Improved High-Performance Liquid Chromatography–Diode Array Detection Method for the Determination of Phenolic Compounds in Leaves and Peels From Different Apple Varieties

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Abstract

Hydroxycinnamic acids derivatives, monomeric and oligomeric flavan-3-ols, flavonols, and dihydrocalcones are four of the major polyphenolic groups found in apples leaves and peels. A simple extraction with minimal pre-treatment and a high-performance liquid chromatography–diode array detection determination are optimized and validated, in order to identify and quantitate the polyphenolic profile of leaves and peels of four apples varieties (Gala, Topaz, Golden Delicious, and Florina). The improved chromatographic method has led to better separation of some known polyphenols in a single course, and diode-array detection has been used for the previsional identification of some polyphenolic compounds not available as standards. Because the mobile phase and the chromatographic column are compatible with a mass spectrometer, this method could investigate the unknown flavanols, flavonols, hydrocinnamic acid derivatives, and chalcone-related compounds found in apple leaves and peel extracts analyzed.

Introduction

Polyphenols have received a great deal of attention because of their wide and abundant presence in fresh products. They seem to protect against cardiovascular disease and have certain potential anticarcinogenic properties, due to their antioxidant activity and their function as free radical scavengers (1–3).

In addition to their beneficial nutritional properties, polyphenols may also contribute to plant resistance against many diseases, such as apple scab (4–6). Some authors studied the polyphenolic pattern in apple tree leaves to find a relation between the levels of some phenolic compounds present in leaves and scab resistance that could be useful to screen resistant and susceptible apple varieties (4,5,7).

Because of the complex chemistry of polyphenols, a series of steps are usually taken to fractionate them (8–10), but these methods require tedious preliminary purification and are time-consuming.

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Moreover, sample preparation causes the primary source of error differences in the results obtained by different laboratories (11). The chromatographic run times of polyphenols analyses often are excessive, and optimization of the gradient is required, in order to develop a routine analysis of samples.

A direct injection, with minimal pre-treatment, could be considered as an alternative when a great number of samples have to be analyzed. This procedure has been assayed in wine, fruit juices, ciders, and apples (12–19).

Reversed-phase high-performance liquid chromatography (HPLC) with C18 columns is the most popular technique for the analysis of polyphenols in different foods. A wide variation exists in the effectiveness of different reversed-phase HPLC columns and mobile phases when used to analyze free and glycosilated flavonoids.

Recently, a LC–mass spectrometry (MS) technique has been playing an important role to identify new polyphenols found in apple samples (20).

The preparation of the sample and the run time needed to be rapid overall when a lot of samples have to be analyzed, and a mass-compatible method allows for the identification of new polyphenolic compounds or the verification of those already known. In this paper, a simple extraction and a chromatographic method applied to leaves and peels of four apple cultivars are described and validated in order to identify the main polyphenolic compounds presents in this plant. The mobile phase and the chromatographic column are compatible with MS applications in order to identify new polyphenolic compounds that could have biological properties or they could be responsible for the resistance against some diseases.

Experimental

Chemicals

Flavanol monomers, chlorogenic acids, *p*-coumaric acid, quercetin-rutinoside, and quercetin-glucoside were supplied by Sigma (Steinheim, Germany), whereas procyanidin B2,

quercetin-galactoside, quercetin-rhamnoside, quercetin, phloretin, and phloridzin were purchased from Extrasynthese (Lyon, France). Acetonitrile, acetone, (Carlo Erba, Milan, Italy), and methanol (Merck, Darmstadt, Germany) were all of HPLC grade. Folin-Ciocalteu phenol reagent was purchased from Merck and formic acid from Carlo Erba (Milan, Italy).

Samples extraction

Leaves

A 0.2-g fresh leaf of different apple varieties (Gala, Topaz, Golden Delicious, Florina) was homogenized with 15 mL of methanol with an Ultraturrax (IKA Works, Wilmington N.C.) for 30 s at 500 rpm. After centrifugation (7800 × *g* for 7 min at 8°C), the solid residue was extracted with 15 mL of acetone and centrifuged again. A third extraction of solid residue was developed with other 15 mL of methanol and centrifuged as described. The three extracts were combined, filtered through a membrane filter (0.45 µm, Chemtek Analytica, Bologna, Italy), and concentrated in a little volume. The extracts were stored at –20°C until HPLC analysis. To ensure complete recovery, a fourth extraction of leaf samples with 15 mL of methanol was developed and analyzed by HPLC separately to the other three extractions.

Before chromatographic analysis, extracts were completely evaporated under N_2, dissolved with 1 mL of methanol–water 1:1 (v/v), and filtered through a 0.22 µm membrane filter (Chemtek Analytica, Bologna, Italy).

Peels

Samples corresponding to approximately 7 to 8 g of frozen peel of different apple varieties (Gala, Topaz, Golden Delicious, Florina) were homogenized three times with 15 mL of acetone–water 70:30 (v/v) as described in the Leaves section. After centrifugation (7800 × *g* for 7 min at 8°C), the three extracts were combined to a final volume of 100 mL of acetone–water 70:30 and stored at –20°C until HPLC analysis. To verify a complete recovery, a fourth extraction of peels samples with 15 mL of acetone–water 70:30 (v/v) was developed and analyzed by HPLC separately to other three extractions. Before chromatographic analysis, 40 mL of extracts were evaporated in a rotavapor at 40°C in order to evaporate acetone. Samples were transferred to 20 mL volumetric flasks with water, filtered through a 0.22-µm membrane filter (Chemtek Analytica, Bologna, Italy), and analized by HPLC.

Total phenolic content based on the colorimetric method

The total amount of polyphenols was measured with an optimized Folin-Ciocalteu (FC) method (21) according to which interfering compounds such as sugars, amino acids, and ascorbic acid were removed by cleanup on a C18 cartridge (Sep-Pak, Waters, Milford) from the evaporated extracts reconstituted in water. The results are expressed as equivalent of (+)-catechin mg/100 g of fresh weight of leaf or peel.

HPLC apparatus and chromatographic conditions

Analyses were performed with a Waters system (Milford, MA), equipped with a 20 µL loop injector, provided with a column oven, two pumps (model 510), a diode array detector (model 996) and Millennium software v.3.2 data module. Separation of polyphenols was carried out on an XTerra MS C18 (250 × 4.6-mm i.d., particle size, 5 µm) column from Waters with a Securityguard C18 ODS (4 × 3 mm) precolumn (Phenomenex, Torrance, CA). The column was thermostated at 40°C, and a flow rate of 1.0 mL/min was used.

The gradient conditions were aqueous 1% formic acid (solvent A) and 100% acetonitrile (solvent B). During the opti-

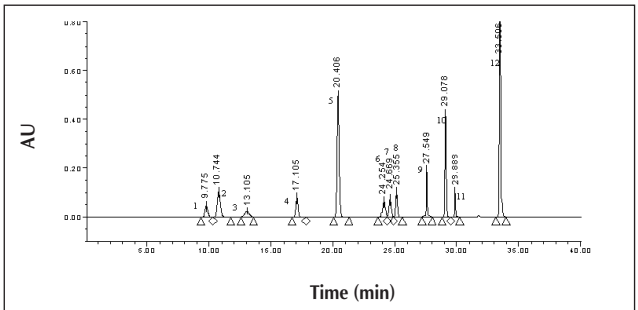

Figure 1. Chromatogram of standard mixture at 280 nm. Peak numbers are: (+)catechin, 1; chlorogenic acid, 2; procyanidin B2, 3; (–)epicatechin, 4; coumaric acid, 5; quercetin-rutinoside, 6; quercetin-galactoside, 7; quercetin-glucoside, 8; quercetin-rhamnoside, 9; phloridzin, 10; quercetin, 11; phloretin, 12.

Table I. Optimized Gradient. Solvent A: 1% Formic Acid–0.5% Methanol and Solvent B: Acetonitrile

Time (min)	A (%)	B (%)
0	91	9
11	91	9
13	85	15
20	83	17
37	40	60
37.5	0	100
38.5	0	100
39.5	91	9

Table II. Concentration (mg/g of Fresh Weight) of the Four Families of Polyphenols (Flavanols, Hydroxycinnamic Acid, Flavonols, and Dihydrochalcones) in Leaves Extracts (*n* = 3)

	Florina	Topaz	Golden Delicious	Gala
Flavanols	0.28 ± 0.10	0.23 ± 0.02	0.39 ± 0.09	0.24 ± 0.001
Hydroxycinnamic acid	0.34 ± 0.04	0.35 ± 0.04	0.51 ± 0.15	0.92 ± 0.02
Flavonols	3.94 ± 1.2	6.83 ± 0.28	12.39 ± 1.52	8.81 ± 1.13
Dihydrochalcones	53.72 ± 4.0	100.44 ± 0.4	106.69 ±1 0.39	90.24 ± 0.38
Total polyphenols	58.28	107.85	119.98	100.21

mization trials, 0.5% methanol was added to solvent A. The gradients conditions are described in Table I.

Phloridzin amount, found in apple leaves samples, exceeded from the detection range; after dilution (1:50) of leaves extracts, samples were re-injected using a rapid chromatographic method, in order to identify and quantify this compound only in 12 min. The system was run with a gradient program: 20–60% B for 11 min, 60–100% B for 1 min, 100% B for 1 min, 100–20% B for 1min.

Detection was performed at 350 nm for the flavonol glycosides, at 320 nm for the hydroxycinnamic acids, and at 280 nm for the rest of phenolic compounds (flavanols, procyanidins, and dihydrochalcones). Spectra were acquired from 200 to 400 nm, and the scanning resolution was of 1.2 nm. Identification of compounds was achieved by comparing their spectra and retention times with those of standards when available.

Results and Discussion

HPLC optimization and application to apple samples

Four families of phenols were taken as references for analytical optimization process: hydroxycinnamic acid and derivatives, monomeric and oligomeric flavanols, flavonols, and dihydrocalcones.

Initial HPLC working conditions were selected on the basis of previous work, where only two classes of polyphenols were determined in a single course (22). Methanol allowed the most rapid separation of phenolic compounds (23), but it was not able to reach a good resolution for some flavonols (19). On the contrary acetonitrile gave the best resolution, but the run time was too long with the chromatographic column used, and it was not able to separate (+)-catechin and chlorogenic acid. In order to overcome these problems, 9% of acetonitrile was used in isocratics conditions for first 10 min of the chromatographic course and 5% of methanol was added in the acidic aqueous phase. The resolution of (+)-catechin and chlorogenic acid peaks were reached, but in the second part of the chromatogram, the increase percentage of acetonitrile was not sufficient to separate flavonols because the methanol percentage was too high. Several concentrations of methanol were evaluated, from 5% to 0.5%, in order to have a good result for all standards analysed. The best separation was found with 0.5% of methanol and 1% of formic acid in a binary gradient with acetonitrile. The final gradient, reported in Table I, permits the separation of 12 standards in less than 35 min (Figure 1). These conditions allowed a good separation between (+)-cathechin and chlorogenic acid, confirming a similar result recently obtained by others authors (15). Formic acid was chosen because it is volatile acid mass compatible, and the optimized mobile phase could be directly applied to LC–MS. Many authors studied the polyphenols pattern of apple peels and pomace (15,23,24,25–27), but only a few authors worked on the apple leaves (5,7,25).

As regards the extraction of samples before HPLC analysis, fresh leaves and frozen peels of different apples varieties were processed. Fresh leaves were chosen because recent data

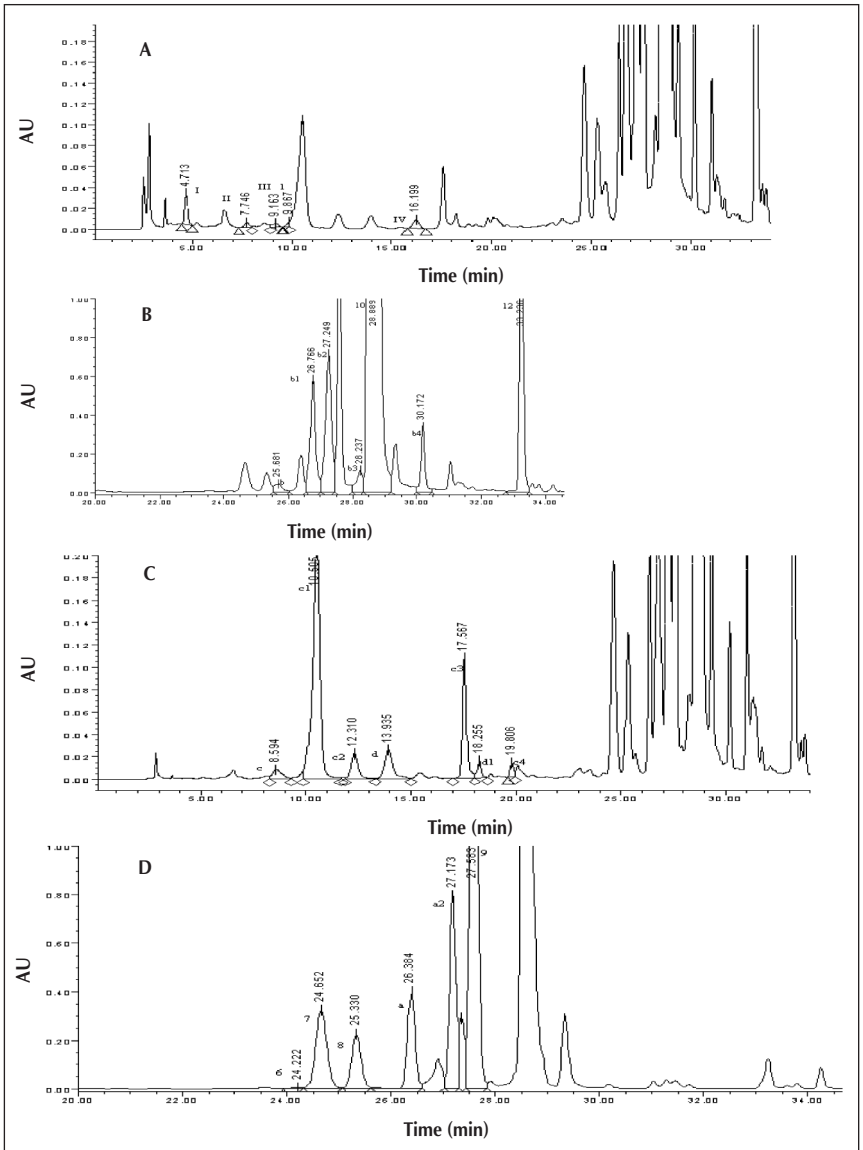

Figure 2. Chromatogram of apple leaf extract. Detection at 280 nm (A). Peak numbers are: procyanidin oligomers, I–IV; (+)-catechin, 1; phloridzin, 10; phloretin, 12; unknown hydroxyphloretin-derivatives, b,b1,b3; phloretin xyloglucoside, b2; unknown phloretin derivatives, b4. A close up of Figure 2A from time (min) 20–34 minutes (B). Detection at 310 nm (C). Peak numbers are: p-coumaroylquinic acid, c3; coumaroyl derivatives, c–c2, and c4; chlorogenic acid derivatives, d and d1. Detection at 350 nm (D). Peak numbers are: quercetin-rutinoside, 6; quercetin-galactoside, 7; quercetin-glucoside, 8; quercetin-rhamnoside, 9; quercetin-glycosides, a; quercetin-arabinofuranoside, a2.

reported in the literature showed that recoveries of polyphenols in fresh leaves were higher than those analyzed after being dried and redissolved (30). While methanol is the solvent that is able to extract a wide range of polyphenols classes in apple leaves (31), the mixture acetone–water, used by many authors (22,24,32) for the peel and pomace extraction, allowed to obtain stable extracts because of the denaturation of polyphenol oxidase (33). In addition, a good extraction of polyphenols, including proanthocyanidins presents prevalently in apple, pomace, was assured using this solvent mix. It was already reported that these compounds can be underestimated when alcoholic or hydro-alcoholic extractions are used because most of them were not extracted (24,34–36).

The fourth extraction of leaves and peels samples didn't show any peaks corresponding to polyphenols (data not shown). Total recovery of these compounds has been achieved with three simple extractions in order to avoid oxidation reactions or the addition of some antioxidant additives that could coelute with polyphenolic compounds (37).

The polyphenolic compounds observed showed a sufficient separation of known and unknown peaks. Identification of the compounds was carried out by comparing their retention times (t_R) and spectra with those of standards when available. Identified peaks were then confirmed by spiking samples with standards mixtures, whereas some unknown chromatographic peaks were tentatively identified through their spectral features and by literature data (15,26,27,38,39).

As regards leaves samples (Figure 2A), (+)-catechin (t_R = 9.9 min) and four peaks (I–IV, t_R = 4.7, 7.7, 9.2, and 16.2 min) with spectra characteristics similar to proanthocyanidins (λ_{max}=280nm) were found, while in apple peels extracts (Figure 3A), the chromatographic profile showed (+)-catechin (t_R = 10.2 min), proantocyanidin B2 (t_R = 12.7 min), (–)-epicatechin (t_R = 17.0 min), and several peaks (I–XVI t_R = 9.1, 9.4, 11.7, 14.0, 14.6, 16.3, 18.2, 18.8, 19.0, 19.5, 20.1, 20.7, 21.2, 21.7, 22.2, and 23.5 min) with spectral characteristics similar to flavanol monomer (λ_{max} = 280 nm). Because acetone is a good solvent for proanthocyanidins extraction and the peel is rich of proanthocyanidins (32), these unknown peaks were identified as flavanols polymers. Usually their estimation by direct HPLC remained incomplete because polymeric forms do not give well-resolved peaks on chromatograms. Some authors used a thiolysis reaction to reach a good resolution in chromatograms (24), but this technique has a drawback, due to the difficulty to assay individual oligomeric procyanidins (36). The chromatographic conditions used and the column chosen in this work could separate with a good efficiency several flavanols and they could be applied to mass detector to verify the presence of monomer and polymer forms in a single course.

Phloridzin (t_R = 28.9 min), phloretin (t_R = 33.2min), and five peaks (b–b4 t_R = 25.7, 26.8, 27.2, 28.2, and 30.2) corresponding to phloretin derivatives with λ_{max}=281.5nm (Figure 2A) were found in leaves extracts,

Table III. Concentration (mg/g of Fresh Weight) of the Four Families of Polyphenols (Flavanols, Hydroxycinnamic Acid, Flavonols, and Dihydrochalcones) in Peel Extracts (*n* = 3)

	Florina	Topaz	Golden Delicious	Gala
Flavanols	2.47 ± 0.22	1.28 ± 0.15	1.05 ± 0.20	0.98 ± 0.02
Hydroxycinnamic acid	0.04 ± 0.01	0.03 ± 0.002	0.18 ± 0.02	0.06 ± 0.01
Flavonols	0.58 ± 0.02	1.76 ± 0.24	1.56 ± 0.25	1.03 ± 0.14
Dihydrochalcones	0.18 ± 0.03	0.06 ± 0.02	0.17 ± 0.02	0.07 ± 0.01
Total polyphenols	3.27	3.13	2.96	2.14

Table IV. Phenolic Compounds Employed in the Optimization of the Gradient Method: Calibration Graph Data

Compound no.	Phenolic compound	Phenolic structure	λmax (nm)	linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Correlation coefficient (r^2)	Slope	Intercept
1	(+) Catechin	Flavonol	280	0.5–90	0.2	0.3	0.9993	1.302	+0.021
2	Chlorogenic acid	Hydroxycinnamic acid	320	0.5–90	0.2	0.3	0.9996	6.507	+1.128
3	Procyanidin B2	Flavonol dimer	280	0.5–90	0.4	0.5	0.9992	1.551	–0.846
4	(–)Epicatechin	Flavonol	280	0.5–90	0.2	0.3	0.9991	1.482	0.723
5	*p*-Coumaric acid	Hydroxycinnamic acid	320	0.5–90	0.2	0.3	0.9998	14.093	+0.225
6	Rutin	Flavonol glycoside	350	0.5–90	0.1	0.2	0.9996	3.035	–0.539
7	Hyperoside	Flavonol glycoside	350	0.5–90	0.1	0.2	0.9994	4.213	–0.754
8	Quercetin-glucoside	Flavonol glycoside	350	0.5–90	0.1	0.2	0.9996	3.616	–0.200
9	Quercetrin	Flavonol glycoside	350	0.5–90	0.1	0.2	0.9996	4.1011	–1.012
10	Phloridzin	Dihydrocalcone	280	0.5–90	0.1	0.2	0.9994	0.488	–0.154
11	Quercetin	Flavonol aglycone	350	0.5–90	0.1	0.2	0.9994	4.653	+2.752
12	Phloretin	Dihydrocalcone	280	0.5–90	0.1	0.2	0.9995	0.790	–27.480

while only phloridzin (t_R = 29.1min) and two peaks (b2, t_R = 27.2 and b3, t_R = 28.2 min) showing spectra characteristics similar to phloridzin, were found in apple peels extracts. The retention time of peaks before phloridzin (phloretin glucoside) indicated a more polar compounds than phloridzin. Therefore compound b, b1, and b3 could be identified as hydroxyphloridzin derivatives, and compound b2 could be identified as phloretin 2-xyloglucoside, also found in apple peels, as reported by others authors (26,39). In leaf extracts, another peak found after phloridzin (b4, t_R = 30.2) was unknown.

Table V. Recovery of Polyphenols Standards in Spiked Apple Leaves Samples (*n* = 3)

Standard added	Amount of analyte (µg/mL) Added	Measured	Recovery (%)
Catechin	4.98	4.87	97.8
Chlorogenic acid	26.50	26.48	99.9
Proantocyanidin B2	5.78	5.55	96.0
p-Coumaric acid	44.00	43.01	97.7
Quercetin-galactoside	20.49	19.73	96.3
Phloretin	38.81	37.83	97.5

Table VI. Recovery of Polyphenols Standards in Spiked Apple Peel Samples (*n* = 3)

Standard added	Amount of analyte (µg/mL) Added	Measured	Recovery (%)
Catechin	4.92	5.02	102.1
Chlorogenic acid	25.96	25.25	97.3
Proantocyanidin B2	5.74	5.86	102.2
p-Coumaric acid	42.56	42.36	99.5
Quercetin-galactoside	20.13	20.18	100.2
Phloridzin	54.36	54.76	100.7

Table VII. Intraday Repeatability of the Method

Compound no.	t_r (min) ± SD	RSD (%)	Area/1000 ± SD	RSD (%)
1	10.04 ± 0.26	2.60	350.68 ± 5.48	1.56
2	10.89 ± 0.35	3.25	1625.42 ± 7.82	0.48
3	13.18 ± 0.42	3.11	384.967 ± 4.19	1.09
4	17.31 ± 0.26	1.50	384.981 ± 2.58	0.67
5	20.67 ± 0.26	1.27	3582.04 ± 36.49	1.02
6	24.24 ± 0.15	0.61	793.75 ± 15.53	1.96
7	24.69 ± 0.12	0.46	1053.38 ± 10.13	0.96
8	25.35 ± 0.13	0.47	917.20 ± 11.07	1.21
9	27.39 ± 0.03	0.10	1071.57 ± 13.20	1.23
10	29.01 ± 0.10	0.34	1184.42 ± 18.35	1.55
11	29.90 ± 0.02	0.07	565.00 ± 16.33	2.89
12	33.17 ± 0.02	0.06	2083.33 ± 34.69	1.67

In a chromatogram of apple leaves, five unknown peaks (c–c4 t_R = 8.6, 10.5, 12.3, 17.6, and 19.8 min) with spectrum similar to *p*-coumaric acid (λ_{max} = 310nm) were found (Figure 2B), and only peak c3, (t_R = 17.6 min) was still found in peels extracts (Figure 3B). This compound could be identified as *p*-coumaroylquinic acid, found in apple peel (15,26). In the leaf extract, two unknown peaks (d–d1 t_R = 13.9 and 18.2 min) with spectrum similar to chlorogenic acid were found. The sample of Figure 2B belonged to scab-resistant cultivar (Topaz), which showed a great amount of hydroxycinnamic acid, studied in the active expression of resistance, as reported by other authors (40,41).

This method is able to separate six flavonols in apple leaves and eight flavonols in peel extracts (Figures 2D and 3C): four of them were identified by their standards (t_R = 24.2 min quercetin-rutinoside, t_R = 24.6 min quercetin-galactoside, t_R = 25.3 min quercetin-glucoside and t_R = 27.6 min quercetin-rhamnoside), whereas the other four peaks were unknown. Following the literature data (5,26,27,28), compound a (t_R = 26.4 min) was tentatively identified as quercetin-xyloside, compound a1 (t_R = 26.9 min) as quercetin-arabinopyranoside, compound a2 (t_R = 27.2 min) as quercetin-arabinofuranoside, and compound a3 (t_R = 27.5 min) was unknown.

Quantitation was performed by three standard curves as reported in the Method validation section. For compounds lacking standards, quantitation was carried out with similar compounds. Thus, unknown proanthocyanidins were quantitated as (–)-epicatechin; acids derivatives with λ_{max} = 326 nm were quantitated as chlorogenic acid; acids derivatives with λ_{max} = 310 nm were quantitated as *p*-coumaric acid; unknown flavonols were quantitated as quercetin-galactoside, and unknown dihydrocalcones were quantitated as phloridzin.

The expected value of the total polyphenols obtained by the Folin-Ciocalteu assay was calculated on the basis of HPLC data as reported by Vrhovsek et al. (33). Our experimental value of the Folin-Ciocalteu (FC) assay should be unbiased from the presence of undesired reacting compounds because a sample pre-treatment was done to separate the potential interfering compounds.

Good agreement between the two sets of independent measures (data not shown) strongly supports the fact that the whole amount of total polyphenols measured by FC assay can be explained by chromatographic analysis. This observation is an indication of the reliability of this HPLC method because the FC assay is generally considered as the method of choice to estimate total phenol contents in plant extracts (29, 42). Total polyphenol amount of leaves and peel extracts analyzed by HPLC were reported in Table II and III.

Method validation

The method linearity was evaluated by the square correlation coefficients (r^2) of calibration curves generated by three repeated injections (n = 3) of standard mixtures at five concentration levels (Table IV). For each

single phenol, the limit of detection was fixed as three times the signal-to-noise ratio (S/N), and for all compounds it was ≤ 0.2 µg/mL, except for proantocyanidn B2 (0.4 µg/mL); the limit of quantitation was ≤ 0.3 µg/mL for all compounds (Table II), except for proantocyanidin B2 (0.5 g/mL).

The recovery of the method was determined by standards addition method on pool of apple leaves and peels samples (Table V–VI). Leaves samples (n = 3) were spiked with a standard solution containing catechin (5 g/mL), chlorogenic acid (25 g/mL), procyanidin B2 (5 g/mL), p-coumaric acid (45 g/mL), quercetin-galactoside (20 g/mL), and phloretin (40 g/mL). Peels samples (n = 3) were spiked with a standard solution containing catechin (5 g/mL), chlorogenic acid (25 g/mL), procyanidin B2 (5 g/mL), p-coumaric acid (45 g/mL), quercetin-galactoside (20 g/mL), and phloridzin (55 g/mL).

Recovery was greater than 96% for all polyphenolic compounds, and the linearity evaluated showed a good relationship between the peak area and concentration.

Regarding the precision, repeatability of peak areas and retention time were calculated by RSD% of six injections carried out in the same day (intraday repeatibility) (Table VII), and the RSD% of six injections carried out in a 30 days working period (interday repeatibility) (Table VIII).

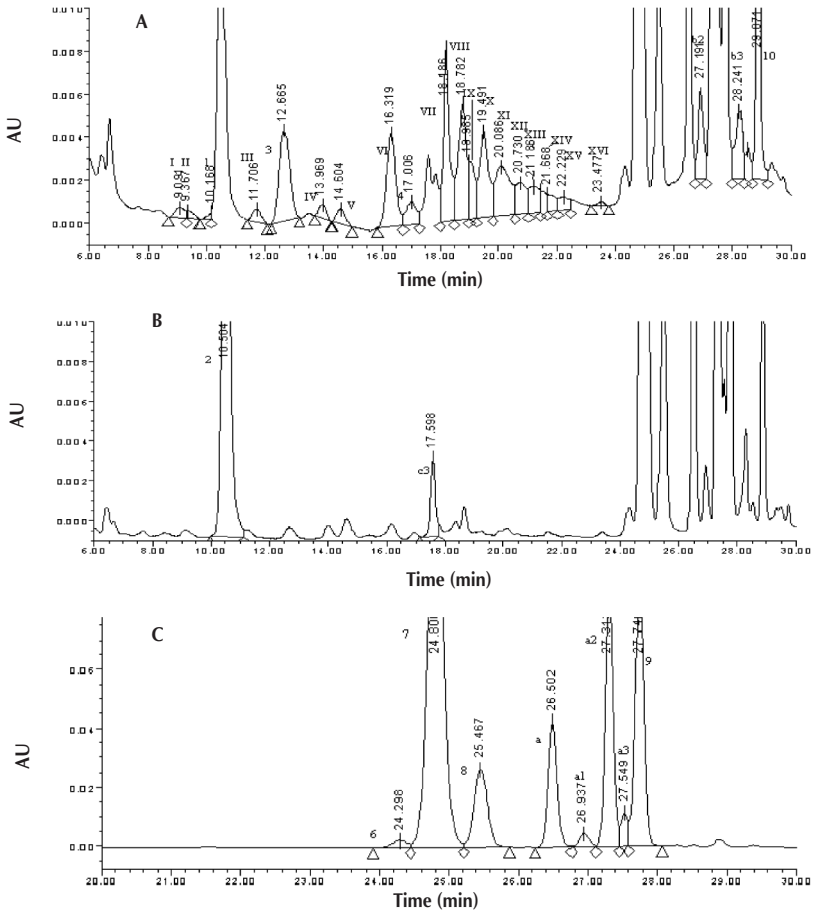

Figure 3. Chromatogram of apple peel extract. Detection at 280 nm (A). Peak numbers are: procyanidin oligomers, I–XVI; (+)-catechin, 1; procyanidin B2, 1; (–)-epicatechin, 4; phloridzin, 10; phloretin xyloglucoside, b2; hydroxyphloretin glycoside derivative, b3. Detection at 310 nm (B). Peak numbers are: chlorogenic acid, 2; *p*-coumaroylquinic acid, c3. Detection at 310 nm (C). Peak numbers are: quercetin-rutinoside, 6; quercetin-galactoside, 7; quercetin-glucoside, 8; quercetin-rhamnoside, 9; quercetin-xyloside, a; quercetin-arabinopyranoside, a1; quercetin-arabinofuranoside, a2; unknown flavonol glycosides, a3.

Table VIII. Interday Repeatability of the Method

Compound no.	t_r(min) ± SD	RSD (%)	Area/1000 ± SD	RSD (%)
1	10.34 ± 0.45	4.34	354.27 ± 17.88	5.05
2	11.08 ± 0.49	4.37	1682.78 ± 86.97	5.17
3	12.99 ± 0.71	5.09	381.56 ± 12.34	3.23
4	17.32 ± 0.28	1.60	408.85 ± 22.94	5.61
5	21.06 ± 0.31	1.46	3658.66 ± 192.30	5.26
6	24.18 ± 0.30	1.19	919.49 ± 48.09	5.23
7	24.67 ± 0.35	1.32	1201.76 ± 54.89	4.57
8	25.37 ± 0.31	1.15	1051.11 ± 50.74	4.83
9	27.58 ± 0.15	0.51	1189.81 ± 62.98	5.29
10	28.98 ± 0.15	0.52	1255.92 ± 51.54	4.10
11	30.13 ± 0.22	0.72	1412.37 ± 88.02	6.23
12	33.21 ± 0.25	0.82	2282.89 ± 142.67	6.25

Conclusion

Many good HPLC methods exist for the separation and quantitation of different polyphenolic groups found in different fruits. Our method showed that good separation could be achieved by using a simple extraction and rapid chromatographic method.

The method was sensitive and selective by using multiple wavelengths corresponding to the different UV–Vis maximum absorptions of the different polyphenolic groups.

The stationary phase and the chormatographic column used in this study display a good separation of the phenolic constituents, isolated from apple leaves and peels, respectively.

As volatile eluents were used, it is possible to combine the HPLC with a MS without changing and down loading the chromatographic conditions.

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