 179, 151	Isorhamnetin glucoside	M/F
51	74.3	254, 349	624	623		477, 315, 300, 285, 151	Isorhamnetin glucosyl-rhamnoside	M/F
52	78.0	262, 346	432	431		285, 255, 151, 133	Kaempferol rhamnoside	M/F

M: male *Peumus boldus*, F: female *Peumus boldus*.

pentosyl-rhamnoside showing MS^2 ions at 447 ($[M-H-pentose]^-$) and 301 (loss of rhamnose moiety) and MS^3 ions at 179 and 151 [41]. Peaks 37 and 39 ($[M-H]^-$ ions at *m/z* 695) had MS^2 ions at *m/z* 549 ($[M-H-rhamnose]^-$) and a kaempferol MS^3 ion at 285 ($[549\text{-di-pentose}]^-$) and were tentatively identified as structural isomers of an kaempferol dipentosyl-rhamnoside. Peaks 38 and 41 provided both a deprotonated molecule at *m/z* 931 which, after the loss of an rutinose moiety (308 u) produced an MS^2 ion at *m/z* 623, which, in turn, lose another rutinose (or neohesperidose) giving the isorhamnetin ion at *m/z* 315, and thus were identified as isomers of a isorhamnetin di-rutinoside or isorhamnetin-rutinoside-neohesperidoside.

Peak 42 was identified as a kaempferol-(di-rhamnosyl)-hexoside, it displayed a $[M-H]^-$ ion at *m/z* 739; and fragment MS ions at *m/z* 593, 447 and 285 resulting from the loss of a first, a second deoxyhexose and a hexose moieties, respectively [41,42]. Peaks 43 and 45 had an $[M-H]^-$ ion at *m/z* 769; which after the loss of rhamnose ($[M-H-deoxyhexose]^-$ at *m/z* 623) and rutinose (308 u), gave the deprotonated isorhamnetin at *m/z* 315 ($[M-H\text{-rhamnose-rutinose}]^-$ and thus were identified as isomers of an isorhamnetin rhamnosyl–rutinoside. Peak 44 can be identified as a quercetin deoxyhexosil-deoxyhexoside ($[M-H]^-$ ion at *m/z* 593), but it was identified as a isorhamnetin pentosyl-deoxyhexoside. It displayed an $[M-H]^-$ ion at *m/z* 593 and an important MS^2 ion at 447 ($[M-H\text{-rhamnose}]^-$, which after a loss of 132 u (dehydrated pentose) gave the diagnostic isorhamnetin fragment at *m/z* 315 (MS^3 ions at 300, 271, 179 and 151) [39]. According to the Dictionary of Natural Products on CDROM [43], some isorhamnetin diglycosides were previously isolated and identified from *P. boldus* leaves: isorhamnetin 3-*O*-

L-arabinopyranosyl-7-*O*-L-rhamnopyranoside with a MW 594; isorhamnetin 3-*O*-D-glucopyranosyl-7-*O*-L-rhamnopyranoside with a MW of 624; and isorhamnetin 3-*O*-(L-rhamnopyranosyl)-L-rhamnopyranoside with a MW 622 (synonym: isorhamnetin 3-di-rhamnoside) as well as kaempferol 7-methoxy-3-*O*-arabinosyl-3′-*O*-L-rhamnopyranoside with a MW of 578. Peak 46 could also be identified as a quercetin deoxyhexosyl-pentoside or an isorhamnetin pentosyl-deoxyhexoside with a MW = 594 u. However, the shape of the UV spectrum (λ max 264, 346) and an MS^3 ion at *m/z* 285, prompted the identification of this compound as an kaempferol hexosyl-rhamnoside (kaempferol rutinoside) as previously reported [36,40,41]. Peak 48 was identified as an isorhamnetin di-rhamnoside with a molecular weight of 608 u showing an $[M–H]^-$ ion at *m/z* 607 in the full scan spectrum and a daughter MS^2 ion at 461 ($[M–H\text{-rhamnose}]^-$ and MS^3 at 315 u $[M–H\text{-di-rhamnose}]^-$. Peak 52 was identified as a kaempferol rhamnoside (deprotonated molecule at *m/z* 431 and MS^2 at 285). Peak 49 was characteristic of quercitrin (quercetin 3-*O* rhamnoside, $[M–H]^-$ at 447 and MS^n ions at *m/z* 301, 179 and 151) [26]. The identity was confirmed with standard quercitrin (Rt, UV and mass spectra). Peak 50 exhibited a pseudomolecular ion mass of 477 u (aglycon + glucose), and a fragment mass at 315 u (MS^3 at 285, 179 and 151) characteristic of the aglycon isorhamnetin [23], identity confirmed with authentic standard, while peak 51 showed a neutral loss of 308 (rutinose) from the deprotonated molecule at *m/z* 623 and also a diagnostic isorhamnetin fragment (315 u). These two compounds were identified as isorhamnetin glucoside and isorhamnetin glucosyl-rhamnoside, respectively [15,29,40].

4. Conclusions

A very simple methodology to detect and identify simultaneously phenolic compounds in boldo leaves infusion is presented. Some 41 phenolic compounds were detected and identified in male and 43 in female *P. boldus* leaves collected in the VII Region of Chile by HPLC-DAD and ESI-MS–MS analysis. Nine quercetin glycosides, eight kaempferol derivatives, nine isorhamnetin glycosides, three phenolic acids and twenty one proanthocyanidins were identified for the first time in the crude drug. The corresponding aglycones (quercetin, isorhamnetin and kaempferol) are known natural antioxidants and this finding adds support to the observation that antioxidant and free-radical scavenging effect of the infusions is mainly related to the plant phenolics. Peaks 1, 13, 19, 23, 28, 39, 40, 45 and 46 were detected only in the male boldo leaves sample while compounds eluting as peaks 8, 10, 12, 18, 32, 35–38, 41 and 42 were detected only in female boldo leaves (Table 1). A complex isorhamnetin-tri-glycoside (isorhamnetin glucosyl-di-rhamnoside peak 43) was the most abundant flavonol glycoside in male boldo infusion, whereas isorhamnetin di-glucosyl-di-rhamnoside (peaks 41) was the most abundant in female boldo infusion (Table 1). However, more studies including a significant number of samples from different locations and altitudinal gradients all over the plant distribution area should be undertaken to disclose the variation in phenolic compounds from the plant as well as to confirm if there is any gender-specific compounds for the species. As almost all studies on phenolic compounds from boldo leaves were performed by spectrophotometry, total phenolic, flavonoid and tannin content were measured and very little has been previously done on the composition/profiling of the plant phenolics. The HPLC profiles reported in this work could be useful for the qualitative and quantitative analysis needed to set improved quality parameters for this important and worldwide used medicinal plant.

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