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Original Article

Determination of phenolic composition and antioxidant activity in fruits, rhizomes and leaves of the white strawberry (*Fragaria chiloensis* spp. *chiloensis* form *chiloensis*) using HPLC-DAD–ESI-MS and free radical quenching techniques

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ABSTRACT

A comparative analysis of methanol extracts from fruits, rhizomes and leaves of the Chilean white strawberry (*Fragaria chiloensis* spp. *chiloensis* var *chiloensis*) was performed by means of reversed phase high-performance liquid chromatography coupled to diode array detection and electrospray ionization mass spectrometry (HPLC–DAD and HPLC–ESI-MS). The total phenolic, total flavonoid and total anthocyanin content of the extracts was measured and compared. For the first time, some 18 phenolic compounds were tentatively identified in rhizomes and 18 in leaves of the Chilean strawberry. The products were mainly procyanidins, ellagitannins, ellagic acid and flavonol derivatives. The different extracts of the native strawberry presented antioxidant activity, which was close to that exhibited by the white fruits. The rhizomes and leaves proved to be a good source of phenolic antioxidants. The obtained information can be used to characterize the local cultivars by metabolite profiling and provide a reference HPLC fingerprint for future comparison of chemical changes associated to the plant response towards environmental factors and pathogens.

1. Introduction

In the southern part of South America, native berries were relevant as a food source for hunter-gatherers during the growing season. *Fragaria chiloensis* ssp. *chiloensis* f. *chiloensis* is a wild species of *Fragaria* endemic to southern Chile which produces light red or "white" strawberries. It is also one of the progenitors of the worldwide known commercial red strawberry (*Fragaria* × *ananassa* Duch.). The leaves and fruits of the Chilean strawberry have been used as food and medicine by the Mapuche aborigines in the Andean region in Chile (Retamales et al., 2005) and Argentina (Ladio et al., 2007), and were also gathered by the extinct Kawashkar culture in the channels and islands of Chilean Patagonia and Tierra del Fuego.

Berries are appreciated all over the world as a pleasant-tasting food often associated with medicinal or health-improving effects (Hancock et al., 2007). Berries such as strawberries (*Fragaria* × *ananassa*), cranberries (*Vaccinium macrocarpon*), raspberries (*Rubus idaeus*), blueberries (*Vaccinium corimbosum*) and cloudberries (*Rubus chamaemorus*) can be consumed raw or after processing as jams, ice creams, liquors and juices, and are a good source of phenolic antioxidants (Chen and Zuo, 2007; Määtä-Riihinen et al., 2003, 2004; Zuo et al., 2002b).

Several reports describe the chemical composition of different berries, mainly focusing on the identity of phenolic and flavonoid compounds and the antioxidant/free radical-scavenging effect of the extracts and isolated/identified compounds. Comparatively little is known about the chemistry of the native Chilean strawberry, and studies are urgently needed to support the efforts to develop this species as a crop (Retamales et al., 2005). The characterization of small molecules such as secondary compounds in crop plants can be used for metabolomic studies with high potential in food chemistry, food component analysis (Wishart, 2008) and to assess selected metabolites in the progeny from breeding programs (McDougall et al., 2008). The impact and

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perspectives of the metabolomic approach in plant science have been discussed by Sumner et al. (2003) as well as by Weckwerth and Fiehn (2002). An approach for the safety assessment of novel plant food (such as the native strawberry) has been proposed by Knudsen et al. (2008).

Previous studies of the fruits of *F. chiloensis* ssp. *chiloensis* f. *chiloensis* have shown the presence of 1-*O*-*E*-cinnamoyl-β-D-rhamnopyranoside, 1-*O*-*E*-cinnamoyl-α-xylofuranosyl-(1 → 6)-β-D-glucopyranose and 1-*O*-*E*-cinnamoyl-β-D-xylopyranoside, the aminoacid tryptophan, ellagic acid and cyanidin-3-*O*-glucoside (Cheel et al., 2005). The antioxidant properties of several cultivars of red strawberries are well known and were described by Asami et al. (2003) and Skupien and Oszmianski (2004), and we have reported a comparison of the antioxidant activity between the red strawberry cultivar Chandler and the white strawberry growing in the same location in Chile (Simirgiotis et al., 2009b).

Several analytical techniques such as high-performance liquid chromatography with UV–vis photodiode array detection (HPLC–DAD) (Chen et al., 2001; Zuo et al., 2002a), gas–liquid chromatography with flame ionization (GC–FID) (Zuo et al., 2008), gas–liquid chromatography coupled with mass spectrometry (GC–MS) (Chen and Zuo, 2007; Zuo et al., 2008, 2002b), capillary electrophoresis with electrochemical detection (CE–ED) (Peng et al., 2008) and capillary electrophoresis coupled to electrospray ionization time-of-flight mass spectrometry (CE–ESI-TOF-MS) (Gómez-Caravaca et al., 2008) have been used to detect, characterize and quantify phenolic compounds present in different food and medicinal plants.

Within this scenario, improved atmospheric pressure ionization (API) methods, especially electrospray ionization mass spectrometry (ESI-MS) coupled to HPLC, have enormously increased the range of natural polar and ionic species in solution amenable to mass spectrometry. Indeed, HPLC using tandem spectrometry (MS/MS) has provided important structural information in the analysis of natural products. In HPLC–DAD and electron spray ionization (ESI) analysis of a complex chemical mixture from a plant extract (e.g. containing flavonol glycosides, phenolic acids and tannins), the polar molecules are separated and detected either in their protonated $[M+H]^+$ or deprotonated $[M-H]^-$ forms, and the structures are characterized by co-chromatography with standard compounds. When the samples of natural products for HPLC analysis are unavailable and when the compounds detected have similar UV spectra, similar molecular ions and similar retention times, the structures of the compounds are characterized by MS–MS analysis. Although definitive structures of all compounds and chemotaxonomic markers in a plant extract forming the protonated or deprotonated molecules detected in the ESI-MS will require more refined analysis by extensive isolation of all the compounds combined with spectroscopy data, ESI tandem mass spectra provide clues to information about the nature of the compounds. The phenolic composition of fruit extracts from the forms *chiloensis* and *patagonica* of *F. chiloensis* was previously compared with that of the commercial strawberry *Fragaria* × *ananassa* cv. Chandler by HPLC–UV detection and mass spectrometry. The phenolic constituents in the three species were mainly proanthocyanidins, hydrolysable tannins, anthocyanins and flavonol glycosides (Simirgiotis et al., 2009b). However, the chemical composition of rhizomes and leaves of this native species has not been previously reported.

Following our studies on the chemistry of South American food plants (Simirgiotis et al., 2009a,b), we have analyzed extracts obtained from Chilean strawberry fruits, rhizomes and leaves by high-performance liquid chromatography with diode array detector coupled with electrospray ion-trap tandem mass spectrometry. In this study we report the phenolic compound composition of the different plant parts as well as the phenolic content, antioxidant properties and chromatographic fingerprints of the methanolic extracts of this native berry.

2. Materials and methods

2.1. Plant material

Rhizomes and leaves of wild growing *F. chiloensis* ssp. *chiloensis* f. *chiloensis* were collected at Las Trancas, Termas de Chillán, VIII Región, Chile, in March 2006. The ripe fruits were harvested in January 2006 in a commercial plantation located in Contulmo, Province of Arauco, VIII Region, Chile. Voucher herbarium specimens were deposited with the number 2865 at the Herbarium of the Universidad de Talca.

2.2. Chemicals

Methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile and formic acid from Merck (Darmstadt, Germany) were used. Ellagic acid was purchased from ChromaDex (Santa Ana, CA, USA). HCl, KCl, Folin-Ciocalteu phenol reagent, sodium acetate, aluminum chloride hexahydrate and sodium carbonate were from Merck (Darmstadt, Germany). Sephadex LH-20 (Pharmacia), thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany), Amberlite XAD-7 (20–60 mesh), diphenylborinic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH•), quercetin, gallic acid, (+) catequin, nitrobluetetrazolium (NBT), xanthine oxidase and hypoxanthine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solvents used for chromatography were HPLC grade.

2.3. Instrumentation

The HPLC system used for DAD analysis of extracts was Merck-Hitachi (LaChrom, Tokyo, Japan) equipment consisting of a L-7100 pump, a L-7455 UV diode array detector, and a D-7000 chromatointegrator. Mass spectra were recorded using an Agilent 1100 LC system connected through a split to an Esquire 4000 Ion Trap LC/MS system (Bruker Daltoniks, Germany). The extracts were dissolved in MeOH-formic acid (99:1) (approximately 3 mg/mL), and submitted to LC–MS. The volume injected was 20 μL. Full scan mass spectra were measured between m/z 150 and 2000 u in positive ion mode for anthocyanins and negative ion mode for other compounds. Nitrogen was used as nebulizer gas at 27.5 psi, 350 °C and at a flow rate of 8 L/min. The mass spectrometric conditions for negative ion mode 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; and the operating conditions for positive ion mode were: 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; capillary exit, 140.6 V. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas.

2.4. Extraction and sample preparation

The sample solutions for HPLC comparison purposes were prepared as previously reported (Simirgiotis et al., 2009b), with some modifications. Approximately 5 g of each freeze-dried plant part was homogenized in a blender with 50 mL MeOH-formic acid (99:1, v/v) and extracted for 1 h three times at room temperature. The fractions were collected; the solution was filtered and concentrated under reduced pressure to evaporate the solvent. Distilled water was added to ca. 10 mL and the solution loaded

onto an Amberlite XAD-7 column (5 cm × 2.5 cm). The column was rinsed with 50 mL distilled water and eluted with 50 mL MeOH-formic acid (99:1, v/v). The eluate was evaporated to dryness under reduced pressure below 40 °C to give 0.085, 0.76 and 0.45 g of extract for fruits, leaves and rhizomes of *F. chiloensis* spp. *chiloensis* f. *chiloensis*, respectively.

2.5. HPLC analysis

Approximately 1 mg of each extract obtained as explained above was dissolved in 1 mL MeOH:formic acid (99:1, v/v) filtered through a 0.45 μm filter and submitted to DAD and HPLC–MS analysis. The compounds were monitored at 254 nm, and UV spectra from 200 to 600 nm were recorded for peak characterization. For anthocyanins, monitoring was at 520 nm and the absorbance was measured between 200 and 600 nm. The HPLC analyses were performed using a linear gradient solvent system consisting of 1% formic acid (A) and acetonitrile (B) as follows: 90–75% A over 30 min; followed by 75–40% A from 30 to 45 min at a flow rate of 1 mL/min. The injected volume was 20 μL. A 250 mm × 4.60 mm i.d., 5 μm C18-RP Luna column (Phenomenex, Torrance, CA, USA) maintained at 25 °C was used.

2.6. Total phenolic, total flavonoid, and total anthocyanin content

For total phenolic (TP), total flavonoid (TF), and total anthocyanin content (TA), a solution of MeOH containing a precisely weighed amount (approximately 1 mg/mL) of each extract obtained as explained in Section 2.4 was used and absorbance was measured using a Heλios R V-3.06 UV/vis spectrophotometer (Unicam spectrometry, Cambridge, UK). The TFs in the samples were determined as previously reported (Simirgiotis et al., 2008). Quercetin was used as a reference for the calibration curve. The absorbance of the reaction mixture was measured at 415 nm. Results were expressed as g quercetin equivalents per 100 g dry weight. Data are reported as mean ± SD for at least three replications. The TPs were determined by the Folin and Ciocalteu's reagent method (Yildirim et al., 2001). Briefly, the appropriate extract dilution was oxidized with the Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 700 nm after 30 min. The calibration curve was performed with gallic acid, and the results were expressed as g of gallic acid equivalents per 100 g of dry weight. The Total anthocyanin content was determined by the pH-differential method (Lee et al., 2005). Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. Anthocyanin concentration is calculated and expressed as mg cyanidin 3-glucoside equivalents/100 g dry weight using the formula:

$$\text{TAC (cyanidin 3-glucoside equivalents, mg/100 g DW)} = \frac{A \times \text{MW} \times \text{DF}}{\varepsilon \times 1}$$

where A ($A510–A700$ nm) pH 1.0–($A510–A700$ 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.

2.7. Determination of antioxidant activity

All extracts obtained as described above were tested for their antioxidant activity using the following two methods.

2.7.1. DPPH• free radical-scavenging assay

The methanolic extracts were assayed for their ability to scavenge radicals in a 96-well microtiter-based DPPH• assay (Smith et al., 1987). All samples (0.6–2.4 mg) were reconstituted in 4 mL methanol to a final concentration of 600 μg/mL. This stock solution was serially diluted from 120 μg/mL to 7.5 μg/mL. DPPH• in methanol (400 μM) was combined with 50 μL test samples to a final well volume of 200 μL. Methanol was used as the negative control, and gallic acid was used as the positive control (IC_{50} = 4.39 ± 0.02 μg/mL). The reaction mixtures, in triplicate, were incubated for 30 min at 25 °C, and absorbance measured at 517 nm. Scavenging of DPPH• radical was evaluated by comparison with a negative control group. Calculated IC_{50} values denoted the concentration of sample required to scavenge 50% DPPH• free radicals. Antiradical DPPH• activity is expressed as IC_{50} in μg/mL. A lower IC_{50} value indicates greater antioxidant activity.

2.7.2. Superoxide anion assay

The enzyme xanthine oxidase is able to generate the superoxide anion by oxidation of reduced products from intracellular ATP metabolism. In this reaction, the xanthine oxidase oxidizes the substrate hypoxanthine generating superoxide anion, which reduces the NBT dye, leading to a chromophore with absorption maxima at 560 nm. Superoxide anion scavengers reduce the generation speed of the chromophore. The activity was measured spectrophotometrically as reported previously (Cheel et al., 2005) using a Genesys-10 UV scanning spectrophotometer. Extracts were evaluated at 50 μg/mL and expressed as percentage, calculated by linear regression analysis. Values are presented as means ± SD of three determinations. Quercetin was used as a reference compound (SA = 62.12% ± 4.20 μg/mL). The percentage of superoxide anion scavenging effect was calculated as follows:

$$\%\ \text{of scavenging activity} = \frac{E - S}{E} \times 100$$

where $E = A - B$ and $S = C - (B + D)$; A is the optical density of the control; B is the optical density of the control blank; C is the optical density of the sample; and D is the optical density of the sample blank.

2.8. Statistical analysis

Each experiment was repeated three times and the results reported are the means of the three trials ±SD. The program software Statistica version 7.0 for windows was used for the statistical analysis, using one way analysis of variance (ANOVA), and Duncan test at a level of significance $P < 0.05$.

3. Results and discussion

3.1. Phenolic composition of the white strawberry and identification of the phenolic compounds by HPLC-DAD–ESI-MS/MS

The combination of DAD and ESI-MS has been shown to be a powerful technique for the fast fingerprinting characterization of fruit extracts (Määtä-Riihinen et al., 2003), food-based products (de Pascual-Teresa et al., 2000), food plants (Vallejo et al., 2004) and food industry by-products (Sanchez-Rabaneda et al., 2003). HPLC-DAD–ESI-MS has also proved to be an excellent method for fast HPLC fingerprinting and analysis of red strawberry (Aaby et al., 2007; Lopes da Silva et al., 2007; Seeram et al., 2006) as well as white strawberry fruit poliphenols (Simirgiotis et al., 2009b). ESI-MS/MS have been used to identify ellagic acid (MW: 302, MS fragments at m/z: 179 and 151) and quercetin (MW: 302, MS fragments at m/z: 257 and 229) sugar derivatives in raspberries (Mullen et al., 2003) red strawberries (Aaby et al., 2007) and white strawberries (Simirgiotis et al., 2009b) since fragmentation of both types of compounds produce fragments at m/z 301 through loss of the sugar moiety.

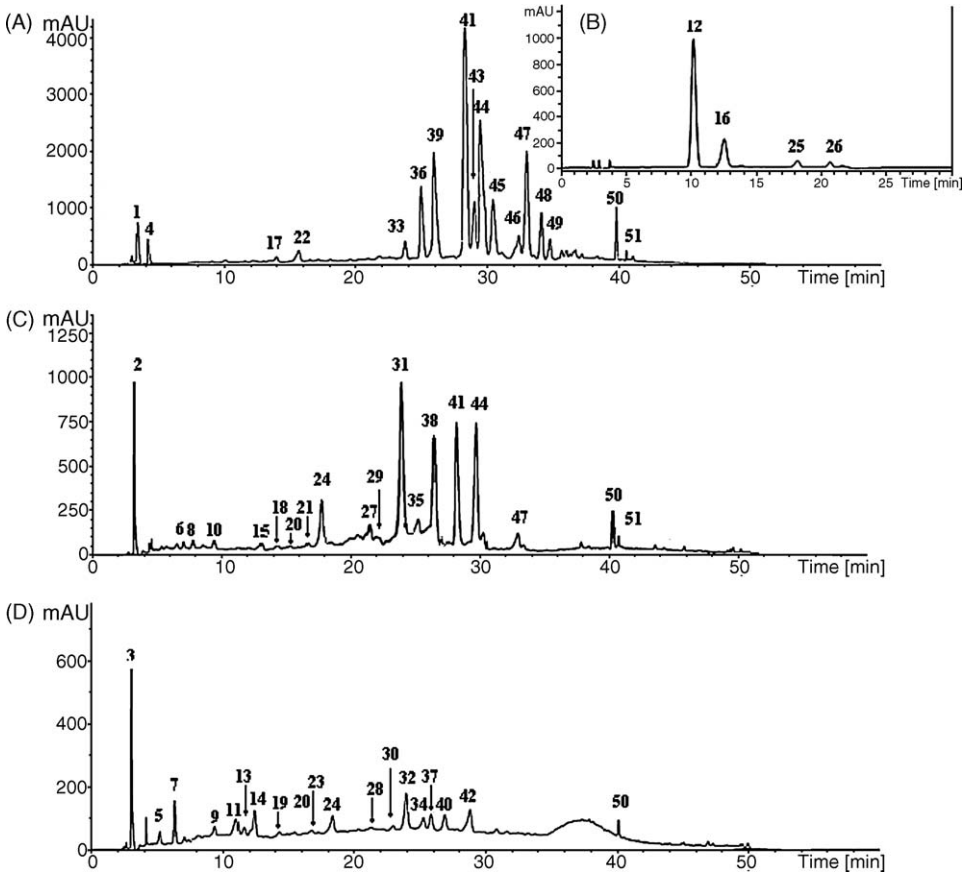

Fig. 1. HPLC–DAD chromatograms of *Fragaria chiloensis* ssp. *chiloensis* f. *chiloensis* MeOH extracts. (A) Fruits, detection at 254 nm, (B) fruits, detection at 520 nm, (C) leaves and (D) rhizomes, detection at 254 nm. Peak numbers refer to Table 1.

In the present work, phenolic compounds in methanol extracts from fruits, rhizomes and leaves of this ancient Mapuche berry were compared by HPLC with ESI tandem mass spectra and classified into three major groups: flavonols, tannins and anthocyanins. Identification was based on chromatographic behavior relative to authentic standards (for some compounds with peak spiking), UV-vis and mass spectral data, as well as by comparison with literature data. The use of low skimmer voltage allowed the detection of single charged molecular ions $[M+H]^+$ or $[M-H]^-$ and the formation of some diagnostic $[2M-H]^-$ artifact peaks. The LC–DAD chromatograms (Fig. 1) of the methanol extracts include all compounds detected, and Table 1 shows the list of the compounds identified with the UV–vis and MS properties, together with the plant part in which each compound was detected. In this comparison, some phenolic compounds were present in the three extracts corresponding to the three plant parts investigated (Table 1). Proposed structures of some of the compounds detected in *F. chiloensis* ssp *chiloensis* f. *chiloensis* plant and the corresponding fragmentations can be observed in Fig. 2. The identification of the compounds is explained below.

3.1.1. Anthocyanins

Anthocyanins present in strawberries are glycosides of cyanidin and pelargonidin in their protonated (flavylium) forms (Lopes da Silva et al., 2007). We have previously reported that cyanidin-3-*O*-glucoside (Fig. 2A) was the major anthocyanin in white strawberries (1.15 mg/100 g fresh weight) followed by pelargonidin-3-*O*-glucoside (0.40 mg/100 g fresh weight) (Simirgiotis et al., 2009b). In the present work no anthocyanins were identified in rhizomes and leaves (Fig. 1C and D). Table 1 shows the HPLC profile of the fruit extract at 520 nm, showing the four main anthocyanins (characterized as cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside cyanidin-malonyl-glucoside and pelargonidin-malonyl-glucoside, peaks 16, 17, 25 and 26, Fig. 1B) responsible in part of the antioxidant activity reported for the fruit (Simirgiotis et al., 2009b).

3.1.2. Tannins

Tannins present in plants are classified into condensed (proanthocyanidins: oligomers and polymers of flavan 3-ol monomer units) and hydrolysable compounds (gallic and ellagic acid or HHDP-based compounds). Even though some tannins including large molecules such as agrimoniin, sanguiin H-6, and various small ellagic acid derivatives have been identified in red strawberry fruits (Aaby et al., 2007; Seeram et al., 2006), red strawberry leaves (Hukkanen et al., 2007; Oertel et al., 2001) white strawberry fruits (Simirgiotis et al., 2009b), the diverse and complex nature of the structures made difficult the isolation and identification of all tannins. In this work, peaks 1–11, 13–15, 17, 21, 23, 24, 27, 29–41 and 46 were tentatively identified as tannins. Some large ellagitannins showed doubly charged quasi-molecular ions on electrospray ionization (Hukkanen et al., 2007; Mullen et al., 2003) However, in the present work all peaks were determined to be singly charged (enlargement of the signals showed that the separation between isotopic peaks was one m/z unit). Sixteen compounds present in rhizomes (peaks 3, 5, 7, 9, 11, 13, 14, 19, 20, 23, 28, 30, 32, 34, 37 and 40) and 4 compounds present in leaves of the white strawberry (peaks 8, 15, 20 and 24) were all identified as proanthocyanidins with different degrees of polymerization (trimers to pentamers) (Aaby et al., 2007; Karonen et al., 2004; Sudjaroen et al., 2005). Peak 17 occurring in fruits was

Table 1
Tentative identification of phenolic compounds in white strawberry (*Fragaria chiloensis* ssp *chiloensis* form *chiloensis*) fruits, rhizomes and leaves by HPLC–DAD, LC–MS and LC–MS/MS data.

Peak #	Rt (min)	λ_{max} (nm)	MW	$[M+H]^+$ or $[M-H]^-$	+/−Ions	$[2M-H]^-$	MS/MS ions	Tentative identification	*F. chiloensis* extract
1	2.9	236, 256	784	783	–		481, 301, 275	Bis-HHDP-glucose isomer	Fruits
2	3.0	235, 275	964	963	–		915, 783, 481, 301	Tetragalloylglucose isomer	Leaves
3	3.1	234, 279	866	865	–		577, 407, 289	Procyanidin trimer	Rhizomes
4	4.0	234, 275	784	783	–		481, 301,	Bis-HHDP-glucose isomer	Fruits
5	5.5	234, 279	866	865	–		577, 407, 289	Procyanidin trimer	Rhizomes
6	6.2	234, 273	784	783	–		481, 301,	Bis-HHDP-glucose isomer	Leaves
7	6.3	237, 278	1154	1153	–		865, 575, 407, 289	Procyanidin tetramer	Rhizomes
8	6.9	237, 278	1156	1155	–		865, 577, 407, 289	Procyanidin tetramer	Leaves
9	9.5	237, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Rhizomes
10	9.7	234, 275	784	783	–		481, 301,	Bis-HHDP-glucose isomer	Leaves
11	11.1	237, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Rhizomes
12	11.4	275, 512	448	449	+		287	Cyanidin-3-O glucoside	Fruits
13	11.8	237, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Rhizomes
14	12.3	234, 279	866	865	–		577, 407, 289	Procyanidin trimer	Rhizomes
15	12.5	237, 278	1154	1153	–		865, 561, 289	Procyanidin tetramer	Leaves
16	13.9	266, 504	432	433	+		271	Pelargonidin-3-O glucoside	Fruits
17	14.6	239, 278	290	289	–	579	123, 149, 221, 245	Catechin	Fruits
18	14.6	236, 255	934	933	–		631, 451, 301	Castalagin/vescalagin isomer	Leaves
19	14.7	237, 278	1154	1153	–		865, 561, 289	Procyanidin tetramer	Rhizomes
20	15.4	234, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Leaves, rhizomes
21	16.5	236, 255	934	933	–		631, 451, 301	Castalagin/vescalagin isomer	Leaves
22	16.5	275, 354	292	291	–	583	247, 191	Unknown	Fruits
23	16.8	237, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Rhizomes
24	18.0	237, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Leaves
25	18.5	276, 512	534	535	+		287, 449	Cyanidin-malonyl-glucoside	Fruits
26	20.9	266, 504	518	519	+		271, 433	Pelargonidin-malonyl-glucoside	Fruits
27	21.2	236, 255	936	935	–		633, 451, 301	Casuarictin/potentillin	Leaves
28	21.3	237, 278	1154	1153	–		865, 695, 577, 407, 289, 287	Procyanidin tetramer	Rhizomes
29	22.0	234, 255	1870	1869	–		1567, 935, 783, 301	Sanguiin H-6/lambertianin A	Leaves
30	23.0	234, 314	1442	1441	–		1151,1137, 863, 575, 289	Procyanidin hexamer	Rhizomes
31	23.6	234, 255	1870	1869	–		935, 783, 633, 433, 301	Agrimoniin isomer	Leaves
32	23.8	237, 278	1730	1729	–		1441, 1153, 865, 577, 433, 407, 289	Procyanidin hexamer	Rhizomes
33	24.5	235, 250	936	935	–		633, 301	Potentillin/casuarictin isomer	Fruits
34	25.0	234, 314	1442	1441	–		1151,1137, 863, 575, 289	Procyanidin pentamer	Rhizomes
35	25.3	235, 256	1870	1869	–		935, 783, 633, 433, 301	Agrimoniin isomer	Leaves
36	25.8	255, 356	434	433	–	867	301, 300, 257, 229	Ellagic acid pentoside	Fruits
37	25.7	234, 314	2003	1441	–		1151,1137, 863, 575, 289	Procyanidin hexamer	Rhizomes
38	26.0	233, 256	1870	1869	–		935, 783, 633, 433, 301	Agrimoniin isomer	Leaves
39	26.7	255, 358	448	447	–	895	357, 300, 257	Ellagic acid rhamnoside	Fruits
40	27.2	237, 278	1730	1729	–		1441, 1153, 865, 577, 433, 407, 289	Procyanidin hexamer	Rhizomes
41	28.1	255, 362	302	301	–		185, 229	Ellagic acid	Fruits, leaves
42	28.6	254, 351	436	435	–	871	303, 185, 179, 151	Quercetin pentoside	Rhizomes
43	29.8	254, 352	464	463	–	927	300, 271, 179, 151	Quercetin hexoside	Fruits
44	30.5	254, 351	478	477	–	955	301, 179, 151	Quercetin glucuronide	Fruits, leaves
45	31.3	254, 351	436	435	–	871	303, 185, 179, 151	Quercetin pentoside	Fruits
46	33.1	235, 349	934	933	–		451, 301	ellagitannin	Fruits
47	33.5	254, 351	434	433	–	867	300, 179, 151	Quercetin pentoside	Fruits, leaves
48	35.0	254, 346	462	461	–	923	285, 257, 163	Kaempferol glucuronide	Fruits
49	35.6	254, 355	492	491	–	983	315, 145, 177	Isorhamnetin-glucuronide	Fruits
50	40.2	254, 316	594	593	–	1187	285, 255	Kaempferol-coumaroyl-hexoside	Fruits, leaves, rhizomes
51	40.5	254, 319	594	593	–				
1187	284, 255	Kaempferol-coumaroyl-hexoside	Fruits						
52	40.8	242, 286	1138	1137	–		487, 797, 469	Unknown	Fruits

identified as the procyanidin monomer (+) catechin (Fig. 2K) (Simirgiotis et al., 2009b). Peaks 3, 5 and 14 showed a deprotonated molecule at *m/z* 865 and were identified as isomers of a procyanidin trimer with a MW of 866 due to characteristic MS^2 ions at *m/z* 577, 407 and 289. Peaks 7, 9, 11, 13, 15, 19, 20, 23, 24 and 28 had an $[M-H]^-$ ion at *m/z* 1153 and they were identified as isomers of a procyanidin tetramer with a MW of 1154 (Fig. 2I) due to MS^2 ions at *m/z* 865, 577, 407 and 289. Peaks 30, 34 and 37 had an $[M-H]^-$ ion at *m/z* 1441 and MS^2 ions at *m/z* 1151, 1137, 865, 577, 407 and 289 and were identified as procyanidin pentamers (MW: 1442). Peaks 1, 2, 4, 6, 10, 18, 21, 27, 33, 36, 39, 41, and 46 were identified as ellagic acid or HHDP (hexahydroxydiphenolic acid, the ellagic acid precursor) based compounds, since all of them produced after fragmentation a fragment at *m/z* 301, which matched that of authentic standard ellagic acid (Fig. 2H, MS fragments at *m/z* 283, 257 and 229). Co-elution and comparison of UV and mass spectra with authentic standard allow the identification of peak 41 as ellagic acid (MW: 302). Peaks 36 and 39 in the fruits were identified as ellagic acid pentoside and rhamnoside, respectively (Simirgiotis et al., 2009b). Peaks 6 and 10 present in leaves and peak 4 identified in fruits (Simirgiotis et al., 2009b) showed a $[M-H]^-$ at *m/z* 783 and MS^2 ions at *m/z* 481

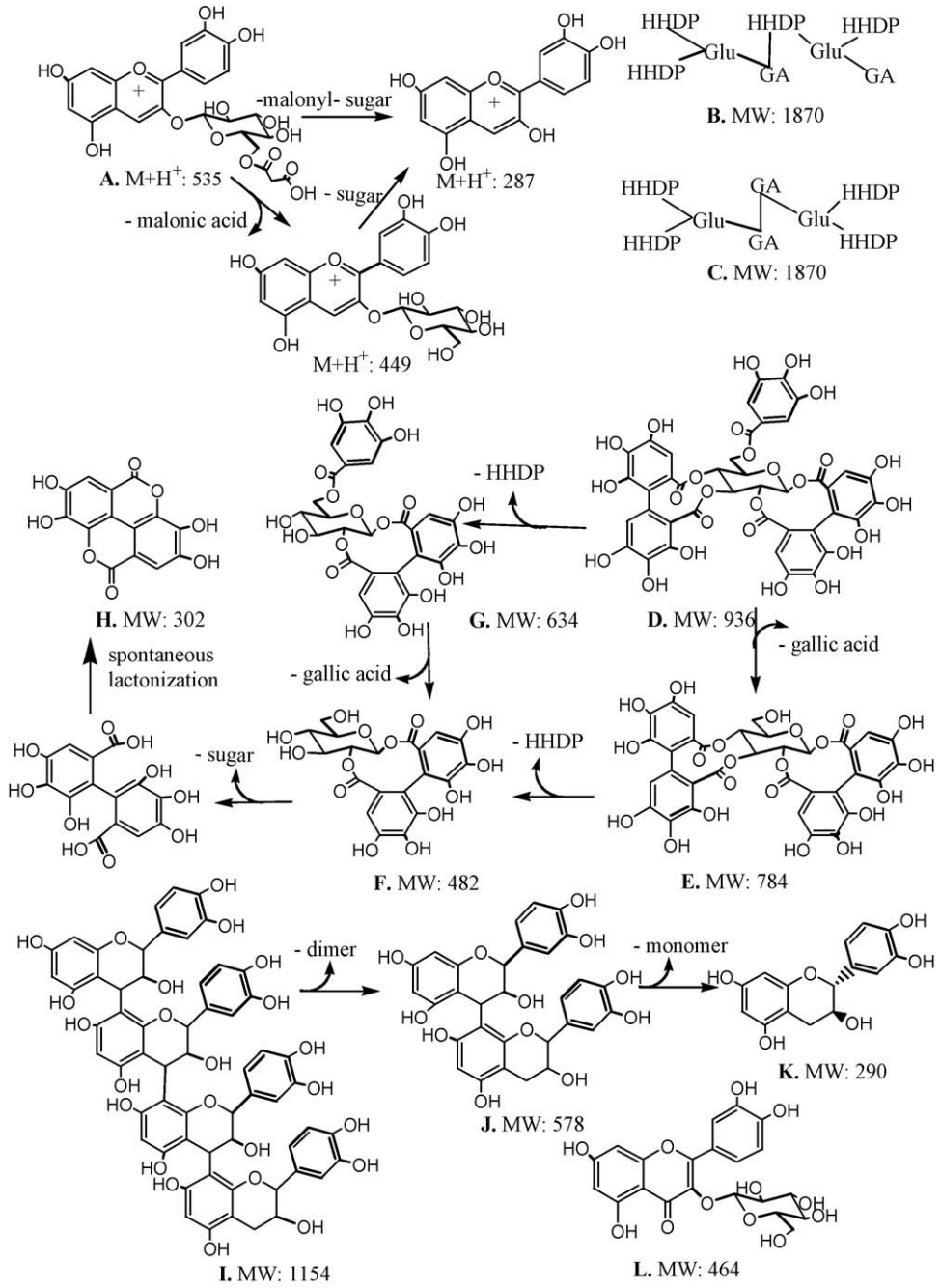

Fig. 2. Proposed structures of some selected compounds detected in *F. chiloensis* ssp. *chiloensis* f. *chiloensis* plant parts and the corresponding fragmentation patterns. HHDP: hexahydroxydiphenolic acid, GA: gallic acid, Glu: glucose, (A) cyanidin-malonyl-glucoside, (B) sanguiin H-6, (C) agrimoniin, (D) galloyl-bis-HHDP-glucoside, (E) bis-HHDP-glucoside, (F) HHDP-glucoside, (G) galloyl-HHDP-glucoside, (H) ellagic acid, (I) procyanidin tetramer, (J) procyanidin dimer, (K) catechin, and (L) quercetin glucoside.

(Fig. 2F, loss of HHDP), 301 (loss of HHDP-glucose) and MS^3 ion at *m*/*z* 301 (loss of glucose from the parent MS^2 ion at *m*/*z* 481), and were tentatively identified as isomers of a bis-HHDP-glucose (Fig. 2E) as reported in literature (Aaby et al., 2007; Hager et al., 2008; Seeram et al., 2006). Peaks 27 and 33 were identified as isomers of galloyl-bis-HHDP-glucose (Fig. 2D, casuarictin and potentillin) because both displayed molecular anions at 935, MS^2 ions at *m*/*z* 633 (Fig. 2G) (loss of 302 u, an HHDP unit) and MS^3 ions at *m*/*z* 301 (loss of 332 u, a galloyl-glucose unit) (Aaby et al., 2007). Peaks 18 and 21 were assigned as castalagin and its isomer vescalagin (MW: 934). They displayed both a [M−H]—at *m*/*z* 933, a MS^2 ion at *m*/*z* 631 (loss of HHDP), an MS^3 ion at *m*/*z* 451 (loss of glucosyl moiety) and a MS^3 ion at 301 (loss of galloyl-glucosyl moiety from the parent MS^2 ion at *m*/*z* 631 (Hager et al., 2008). The mayor peak (peak 31) in the HPLC–DAD spectra (Fig. 1) of the leaves as well as peaks 35 and 37 were identified as isomers of a galloyl-bis-HHDP-glucose dimer, agrimoniin (Fig. 2C). They showed an $[M-H]^-$ion at *m*/*z* 1869, an MS^2 ion at *m*/*z* 935 (loss of HHDP) and MS^3 ions at *m*/*z* 433 and 301, data which is in concordance with HPLC–MS data reports (Aaby et al., 2007; Okuda et al., 1992) and the MS data of a major compound detected in red strawberry leaves (Hukkanen et al., 2007; Oertel et al., 2001). Peak 29 detected in leaves was tentatively identified as sanguiin H-6 (Fig. 2B) or its isomer lambertianin A. It also displayed a pseudomolecular ion at *m*/*z* 1869, and characteristic MS^2 ion at *m*/*z* 1567 ($[M-H]^-$-HHDP) and MS^3 ions at 935, 783, and *m*/*z* 301,

Table 2
Free radical-scavenging activity measured by the DPPH• bleaching test, superoxide anion scavenging activity (SA) total anthocyanin (TA), total flavonoid (TF) and total phenolic content (TP) of MeOH extracts of the fruits, leaves and rhizomes of the white strawberry *Fragaria chiloensis* spp. *chiloensis* f. *chiloensis*.

Extract	Yield (%)	TP	TF	TA	SA (%)	DPPH•
Fruits	1.72	2.72 ± 0.01	2.00 ± 0.01	43.6 ± 0.01	79.3 ± 0.76	38.7 ± 0.80
Leaves	15.32	1.99 ± 0. 01	0.83 ± 0.01	0.00	67.60 ± 1.01	49.40 ± 0.81
Rhizomes	9.03	1.45 ± 0. 02	0.55 ± 0.00	0.00	55.0 ± 1.50	64.8 ± 1.54

All measurements are expressed as mean ± SD ($n = 3$). Yield expressed as g/100 g dry weight. Superoxide anion scavenging activity (SA) is presented as percent inhibition evaluated at 50 μg/mL. Antiradical DPPH• activity is expressed as IC_{50} in μg/mL. TA expressed as mg cyanidin 3-glucoside equivalents/100 g dry weight, TF expressed as g quercetin equivalents/100 g dry weight, TP as g gallic acid equivalents/100 g dry weight. All values in the same column are significantly different ($P < 0.05$).

which is consistent with previously published data for a compound detected in red strawberry (Seeram et al., 2006) and blackberry species (Mertz et al., 2007).

3.1.3. Flavonols

Peak 42 present in rhizomes and peak 47 present in leaves had a $[M-H]^-$ ion at m/z 435 and an artifact $[2M-H]^-$ ion at m/z 871. The molecular anion was subsequently fragmented giving a quercetin aglycone fragment at m/z 301, which fragmented further to produce MS^3 ions at m/z 185, 179 and 151. A compound with the same UV and MS spectral data but different retention time (peaks 45) was identified in the fruits as a quercetin pentoside (Simirgiotis et al., 2009b). Therefore, peaks 42 and 47 were also identified as quercetin pentosides. Peaks 43, 44, 48, 49 and 51 present in fruits were identified as quercetin hexoside (Fig. 2L), quercetin glucuronide, kaempferol glucuronide, isorhamnetin glucuronide (methyl-quercetin glucuronide) and kaempferol-coumaroyl-hexoside, respectively (Simirgiotis et al., 2009b). Peak 50 present in rhizomes and leaves was identified as a kaempferol-coumaroyl-hexoside (a $[2M-H]^-$ ion at m/z 1187 and a $[M-H]^-$ion at m/z 593, which fragmented to kaempferol MS ions at m/z 285 and 255) (Aaby et al., 2007).

3.1.4. Unidentified compounds

Peak 22 (t_R = 16.5 min, λ_{max} = 354 nm, $[M-H]^-$ion at m/z 291 and MS^2 fragments at m/z 247 and 191), and peak 52 (t_R = 40.8 min, λ_{max} = 286 nm, $[M-H]^-$ ion at m/z 1137 and MS^2 fragments at m/z 487, 797, and 469), occurring in fruits (Simirgiotis et al., 2009b) remain unidentified.

3.2. Total phenolic, flavonoid and anthocyanin content of F. chiloensis f chiloensis fruits, leaves and rhizomes

The methanolic fruit extract showed a total phenolic content of 2.72 g gallic acid equivalents (GAE)/100 g dry weight while the leaves and rhizome extracts presented values of 1.99 g and 1.45 g GAE/100 g dry weight, respectively. The extraction yield for the leaves and rhizomes (15.32 and 9.03%, respectively) was higher than that of the fruits (0.17%).

Total phenolic content of white strawberry leaves was close to those reported for *Fragaria* × *ananassa* Duch. cultivars Elsanta (1.95 g/100 g dry weight), Senga (2.07 g/100 g dry weight) and Dukar (1.95 g/100 g dry weight) measured after acid hydrolysis (Skupien and Oszmianski, 2004). The total flavonoid content presented the same trend as total phenolic and was higher in the fruit extract than in the leaves or rhizomes (Table 2).

Anthocyanins were detected only in fruits in low amounts: 43.6 mg per 100 g dry weight, corresponding approximately to 4.4 mg per 100 g fresh weight, measured by the pH differential method. The reported anthocyanin content of commercial red strawberries (*Fragaria* × *ananassa* Duch.) varies according to the cultivars as follows (mg per 100 g fresh fruits): Dukat (36.14), Elkat (30.9), Selva (32.8), Elsanta (25.26) and Kent (39.77) from Poland (Skupien and Oszmianski, 2004) and cultivars Camarosa (32.9) Carisma (26.7), Eris (22.5) Oso grande (34.5) and Tudney (52.5) cultivated in Spain (Lopes da Silva et al., 2007).

3.3. Antioxidant properties of F. chiloensis form chiloensis

The white strawberry fruit extract showed the highest superoxide anion scavenging effect at 50 μg/mL (79.3%). The value was similar to that found for leaves (67.60%) while lower activity was found for the rhizome extract (55%). Anthocyanin pigments do not contribute much to the antioxidant activity of the fruit due to the low content (43.6 mg c3g equivalents/100 g dry weight) in comparison with the anthocyanin amount reported in commercial red strawberries (Lopes da Silva et al., 2007; Simirgiotis et al., 2009b; Skupien and Oszmianski, 2004). The DPPH• radical-scavenging effect was higher in the fruits (IC_{50} = 38.7 μg/mL) and lower in the rhizomes extract (IC_{50} = 64.8 μg/mL) and can be related to the higher phenolic content of the fruit extract. The main phenolic compounds of the fruit were aglycone and glycosylated ellagic acid and flavonoids with known antioxidant/free radical-scavenging effect. The same products and related compounds have been detected and identified in other berries. Chen and Zuo (2007) and Chen et al. (2001) reported several phenolic acids, quercetin free and glycosylated as well as myricetin from cranberries. The ellagitannin composition of blackberry was determined by Hager et al. (2008) who also investigated the compounds occurring in different parts of the fruit.

Recent studies on the chemistry and antioxidant activity of the Chilean strawberry indicates that the main antioxidants from the fruits are anthocyanins, flavonoids and ellagic acid (Simirgiotis et al., 2009b), and that the identity and amount of fruit phenolics between the different forms of *F. chiloensis* (forms *chiloensis* and *patagonica*) allowed a clear chemical differentiation between the wild and commercial strawberry. A comparative study on the phenolic content and free radical-scavenging activity of the native Chilean strawberry, *F. vesca* and *F.* × *ananassa* cv. Chandler was reported by Cheel et al. (2007). The main antioxidant phenolics from *F. chiloensis* ssp. *chiloensis* were isolated and identified as E-cinnamic acid derivatives, cyanidin glycosides and ellagic acid (Cheel et al., 2005). Related products were also found in the commercial strawberry *Fragaria* × *ananassa* by Aaby et al. (2007). The genetic role on the chemistry and antioxidant activity of different strawberry cultivars was described by Tulipani et al. (2008). The authors outlined the relevance to obtain the chemical and antioxidant profiles of different genotypes to assess the differences between cultivars or accessions with implications in the diet and quality of the fruit.

4. Conclusions

The LC–DAD and ESI-MS/MS method employed was successfully applied to separate and identify the main and minor polyphenols from rhizomes and leaves and allowed HPLC comparison with the fruits of the native white strawberry. In this

report, 12 compounds were identified in fruits, 18 phenolic compounds were identified in rhizomes and a further 18 phenolic compounds were identified in the leaves of *F. chiloensis* spp. *chiloensis* form *chiloensis*, making a total of 52 different metabolites. Methanol extracts of the non-edible parts of the native strawberry (leaves and rhizomes) showed a complex composition and presented antioxidant activity, which was close to that exhibited by the extract of the edible fruits. The chemical information and analytical data obtained are relevant as a starting point for studies on the plant chemical changes elicited as a response against pathogens, growing under different environmental conditions and for metabolomic studies. The study of wild berries gathered by native South Americans opens new possibilities to develop selected species as new crops that contain phytochemicals little influenced by selection or commercial breeding, as outlined by Kraft et al. (2008) for some species consumed by native North Americans.

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