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Analytical Methods

Identification of phenolic compounds from the fruits of the mountain papaya *Vasconcellea pubescens* A. DC. grown in Chile by liquid chromatography–UV detection–mass spectrometry

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ABSTRACT

The quercetin glycosides rutin and manghaslin were isolated from the fruits of the mountain papaya *Vasconcellea pubescens* A. DC. grown in Chile by selective fractionation using the bleaching of the free radical scavenger 1,1-diphenyl-2-picrylhydrazyl (DPPH·) as the guiding assay. The structures were characterized by spectroscopic methods. Furthermore, 19 phenolic compounds were identified for the first time in the edible fruits by HPLC with UV and ESI–MS–MS detection. Ten of the compounds detected in the fruits and active fractions were tentatively characterized as hydroxycinnamic acid glycosides and nine as quercetin glycoside derivatives. The results provide relevant information on the low molecular weight constituents of this important fruit crop.

1. Introduction

The mountain or highland papaya *Vasconcellea pubescens* A. DC. (synonyms: *Carica pubescens* (A. DC.), *C. candamarsensis* Hook. F., *C. chiriquensis* Woodson and *V. cundinamarsensis* Linden) (Badillo, 2000; Van Droogenbroeck et al., 2002) is a tree belonging to Caricaceae and is native to South America. The plant grows 8–10 m in height with few branches and large leaves that bears yellow egg-shaped fruits 5–6 cm in diameter and 6–14 cm long with many seeds enclosed in the central cavity of the fruit. It is also called cold papaya because it grows in cooler climates than the popular and widely cultivated *Carica papaya* L. and could be found in valleys up to 2000 m above sea level. In South America, *V. pubescens* is distributed from Panama to Bolivia and is cultivated in Chile. The fruits, which are consumed after processing, are very popular and are used for the production of jams, preserves, cold drinks and cocktails (Idstein, Keller, & Schrejer, 1985; Moya-León, Moya, & Herrera, 2004). The fruits have a juicy yellow flesh with a strong and aromatic flavor, characterized by a high content of the proteolytic enzyme papaine, which is widely used in the industry as a meat tenderizer (Moya-León et al., 2004). Previous studies on the fruits of *V. pubescens* have shown the presence of 6′-*O*-malonylated *a*-D-glucopyranosides of benzyl alcohol, 2-phenylethanol and geraniol (Withopf, Richling, Roscher, Schwab, & Schreier, 1997) and a series of aliphatic 3-hydroxyesters as the volatile constituents responsible for the aroma of Chilean samples (Balbontın, Gaete-Eastman, Vergara, Herrera, & Moya-Leon, 2007), as well as several alcohols, aldehydes, ketones and glucosides of ethyl and butyl 3-hydroxybutanoates from samples collected in Chile (Idstein et al., 1985) and Colombia (Krajewski, Duque, & Schrejer, 1997; Morales & Duque, 1987). Several volatile compounds such as methyl *cis*-hex-3-enoate, isopentyl acetate, methyl 3-hydroxyhexanoate and ethyl nicotinoate were reported for the Chilean fruits that were not detected in the mountain papaya grown in Colombia (Morales & Duque, 1987). On spite of the relevance of this crop, the phenolic constituents of mountain papaya fruits have not been reported so far. HPLC coupled to diode array detector with mass spectrometry (DAD-MS–MS) has proved to be a very useful method in the identification of phenolics in several edible fruits extracts (Aaby, Ekeberg, & Skrede, 2007) as well as in the identification of complex glycosylated flavonoids (Vallejo, Tomás-Barberán, & Ferreres, 2004). Continuing our program to find active antioxidant phenolics from plants (Cheel, Theoduloz, Rodriguez, & Schmeda Hirschmann, 2005; Cheel et al., 2005) we investigated the constituents of the ripe fruits of *V. pubescens* and report herein the isolation of the main antioxidant compounds as well as the characterization of polyphenols ocurring in the MeOH fruit extract by HPLC–DAD and HPLC–MS.

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2. Materials and methods

2.1. Plant material

Ripe mountain papaya (*V. pubescens*) fruits were harvested in october 2007 in a commercial plantation located at Lipimavida, (34°51′S; 72°08′W), Provincia de Curicó, VII Region, Chile. The samples were immediately processed upon arrival to the laboratory. A voucher herbarium specimen is deposited at the Herbarium of the University of Talca under reference number 3143.

2.2. Chemicals

Methanol and 1-butanol were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Caledon Lab. (Georgetown, Canada), respectively. Aluminum-coated silica gel TLC plates, acetic acid and formic acid from Merck (Darmstadt, Germany). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, USA). Amberlite XAD-7, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH·), gallic acid and rutin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HCl, KCl, Folin–Ciocalteu phenol reagent, sodium acetate, aluminum chloride hexahydrate and sodium carbonate were from Merck (Darmstadt, Germany). The solvents used for chromatography were HPLC grade.

2.3. Extraction and isolation of phenolic compounds

2.3.1. Extraction

Fresh fruits (25 kg) were carefully washed, pulp and seeds manually separated to yield 17 kg of pulp, which was homogenized in a blender and extracted three times with MeOH (3 × 20 L each) at room temperature in the dark for 1 h per extraction. Extracts were filtered, combined and concentrated under reduced pressure below 50 °C. A representative sample from the initial extraction was lyophilized; the w/w yield in terms of fresh starting material was 3.4%. The resulting aqueous extract was diluted with distilled water to ca. 2 L, filtered and loaded onto an Amberlite XAD-7 column (33.5 × 6.8 cm). The column was rinsed with 2 L of distilled water and eluted with 2 L MeOH. The eluate was concentrated under reduced pressure and lyophilized to give 20.2 g (0.12% w/w yield) of a brown extract.

2.3.2. Isolation

The obtained extract was subjected to repeated permeation over Sephadex LH-20 (column length 60 cm, internal diameter 3 cm) using MeOH:H_2O 7:3 as eluent and 75 fractions of 10 mL each were collected. Fractions with similar TLC patterns were pooled and four subfractions (X-1 to X-4) were obtained. All four DPPH· bleaching active fractions (Table 1) were subjected to high speed centrifugal countercurrent chromatography (HSCCC). The HSCCC instrument used (P. C. Inc., Potomac, MD, USA) was equipped with a multilayer coil of 1.68 mm i.d. polytetrafluoroethylene (PTFE) tubing of approximately 240 mL with a total capacity of 320 mL. The revolution radius (R), was 10.5 cm, and the β value was 0.76 (β) r/R, where r is the distance from the coil to the holder shaft. The speed varied between 0 and 1200 rpm. The flow rate was controlled with a DC Analytic Gearmotor (Bodine Electric Company, Chicago, IL, USA). The sample was injected with a P. C. Inc., injection module with a 10 mL sample injection loop. The solvent system used was BuOH:EtOAc:H_2O (3:2:5, v/v/v). It was thoroughly equilibrated in a separation funnel at room temperature, and the two phases were separated shortly before use. The solvent system provided an ideal range of the partition coefficient (K) for the applied sample and a desirable settling time (28 s), 36% of the stationary phase was retained in the coil. First, the coiled column was entirely filled with the stationary phase (upper phase, 320 mL). Then, the apparatus was rotated forward at 800 rpm, while the mobile phase (lower phase) was pumped into the column in a head to tail direction at a flow rate of 2.5 mL/min. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 10 mL of filtered sample solution (dissolved in 5 mL of upper phase and 5 mL of lower phase) was injected through the injection module. The effluent of the column was continuously monitored by TLC on silica gel (mobile phase: BuOH:AcOH:H_2O, 80:20:4, v/v/v). The spots were observed under ultraviolet light (254 nm). HSCCC of fraction X-3 (5.24 g, 0.03% w/w yield), yielded 50 fractions of 8 mL each run and 532 mg of a compound labeled as peak 20 in the HPLC trace was obtained after 11 runs (elution volume 224–280 mL, peak 20, Table 2). From fraction X-2 (3.52 g, 0.02% w/w yield) HSCCC afforded 47 fractions of 8 mL each run which gave after seven runs 625.2 mg of a compound labeled as peak 17 in the HPLC trace (elution volume 176–216 mL, Table 2).

2.3.3. Unresolved fractions

TLC and HPLC UV–MS analysis of the unresolved fractions X-1 and X-4 revealed that they contained mainly waxes and hydroxycinnamic acid derivatives (peaks 6, 7, 9, 11 and 12, Table 2) and complex quercetin glycoside derivatives (peaks 14, 16, 18 and 19, Table 2), respectively, Fraction X2 also contained peaks 14 and 16, while fraction X3 contained also peaks 18 and 19. However, we were not able to isolate all of those minor polar compounds even using HSCCC with different solvent systems due to the low amount of the compounds in the extract and fractions and the hydrolysis of the acyl groups as in the case of the quercetin glycosides identified with peaks 16 and 18 in the HPLC trace, (Table 2, Figs. 6 and 7) as both compounds could easily loose the acyl groups to produce peak 20.

2.3.4. Identification of compounds

2.3.4.1. NMR analysis. The structures of purified compounds from *V. pubescens* fruits were determined by NMR. ^1H and ^{13}C, DEPT, COSY, NOESY, gHMQC, and gHMBC NMR spectra (g = gradient enhanced) of compounds identified wit peaks 17 and 20 were measured on a Bruker AMX spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C. All spectra were obtained in CD_3-OD with chemical shifts expressed in ppm and coupling constant (J) in Hertz (Hz).

2.3.4.2. LC–DAD and LC–ESI MS analysis. The Amberlite XAD-7 retained fraction of the MeOH fruit extract was dissolved in MeOH:H_2O:formic acid (7:3:0.1% v/v, aprox. 1.5 mg/mL) filtered through a 0.45 μm PTFE filter (Waters), and submitted to HPLC–

Table 1
Results of antiradical DPPH· bleaching activity, total flavonoid (TF) and total phenolic content (TP) of the MeOH extract, fractions and compounds 17 and 20 isolated from the fruits of *Vasconcellea pubescens* A. DC.

Fraction/compound	Yield	DPPH	TP	TF
MeOH	3400.3	114.52 ± 2.43	2.31 ± 0.32	0.34 ± 0.00
X	120.1	62.34 ± 2.02	15.68 ± 0.24	1.35 ± 0.01
X1	48.2	53.55 ± 2.10	26.62 ± 2.15	0.99 ± 0.00
X2	20.0	25.26 ± 0.69	41.56 ± 1.36	24.83 ± 0.03
X3	30.1	48.06 ± 1.44	18.96 ± 0.91	6.77 ± 0.02
X4	24.2	75.25 ± 2.12	4.62 ± 0.56	3.89 ± 0.01
17	2.7	56.05 ± 1.28	nt	nt
20	3.1	67.64 ± 2.32	nt	nt

All measurements are expressed as mean ± S.D. (n = 3). NT: Not tested. Yield expressed as mg/100 g fresh weight. Antiradical DPPH· bleaching activity is expressed as IC_{50} in μg/ml for extracts or fractions and μM for pure compounds. TF expressed as g quercetin equivalents/100 g dry weight, TP as g gallic acid equivalents/100 g dry weight. X = XAD7 retained fraction of the MeOH extract and X1–X4, fractions obtained in the fractionation. Seventeen manghaslin; 20, rutin.

Table 2
Tentative identification of phenolic compounds in mountain papaya fruits by LC-DAD, LC-MS and MS/MS data.

Peak #	Rt_t (Min)	λMax (nm)	MW	$[M-H]^-$ (m/z)	$[2M-H]^-$ (m/z)	MS/MS ions (m/z) (%)		Tentative identification
						MS^2	MS^3	
1	6.5	234, 293sh, 324	966	965		947 (100), 643 (30)	625 (100), 539 (25), 480 (25)	Unknown
2	18.1	234, 293sh, 324	372	371	743	209 (100), 191 (30)	191 (100), 163 (10), 132 (10)	Hydroxy-FA-hexoside
3	21.9	234, 293sh, 326	372	371	743	209 (100), 191 (30)	191 (100), 163 (10), 132 (10)	Hydroxy-FA-hexoside
4	25.0	234, 293sh, 324	372	371	743	209 (100), 191 (30)	191 (100), 163 (10), 132 (10)	Hydroxy-FA-hexoside
5	29.6	234, 293sh, 326	372	371	743	209 (100), 191 (30)	191 (100), 163 (10), 132 (10)	Hydroxy-FA-hexoside
6	31.2	234, 293sh, 324	356	355	711	209 (50), 191 (100)	191 (100), 163 (10), 145 (30)	Hydroxy-FA-hexoside
7	35.6	234, 293sh, 322	386	385	771	223 (100), 205 (20), 191(20)	205 (100), 191 (25) 129 (20)	Sinapic acid-hexoside
8	36.7	234, 293sh, 324	356	355	711	209 (50), 191 (100)	191 (100), 163 (10), 145 (30)	Hydroxy-FA-rhamnoside
9	37.5	234, 293sh, 324	386	385	771	223 (30), 205 (3), 191(100)	205 (100), 191(20)	Sinapic acid-hexoside
10	40.0	254, 351	918	917		755 (100)	591 (50), 300 (100), 271 (30), 179 (10), 151 (10)	Q-3-*O*-(2′ rhamno)-rutinosyl-7-*O*-hexoside
11	40.9	234, 293sh, 328	386	385		223 (100), 205 (20), 191(20)	205 (100), 191 (25) 129 (20)	Sinapic acid-hexoside
12	42.5	254, 351	788	787		625 (100)	301 (100), 271 (30), 179 (5), 151 (5)	Q-3-*O*-(2' hexo)-hexosyl-7-*O*-hexoside
13	44.5	240, 291sh, 326	386	385	771	223 (30), 205 (3), 91(100)	205 (100), 191(20)	Sinapic acid-hexoside
14	45.6	256, 291sh, 317	918	917		755 (100)	609 (20), 591 (30), 300 (100), 271 (30), 179 (10), 151 (10)	Q-3-*O*-(2' coumaro)- rutinosyl-7-*O*-hexoside
15	53.6	254, 351	772	771		609 (20), 301 (100)	301 (100), 179 (5), 151 (5)	Q-3- *O* -rutinosyl-7- *O* -hexoside
16	54.8	254, 291sh, 326	772	771		609 (20), 591 (30) 301 (100)	301 (100), 271 (30), 179 (10), 151 (10)	Q-3- *O* -(2' caffeoyl)-rutinoside
17	60.7	254, 302sh, 355	756	755	1511	591(30), 300 (100)	301 (100), 271 (20), 179 (5), 151 (5)	Q-3- *O* -(2' rhamnosyl)-rutinoside (manghaslin)*
18	61.7	252, 291sh, 317	756	755		609 (5), 591 (30), 300, (100)	301(100), 271 (30), 179 (20), 151 (5)	Q-3- *O* -(2' coumaroyl)-rutinoside
19	62.9	254, 290sh, 349	626	625		301 (100)	271 (20), 179 (10), 151 (10)	Q-3- *O* -(2' hexosyl)-hexoside(Q-3-*O*-sophoroside)
20	68.7	254, 302sh, 351	610	609	1219	301 (100)	271 (20), 179 (10), 151 (10)	Q-3-*O*-rutinoside (rutin)*

* Structure unambiguously determined by 1 D and 2 D NMR spectroscopy. Q, quercetin; FA, ferulic or isoferulic acid; sh, shoulder in the spectrum.

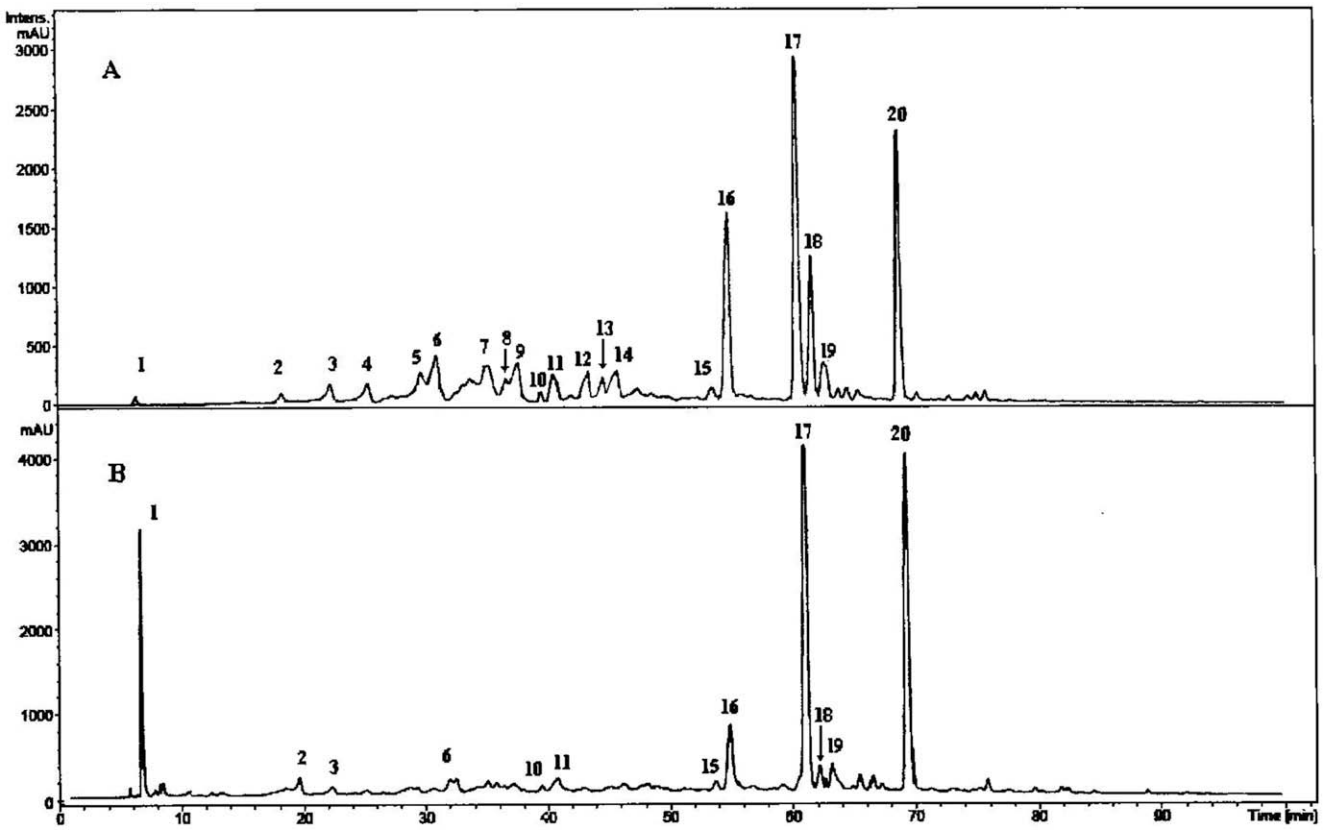
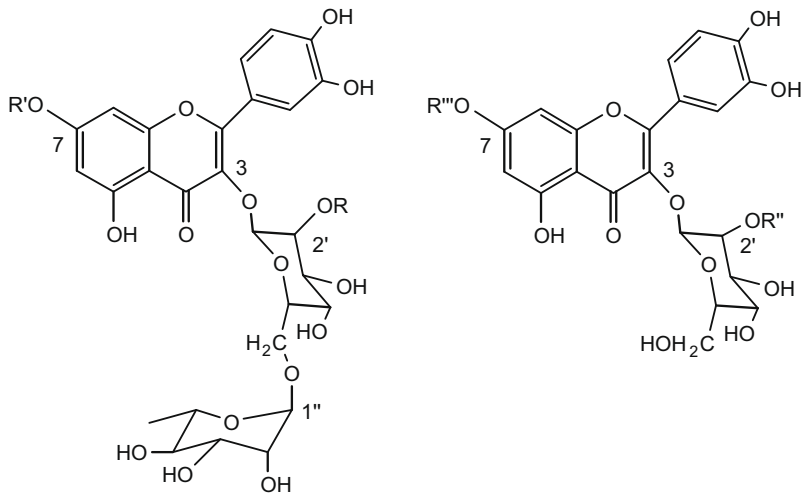


Fig. 1. HPLC–DAD chromatogram of mountain papaya fruit MeOH extract at 320 nm (A) and 254 nm (B).



10. R =α- Rhamnose, R' = Hexose
14. R = Coumaric acid, R' = Hexose
15. R = H, R' = Hexose
16. R = Caffeic acid, R' = H
17. R =α- Rhamnose R' = H
18. R = Coumaric acid, R' = H
20. R = R' = H

12. R" = hexose, R"' = H
19. R" = R"' = hexose.

Fig. 2. Proposed structures of flavonol derivatives tentatively identified in mountain papaya fruits cultivated in Chile. HPLC peaks 10, 12, and 14–20. Compounds identified with peaks 17 (manghaslin) and 20 (rutin) were unambiguously identified by NMR spectroscopy.

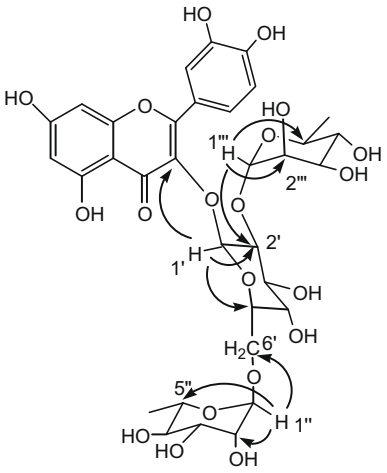


Fig. 3. Important HMBC correlations for Manghaslin (peak 17).

DAD and HPLC–ESI–MS analysis. HPLC–DAD analysis was performed using a Merck–Hitachi (LaChrom, Tokio, Japan) equipment consisting of an L-7100 pump, an L-7455 UV diode array detector, and D-7000 chromatointegrator. The compounds were monitored at 256 and 320 nm, and UV spectra from 200 to 600 nm were recorded for peak characterization. HPLC coupled to mass spectrometry was performed using a Squire 4000 plus ion trap spectrometer fitted with electrospray ionization (Bruker Daltonics, Billerica, MA, USA). The capillary voltage was 4000 V. Nitrogen was used as nebulizer gas at 350 °C at a flow of 8.0 L min^{-1}, nebulizer pressure was 27.5 psi. Spectra were recorded between *m/z* 150 and 2000 in negative ion mode. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas.

HPLC–DAD and HPLC–MS analysis were carried out using a 250 mm × 4.60 mm i.d., 5 μm C18-RP Luna column (Phenomenex, Torrance, CA, USA) maintained at 25 °C with a linear gradient solvent system consisting of 1% formic acid (A) and MeOH (B) as follows: 90% to 75% A over 60 min; followed by 75–40% A from 60 to 90 min at a flow rate of 0.5 mL/min. The injected volume was 20 μL.

2.3.4.3. Alkaline hydrolysis. In order to verify the acylation of the quercetin glycoside derivatives identified with peaks 14, 16 and 18 in the HPLC trace, 3 mg of the Amberlite XAD-7 retained fraction was dissolved in 2 ml of MeOH: H_2O 7:3% v/v and saponificated with 2 mL of sodium hydroxide 2 N. The mixture was kept for 16 h at room temperature, acidified with concentrated hydrochloric acid (up to pH 1–2), filtered and directly analyzed by HPLC–DAD and ESI–MS–MS as suggested by Ferreres et al. (2008).

2.4. Antioxidant activity – DPPH· scavenging

The mountain papaya fruits MeOH extract, fractions and purified compounds were tested for their ability to scavenge radicals in a 96-well microtiter-based DPPH· assay (Yang et al., 2003). 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 volume of 200 μl. Methanol was used as the negative control, and gallic acid was used as the positive control (IC_{50} = 25.2 μM). The reaction mixtures, in triplicate, were incubated for 30 min at 25 °C, and absorbance measured at 517 nm in a Biotek Instruments Inc., Universal Microplate Reader (ELX 800, Winooski, VT, USA). Scavenging of DPPH· radical was evaluated by comparison with a negative control group (dissolved sample without DPPH·). Calculated IC_{50} values indicate the concentration of sample required to scavenge 50% DPPH· free radicals. DPPH· bleaching activity is expressed as IC_{50} in μg/ml for extracts or fractions and μM for pure compounds.

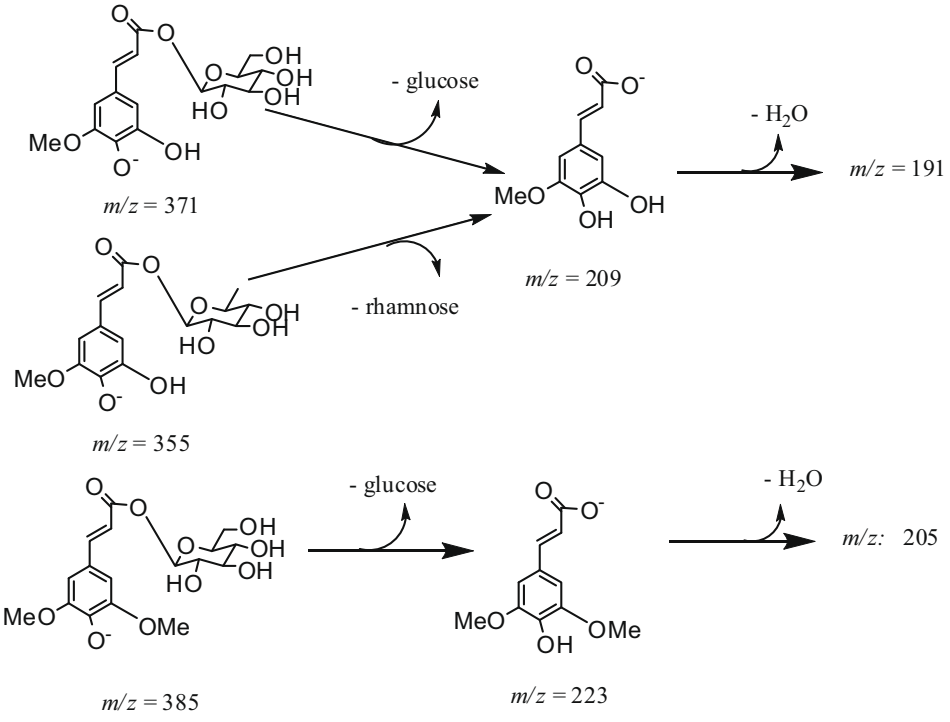


Fig. 4. Proposed fragmentation of cinnamic acid derivatives (*m/z* = 371, 355 and 385) tentatively identified in mountain papaya fruits cultivated in Chile.

2.5. Total phenolic (TP) and total flavonoid (TF) content

For total phenolic (TP) and total flavonoid (TF) content, a solution of 10 mL MeOH containing a precisely weighed amount (aprox. 1 mg/mL) of each extract or fraction was used. The TFs in the samples was 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, Mavi, & Kara, 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 using a Heλios UV/vis spectrophotometer (R V-3.06, Unicam, Germany). 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.

3. Results and discussion

3.1. Isolation and characterization of phenolic constituents of mountain papaya fruits

Two quercetin glycosides were isolated from a MeOH extract of mountain papaya fruits cultivated in Chile using the bleaching of the DPPH· radical as guiding assay. The compounds were identified by ^{1}H NMR, ^{13}C NMR, 2 D NMR and ESI–MS–MS. Furthermore, HPLC with DAD detection and ESI-MS^{n} was used to generate a phenolic profile of the MeOH fruit extract. Fig. 1 shows the HPLC–DAD traces of the extract recorded at 320 nm (A) and 254 nm (B). Table 2 shows the identification of the phenolic compounds labeled as peaks 1–20 following the elution order in the HPLC–DAD chromatograms. Fig. 4 shows the proposed fragmentation while Fig. 5 shows full ESI scan mass spectra of cinnamic acid derivatives (*m/z* = 371, 355 and 385). Fig. 6 shows the proposed fragmentation while Fig. 7 shows full scan ESI mass spectra of quercetin derivatives, peaks 15–18 and 20 identified in mountain papaya fruits cultivated in Chile.

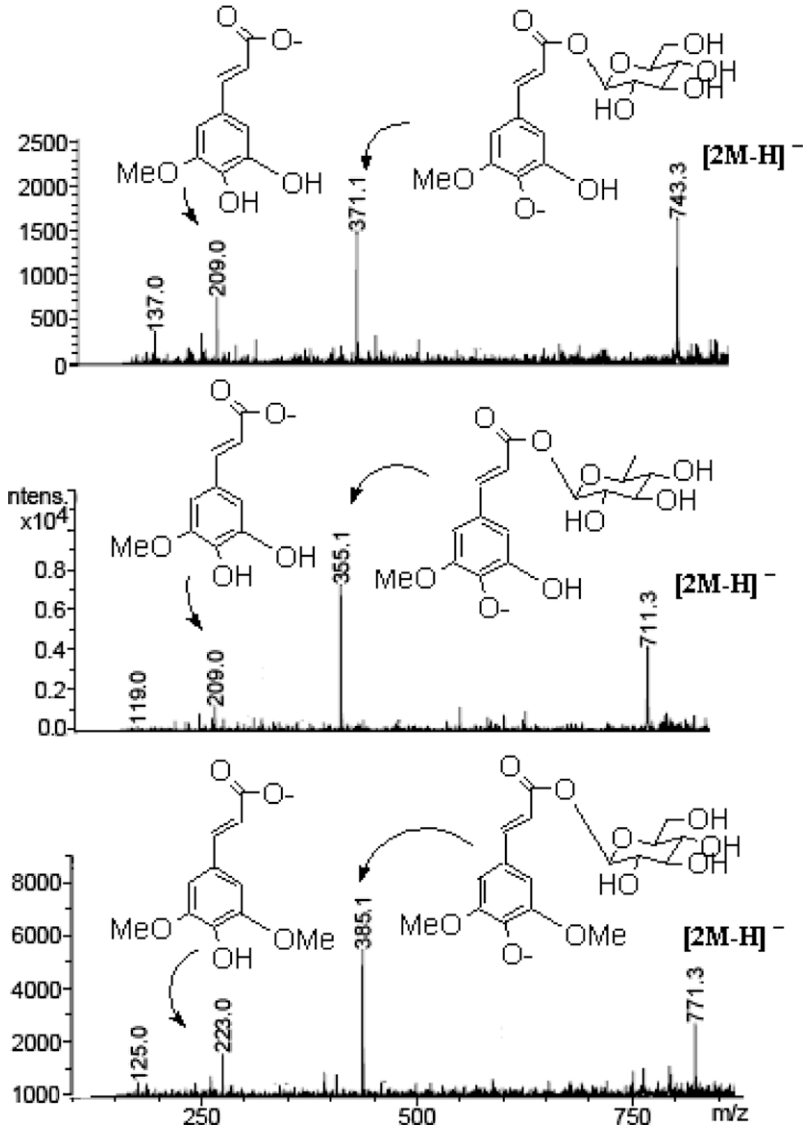


Fig. 5. Full scan ESI mass spectra of cinnamic acid derivatives (*m/z* = 371, 355 and 385) tentatively identified in mountain papaya fruits cultivated in Chile.

Peak 20 was identified as quercetin 3-*O*-α-rhamnopyranosyl (1→6)-β -glucopyranoside (rutin, Table 2, Figs. 2 and 7) by TLC, DAD–HPLC (*R*t and UV spectrum) and HPLC–ESI–MS comparison ($[M-H]^-$ ion at *m/z* = 609, MS^2 ion at *m/z* 301), with a standard sample of rutin. The structure was confirmed by NMR spectroscopy.

Peak 17, ($[M-H]^-$, *m/z* 755, Figs. 2 and 7), 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 mayor MS^2 ion at *m/z* 300 (M–H- 448) and MS^3 fragments at *m/z* = 179 and 151, characteristic of quercetin. This analysis also showed that compound 17 contained one additional deoxyhexose sugar unit (MS^2 ion at *m/z* 591$[M-H-164]^-$). The loss of the deoxyhexose moiety as an entire sugar molecule (164 u) rather than a residue (146 u for deoxyhexose) suggested that this additional sugar moiety could be linked at C-2 of the primary sugar, as previously reported for flavonols bearing a branched triglycoside at C-3 with glucose or galactose as the primary sugar (Kite, Stoneham, & Veitch, 2007). The ^1H NMR and ^{13}C NMR spectra of 17 were similar to that of rutin, but revealed a set of additional resonances corresponding to the third sugar moiety, including an anomeric proton signal at δ 5.23 d (1H, *J* = 1.7 Hz, δ_C = 100.85) and a methyl group signal at δ 1.27 d (*J* = 6.4 Hz, δ_C 16.42), that were attributed to an additional α-rhamnose unit. The assignment of all the resonances of the sugar units was accomplished by detailed examination of its 1D (^1H, ^{13}C and DEPT) and 2D NMR (COSY, NOESY, HMBC, HMQC) spectra. The 0.3 ppm downfield shift for the anomeric proton (H-1′: δ5.61, *J* = 7.63 Hz, δ_C = 101.23) of β-glucose in the ^1H NMR spectrum of compound 17 compared to that of rutin (H1′: δ5.32, *J* = 7.62 Hz, δ_C = 101.02), the correlation between H-1‴ and the carbon signal at δ 79.0 (C-2′) and the crosspeaks between H-2′ and C-3′, C-2′ and C-1‴ in the HMBC spectrum of compound 17 (Fig. 3) confirmed the substitution of the additional α-rhamnose at C-2′ of glucose. Therefore, the structure of the compound has been established as quercetin 3-*O*-α-rhamnopyranosyl (1→2) [α-rhamnopyranosyl (1→6)]-β-glucopyranoside. This structure was previously reported and elucidated by UV, IR spectroscopy and MS spectrometry of chemical derivatives and named manghaslin (peak 17 in the HPLC trace, Table 2, Figs. 2 and 7, Sakushima, Hisada, Ogihara, & Nishive, 1980). The isolated quercetin glycosides are known antioxidants (Joubert, Winterton, Britz, & Ferreira, 2004; Quiu, Chen, Pei, Matsuda, & Yoshikawa, 2002), and rutin is a common flavonoid present in many edible fruits and vegetables. In this study, total phenolic, total flavonoid content and free radical scavenging effect of the XAD-7 retained fraction and fractions X1–X4 as well as the DPPH· bleaching activity of the pure compounds (peaks 17 and 20) obtained in the chromatography of the MeOH extract from the fruits of *V. pubescens* are shown in Table 1. Fractions X2 and X3 showed remarkable scavenging activity (25.2 and 48.2 μg/mL, respectively) in the DPPH· assay. The TPs were in the order: fraction X2 > X1 > X3 > X4, and the TFs in the order: fraction X2 > X3 > X4 > X1.

3.2. Identification of phenolic compounds by UV-DAD and ESI-MS^n spectra

Ten of the compounds detected in the HPLC–DAD chromatogram (Fig. 1) were identified as hydroxycinnamic acid sugar derivatives (Table 2, Figs. 4 and 5) and nine as quercetin glycoside derivatives (Table 2, Figs. 6 and 7). Peaks 2–5 showed all a deprotonated molecular ion at *m/z* 371 and a $[2\ M-H]^-$ ion at *m/z* 743 and were tentatively identified as isomeric forms of cinnamic acid sugar derivatives (MW = 372), (Mansouri, Embarek, Kokkalouc, & Kefalas, 2005), substituted with one CH_3O– and two HO– groups in the aromatic ring (hydroxy-ferulic or hydroxy-isoferulic acid). They yielded MS^2 ions at *m/z* 209 ($[M-H-162]^-$) and *m/z* 191 ($[M-H-162-H_2O]^-$) characteristic of a loss of a hexose sugar unit. The different retention times of these compounds (18.1, 21.9,

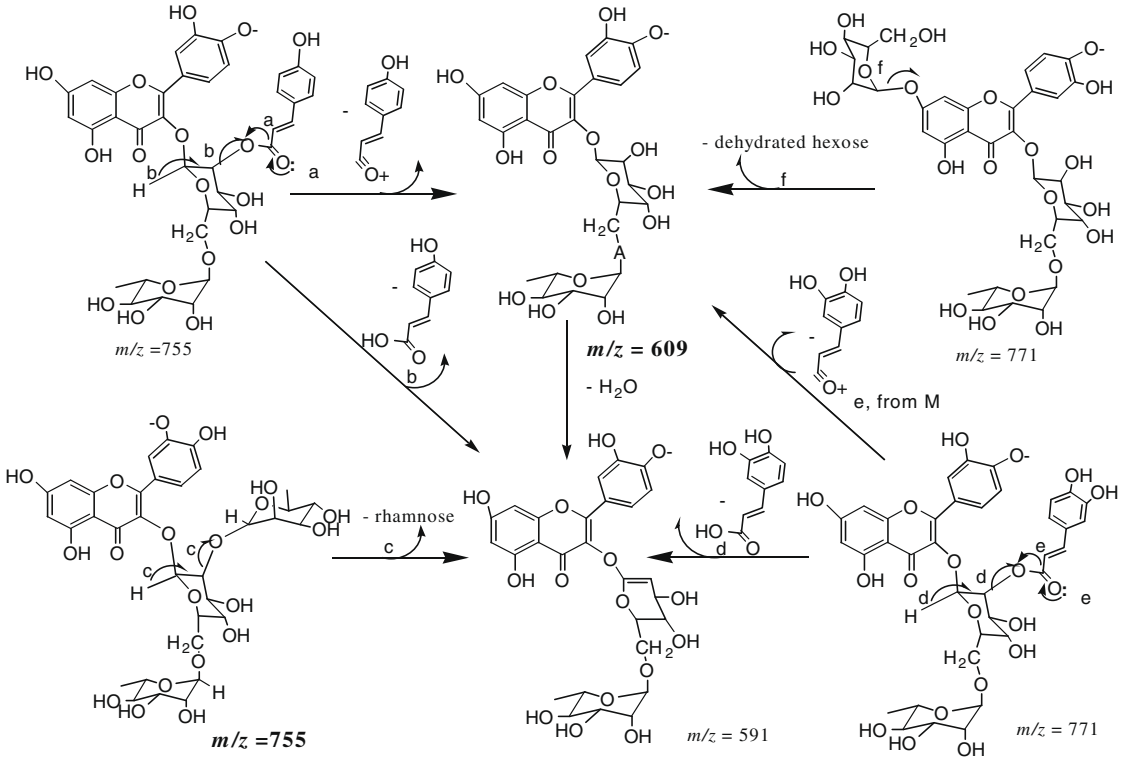


Fig. 6. Proposed fragmentation (a–f) of quercetin derivatives (peaks 10, 12, 14–20) tentatively identified in mountain papaya fruits cultivated in Chile. Two of the structures: *m/z* = 609 (compound 20) and 755 (compound 17), shown in bold, were elucidated by NMR spectroscopy as rutin and manghaslin, respectively.

25.0, and 29.6 min, respectively, Table 2) could be associated to differences in the point of attachment of the CH_3O– and OH groups to the cinnamoyl-hexoside structure and/or the identity of the sugar moieties. Peaks 6 and 8 (λ_{max} = 234, 293sh, 324) showed both a $[M–H]^-$ ion at *m/z* = 355 and a $[2\ M–H]^-$ ion at *m/z* 711 which matches a deprotonated molecular ion for glucoferulic acid, $C_{16}H_{20}O_9$, (MW = 356, Belles et al., 2008). However, these two compounds were identified as cinnamic acids substituted with one

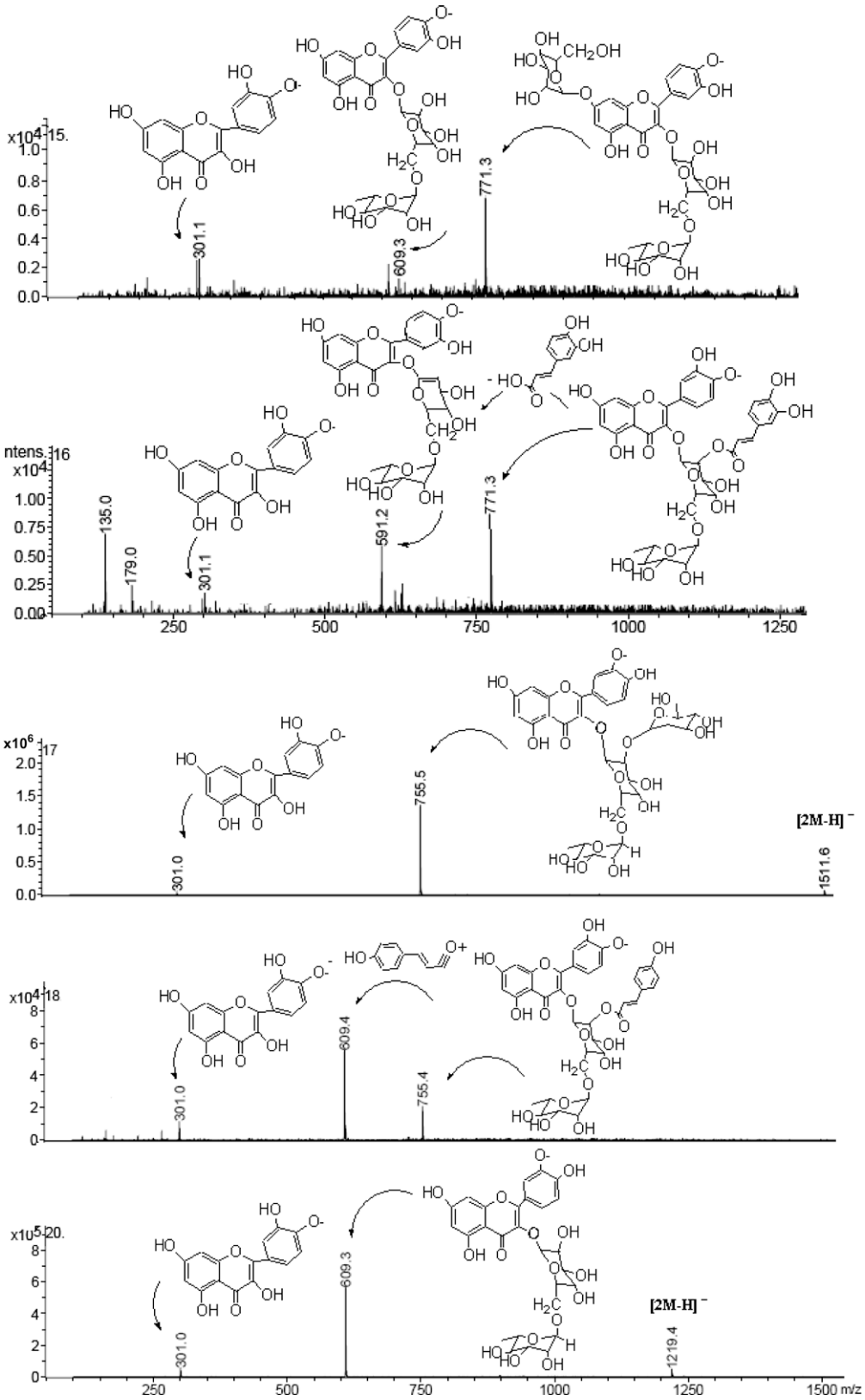


Fig. 7. Full scan ESI mass spectra of the quercetin derivatives, peaks 15–18 and 20 tentatively identified in mountain papaya fruits cultivated in Chile.

CH_3O– two OH groups (hydroxyferulic acid) and one rhamnose moiety due to MS^2 ions at *m/z* = 209 ([M–H- deoxihexose]$^-$) and 191([M–H- deoxihexose- H_2O]$^-$. Similarly, peaks 7, 9, 11 and 13 showed [M–H]$^-$ ions at *m/z* 385 and adduct [2 M–H]$^-$ ions at *m/z* 771 that could be assigned as isomeric forms of ferulate derivatives (MW = 386) of galactaric acid, glucaric acid or altraric acid (Lin, Harnly, Pastor-Corrales, & Luthria, 2008; Risch, Hermann, Wray, & Grotjahn, 1987) However, they were identified as cynnamoyl hexosides bearing two CH_3O– and one OH group in the aromatic moiety (sinapic acid) due to MS^2 ions at *m/z* = 223 ([M–H- hexose]$^-$) and 205 ([M–H- hexose- H_2O]$^-$, which is in concordance with the data reported for sinapic acid glucoside (Ferreres et al., 2006). The proposed MS fragmentation pattern of these hydroxycinnamic derivatives is shown in Fig. 4. Peak 1 could be also identified as a hydroxycinnamic acid derivative (λ_{max} = 234, 293sh, 324) but the tentative structure remains unknown.

Peaks 10, 12, 15, 17, 19 and 20 (Table 2) were tentatively identified as flavonoid glycoside derivatives. MS–MS experiments of all of these compounds produced fragments at *m/z* 179 and 151 which matches the fragmentation pattern of quercetin (Aaby et al., 2007). Tentative identification of these compounds was based on the analysis of the UV and ESI-MS^n spectra and comparison with those of the quercetin glycosides rutin and manghaslin (peaks 17 and 20, R_t = 60.7 and 68.7 min, respectively, Table 2), unambiguously identified by NMR spectral data. Peak 15 had a quasi-molecular ion at *m/z* 771 in the ESI–MS spectrum, suggesting that an additional hexose unit is attached to the rutin skeleton. The shape of the UV spectrum and the loss of 162 u from the [M–H]$^-$ ion by elimination of a dehydrated hexose (ion at *m/z* 609) instead of an entire moiety as in the MS^2 spectrum of compound 17 suggested the additional *O*-glycosylation at C-7 (Ferreres et al., 2008; Kite et al., 2007; Mabry, Markham, & Thomas, 1970; Vallejo et al., 2004) Thus, peak 15 was tentatively identified as a quercetin 3-*O*-rutinosyl-7-*O*-hexoside. Tiberti et al. have reported the identification using HPLC UV–ESI-MS^n of two quercetin tetraglycosides from *Maytenus aquifolium* (Celastraceae) leaves with an [M–H]$^-$ ion at *m/z* 917 and MS–MS ions at *m/z* 300 and 271 that were tentatively identified as two quercetin 3-*O*-(di)-rhamnosyl-hexosides (Tiberti, Yariwake, Ndjoko, & Hostettmann, 2007). Peak 10 showed a [M–H]$^-$ ion at *m/z* 917 but was identified as a quercetin 3-*O*-(2′ rhamno)-rutinosyl-7-*O*-hexoside based on MS^2 ions at *m/z* 755 (loss of 162 u, dehydrated hexose from C-7), 609 (loss of 164 u, an entire rhamnose moiety from C-2′ as in compound 17), 301 (loss of rutinose) and the MS^3 quercetin fragments at *m/z* 271, 179 and 151. Peak 18 had a [M–H]$^-$ ion at *m/z* 755 and MS^2 ions at *m/z* 609, 591, 445, 301. This peak could be assigned as an isomer of manghaslin, with differences in the position of attachment of the sugar moieties. However, the different shape of the UV spectrum (λ_{max} = 252, 291sh, 317) suggested that a coumarate residue was attached to the sugar moiety of the quercetin-glycoside structure (Ferreres et al., 2008; Prieto, Braca, Morelli, Barker, & Schaffner, 2004). Thus, peak 18 was tentatively identified as a quercetin-3-*O*-(2' coumaroyl)-rutinoside (Figs. 2 and 7). Similarly, Peak 16 (λ_{max} = 254, 291sh, 326; Ferreres et al., 2008), was assigned as a quercetin 3-*O*-(2′ caffeoyl)-rutinoside (Figs. 2 and 7), based on the [M–H]$^-$ ion at *m/z* 771, and MS^n ions at *m/z* 591([M–H-caffeic acid]$^-$), 301, 179 and 151, while peak 14 was identified as a quercetin 3-*O*-(2′ coumaro)-rutinosyl-7-*O*-hexoside (λ_{max} = 256, 291 sh, 317) due to a deprotonated molecule at *m/z* 917, MS^2 fragments at *m/z* 755 ([M–H-hexoside]$^-$), 591 ([M–H-hexoside-coumaric acid]$^-$), and quercetin MS^n fragments at *m/z* = 301, 271, 179, and 151. The acylated derivatives identified with peaks 14, 16 and 18 were not detected by HPLC DAD and MS–MS analysis after saponification of a portion of the extract. Peak 19 (λ_{max} = 254, 302 sh, 351) had a [M–H]$^-$ ion at *m/z* 625, yielded a MS^2 fragment at *m/z* 301([M–H-hexosyl-hexoside]$^-$), and was identified as a quercetin-3-*O*-sophoroside (Ferreres et al., 2007). Peak 12 (λ_{max} = 254, 351) had a [M–H]$^-$ ion at *m/z* 787 that lost 162 u yielding a MS^2 fragment at *m/z* 625 ([M–H-hexoside]$^-$), which yielded an MS^3 fragment at *m/z* 301([M–H-hexose-sophoroside]$^-$), and thus was identified as a quercetin 3-*O*-(2' hexo)-hexosyl-7-*O*-hexoside (quercetin 3-*O*-sophorosyl-7-*O*-glucoside), in agreement with literature data (Ferreres et al., 2007; Ferreres et al., 2008).

4. Conclusions

Two main antioxidant phenolic compounds were isolated from the MeOH extract of a Chilean sample of the edible mountain papaya fruits by selective fractionation using the bleaching of the DPPH· radical and identified by NMR spectroscopy as well as 19 phenolic compounds present in it by HPLC–DAD–ESI–MS analysis. The combination of NMR spectroscopy and HPLC with DAD detection coupled to ESI–MS–MS has proved to be a highly advanced and valuable tool on the determination of the complex phenolic composition of the edible fruits of *V. pubescens*.

The identification of polyphenols such as flavonols and carotenoids in the fruits and leaves of the widely known *Carica papaya* by HPLC–PDA (Lako, Wahlqvist, Wattanapenpaiboon, Sotheeswaran, & Premier, 2007) and GC–MS (Canini, Alesiani, D'Arcangelo & Tagliatesta, 2007), have been reported but no complex antioxidant flavonoids as compounds 17 and 20 were reported to occur in *C. papaya* and thus this information could be useful in the differentiation between the edible fruits of both papaya species and can be used for authentication of *V. pubescens* and comparison of different orchards in Chile as well as in other growing places.

A chromatographic fingerprint for the MeOH extract of this fruit was established and these results provide important information on the constituents of mountain papaya fruits that are grown commercially in Chile. The metabolite profiling of papaya fruits and specially the confirmation of the identity by NMR spectroscopy of the two mayor phenolics in the fruits (peaks 17 and 20 in the HPLC trace) which could be used as chemical markers, is crucial for the authentication of Chilean papaya-based food products, very important in the fruit market of Chile.

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References

Aaby, K., Ekeberg, D., & Skrede, G. (2007). Characterization of phenolic compounds in strawberry (*Fragaria x ananassa*) fruits by different HPLC detectors and contribution of individual compounds to total antioxidant capacity. *Journal of Agricultural and Food Chemistry, 55*, 4395–4406.

Badillo, V. M. (2000). *Carica* L. vs *Vasconcellea* St. Hil. (Caricaceae): Con la rehabilitacion de este último. *Ernstia, 10*, 74–79.

Balbontın, C., Gaete-Eastman, C., Vergara, M., Herrera, R., & Moya-Leon, M. A. (2007). Treatment with 1-MCP and the role of ethylene in aroma development of mountain papaya fruit. *Postharvest Biology and Technology, 43*, 67–77.

Belles, J. M., Lopez-Gresa, M. P., Fayos, J., Pallas, V., Rodrigo, I., & Conejero, V. (2008). Induction of cinnamate 4-hydroxylase and phenylpropanoids in virus-infected cucumber and melon plants. *Plant Science, 174*, 524–533.

Canini, A., Alesiani, D., D'Arcangelo, G., & Tagliatesta, P. (2007). Gas chromatography–mass spectrometry analysis of phenolic compounds from *Carica papaya* L. leaf. *Journal of Food Composition and Analysis, 20*, 584–590.

Cheel, J., Theoduloz, C., Rodriguez, J., Saud, G., Caligari, P. D. S., & Schmeda-Hirschmann, G. (2005). E-cinnamic acid derivatives and phenolics from Chilean strawberry fruits, *Fragaria chiloensis* ssp. *chiloensis*. *Journal of Agricultural and Food Chemistry, 53*, 8512–8518.

Cheel, J. A., Theoduloz, C., Rodriguez, J., & Schmeda Hirshmann, G. (2005). Free radical scavengers and antioxidants from lemongrass (*Cymbopogon citratus* (DC.) Stapf.). *Journal of Agricultural and Food Chemistry, 53*, 2511–2517.

Ferreres, F., Sousa, C., Valentao, P., Pereira, J. A., Seabra, R. M., & Andrade, P. B. (2007). Tronchuda cabbage flavonoids uptake by *Pieris brassicae*. *Phytochemistry, 68*, 361–367.

Ferreres, F., Sousa, C., Vrchovska, V., Valentao, P., Pereira, J. A., Seabra, R. M., et al. (2006). Chemical composition and antioxidant activity of tronchuda cabbage internal leaves. *European Food Research and Technology, 222*, 88–98.

Ferreres, F., Valentao, P., Pereira, J. A., Bento, A., Noites, A., Seabra, R. M., et al. (2008). HPLC–DAD–MS/MS–ESI screening of phenolic compounds in *Pieris brassicae* L. reared on *Brassica rapa* var. rapa L.. *Journal of Agricultural and Food Chemistry, 56*, 844–853.

Idstein, H., Keller, T., & Schrejer, P. (1985). Volatile constituents of mountain papaya (*Carica candamarcensis* synonym *Carica pubescens*) fruit. *Journal of Agricultural and Food Chemistry, 33*(4), 663–666.

Joubert, E., Winterton, P., Britz, T. J., & Ferreira, D. (2004). Superoxide anion and a, a-diphenyl-b-picrylhydrazyl radical scavenging capacity of rooibos (*Aspalathus linearis*) aqueous extracts, crude phenolic fractions, tannin and flavonoids. *Food Research International, 37*, 133–138.

Kite, G. C., Stoneham, C. A., & Veitch, N. C. (2007). Flavonol tetraglycosides and other constituents from leaves of *Styphnolobium japonicum* (Leguminosae) and related taxa. *Phytochemistry, 68*, 1407–1416.

Krajewski, D., Duque, C., & Schrejer, P. (1997). Aliphatic β-D glucosides from fruits of *Carica pubescens*. *Phytochemistry, 45*(8), 1627–1631.

Lako, J. V. T. C., Wahlqvist, M., Wattanapenpaiboon, N., Sotheeswaran, S., & Premier, R. (2007). Phytochemical flavonols, carotenoids and the antioxidant properties of a wide selection of Fijian fruit, vegetables and other readily available foods. *Food Chemistry, 101*, 1727–1741.

Lin, L.-Z., Harnly, J. M., Pastor-Corrales, M. S., & Luthria, D. L. (2008). The polyphenolic profiles of common bean (*Phaseolus vulgaris* L.). *Food Chemistry, 107*, 399–410.

Mabry, T. J., Markham, K. R., & Thomas, M. B. (1970). *The systematic identification of flavonoids*. Berlin: Springer-Verlag.

Mansouri, A., Embarek, G., Kokkalouc, E., & Kefalas, P. (2005). Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (*Phoenix dactylifera*). *Food Chemistry, 89*, 411–420.

Morales, A. L., & Duque, C. (1987). Aroma constituents of the fruit of the mountain papaya (*Carica pubescens*) from Colombia. *Journal of Agricultural and. Food Chemistry, 35*(4), 538–540.

Moya-León, M. A., Moya, M., & Herrera, R. (2004). Ripening of mountain papaya (*Vasconcellea pubescens*) and ethylene dependence of some ripening events. *Postharvest Biology and Technology, 34*, 211–218.

Prieto, J. M., Braca, A., Morelli, I., Barker, A. B., & Schaffner, U. (2004). A new acylated quercetin glycoside from *Ranunculus lanuginosus*. *Fitoterapia, 75*, 533–538.

Quiu, Y., Chen, Y., Pei, Y., Matsuda, H., & Yoshikawa, M. (2002). Constituents with radical scavenging effect from *Opuntia dillenii*: Structures of new α-pyrones and flavonol glycoside. *Chemical and Pharmaceutical Bulletin, 50*, 1507–1510.

Risch, B., Hermann, K., Wray, V., & Grotjahn, L. (1987). (E)-*O*-*p*-coumaroyl-derivatives of glucaric acid in citrus. *Phytochemistry, 10*, 3307–3309.

Sakushima, A., Hisada, S., Ogihara, Y., & Nishive, S. (1980). Studies on the constituents of Apocinaceae plants. Gas chromatography mass spectrometry determination of new flavonoid triglycosides from the leaves of *Cervera manghas* L. (2). *Chemical and Pharmaceutical Bulletin, 28*, 1219–1223.

Simirgiotis, M. J., Adachi, S., To, S., Yang, H., Reynertson, K. A., Basile, M. J., et al. (2008). Cytotoxic chalcones and antioxidants from the fruits of *Syzygium samarangense* (Wax Jambu). *Food Chemistry, 107*, 813–819.

Tiberti, L. A., Yariwake, J. H., Ndjoko, K., & Hostettmann, K. (2007). Identification of flavonols in leaves of *Maytenus ilicifolia* and *M. aquifolium* (Celastraceae) by LC/UV/MS analysis. *Journal of Chromatography B, 846*, 378–384.

Vallejo, F., Tomás-Barberán, F. A., & Ferreres, F. (2004). Characterisation of flavonols in broccoli (*Brassica oleracea* L. var. italica) by liquid chromatography-UV diode-array detection-electrospray ionisation mass spectrometry. *Journal of Chromatography A, 1054*, 181–193.

Van Droogenbroeck, B., Breyne, P., Goetghebeur, P., Romeijn-Peeters, E., Kyndt, T., & Gheysen, G. (2002). AFLP analysis of genetic relationships among papaya and its wild relatives (Caricaceae) from Ecuador. *Theoretical and Applied Genetics, 195*, 289–297.

Withopf, B., Richling, E., Roscher, R., Schwab, W., & Schreier, P. (1997). Sensitive and selective screening for 6'-*O*-malonylated glucoconjugates in plants. *Journal of Agricultural and Food Chemistry, 45*, 907–911.

Yang, H., Protiva, P., Cui, B., Ma, C., Baggett, S., Hequet, V., et al. (2003). New bioactive polyphenols from *Theobroma grandiflorum* (cupuacu). *Journal of Natural Products, 66*, 1501–1504.

Yildirim, A., Mavi, A., & Kara, A. (2001). Determination of antioxidant and antimicrobial activities o*f Rumex crispus* L. extracts. *Journal of Agricultural and Food Chemistry, 49*, 4083–4089.