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ARTICLES

Improved HPLC Determination of Phenolic Compounds in Cv. Golden Delicious Apples Using a Monolithic Column

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A rapid HPLC-DAD determination of phenols in apple using an RP monolithic column is reported. Because of the hydrodynamic advantages offered by this kind of column and the use of acidified acetonitrile as eluent, assays of apple extracts can be performed in <21 min. Assays of pulp and peel extracts were carried out without the need for time-consuming sample pretreatment except filtration. Several flavanols, hydroxycinnamic acids, dihydrochalcones, and six quercetin glycosides were identified and quantified. A seventh quercetin derivative, two chalcone-related compounds, and three hydroxycinnamic derivatives were also found. Peels proved to be richer in phenols than pulps, the former being composed mainly of (−)-epicatechin, procyanidin B2, chlorogenic acid, phloridzin, hyperin, and avicularin. In pulps, where the chlorogenic acid was the principal phenolic compound, quercetin glycosides were found in very low amounts.

KEYWORDS: HPLC-DAD; monolithic column; phenolics; apple; Golden Delicious; analysis

INTRODUCTION

During the past decade, apple phenols have been subjected to a number of investigations due to their presence in the human diet (*1*) and beneficial effects on health (*2*). Moreover, phenols are involved in apple browning, which is one of the main problems in juice processing (*3*, *4*). Phenols are also important because of their contribution to the color, taste, and flavor of the fruits (*5*).

Apple phenolic composition appears to be largely made up of flavanols, hydroxycinnamic acids, flavonol glycosides, and dihydrocalchones (*6*−*10*). For their determination, RP-HPLC has proved to be the most appropriate technique, being sufficiently sensitive and precise. Unfortunately, HPLC run times are often excessive and sometimes preceded by a time-consuming sample cleanup step (*6*, *10*−*14*). Only recently has a fast separation of apple polyphenols been reported (*9*, *15*), the determination of the main phenols taking <25 min using a conventional RP C18 column.

However, nonconventional monolithic supports for column packing are increasingly attracting the interest of researchers (*16*, *17*). Due to their rigid and porous structure, they enable higher solvent flows, shorter assay times, and fast column re-equilibration between runs (*18*). The present paper reports a simple and fast method for the determination of the largest number of phenolic compounds in apple peels and pulps using a monolithic column and a diode array detector.

Table 1. Standard Compounds, Concentration Ranges, and Wavelengths Used To Optimize the Gradient Method

compound	chemical class	detection wavelength (nm)	linear range (μg/mL)
gallic acid	hydroxybenzoic acid	280	0.6–40.5
(+)-catechin	flavanol	280	0.5–40.0
chlorogenic acid	hydroxycinnamic acid	320	0.6–45.0
procyanidin B2	flavan dimer	280	1.0–90.0
p-coumaric acid	hydroxycinnamic acid	320	0.5–50.0
(−)-epicatechin	flavanol	280	0.6–40.5
ferulic acid	hydroxycinnamic acid	320	0.6–45.0
hyperin	flavonol glycoside	350	1.0–90.0
rutin	flavonol glycoside	350	0.5–40.5
phloridzin	dihydrochalcone	280	0.6–42.0

MATERIALS AND METHODS

Reagents and Solvents. Solvents were from Merck and of analytical or HPLC grade. Flavanol, hydroxycinnamic acids, and dihydrochalcones (**Table 1**) were from Sigma (St. Louis, MO), whereas procyanidin B2, hyperin (quercetin 3-galactoside), rutin (quercetin 3-rutinoside), quercitrin (quercetin 3-rhamnoside), and isoquercitrin (quercetin 3-glucoside)

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were purchased from Extrasynthese (Lyon, France). In all cases, water was of HPLC quality and purified in a Simplicity system (Millipore, Bedford, MA).

Samples. Cv. Golden Delicious apples were harvested in Pergine di Valsugana (Trent, Italy) on September 25, 2002, and stored for 20 days at 4 °C to provide a comparable degree of maturation. Ten trees were sampled, and six apples per tree were used to obtain representative and uniform starting material. Peels were separated from pulps using a manual device for a reproducible peel thickness of 0.5 cm. For each apple, separate portions of pulp slices and peels, of ~2.5 g, were exactly weighed, freeze-dried, and stored until extraction.

Extraction Procedure. Samples corresponding to ~15 g of fresh peel or pulp (representing six apples from the same tree) were extracted at room temperature using an ultrasonic bath after the method of Escarpa and Gonzalez (*15*), with minor modifications. Samples were extracted three times with aqueous 95% methanol containing 300 mg/L sulfur dioxide as follows: 20 mL of solvent for 1 h, 10 mL for 1 h, and 10 mL for 30 min. The three extracts were combined and stored at −18 °C until analysis. All of the samples were filtered through a membrane filter (0.22 μm) before HPLC runs.

HPLC Apparatus. HPLC separation was conducted using a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 1580), a 20-μL loop, a Reodyne valve (Cotati, CA), a photodiode detector (PU MD 910), and a column oven. The column was a Chromolith Performance RP-18e (100 × 4.6 mm i.d.) (Merck, Darmstadt, Germany). All runs were acquired and processed using Borwin 5.0 software (JMBS Developments, Grenoble, France).

Chromatography. Detection was performed at 280 nm for gallic acid, flavanols, and hydrochalcones, at 320 nm for hydroxycinnamic acids, and at 350 nm for flavonol glycosides. Spectra were acquired from 200 to 400 nm with the highest scanning resolution allowed by the acquisition software (1 nm). The elution solvents were aqueous 0.01 M phosphoric acid (solvent A) and 100% acetonitrile (solvent B). During the optimization trials, 0.5% methanol was added to solvent A. The column was thermostated at 25 °C with a flow of 2.5 mL/min.

Compounds Identification and Quantification. Identification of the compounds was carried out by comparing their retention times and spectra to those of standards when available. Identified peaks were then confirmed by spiking samples with standard mixtures. Unknown chromatographic peaks were tentatively identified via their spectral features and by literature data.

Quantification was performed according to an external standard method. Six standard mixtures, at the concentration ranges shown in **Table 1**, were injected in triplicate to obtain calibration curves (**Table 3**). For quercitrin and isoquercitrin the amount at our disposal was too small, and they were thus quantified as hyperin. For compounds lacking standards, quantification was carried out with similar compounds. Thus, phloretin 2-xyloglucoside was quantified as phloridzin, *p*-coumaroylquinic acid was quantified as coumaric acid, and reynoutrin (quercetin 3-xyloside) and avicularin (quercetin-3-arabinofuranoside) were quantified as hyperin.

Method Validation. Method linearity was evaluated by the square correlation coefficients (R^2) of the calibration curves generated with six standard mixtures. Repeatability of peak areas was calculated by the RSD of five injections carried out on the same day, whereas, for method reproducibility, five injections were randomly executed in a 20-day period. To evaluate method recovery, actual samples were spiked with mixtures of phenolic compounds ($n = 5$) and the percent ratio between the observed and expected concentrations was calculated. For each single phenol, the limit of detection (LOD) was fixed as 3 times the signal-to-noise ratio (S/N).

RESULTS AND DISCUSSION

Method Optimization. Escarpa and Gonzalez compared both acidified acetonitrile and methanol as eluents for rapid RP-HPLC determination of phenols in apples and pears (*15*). Their results showed that methanol allows the most rapid separation of phenolic compounds, whereas acetonitrile gives the best resolution. It was thus decided to join the hydrodynamic advantages offered by the monolithic column with the better resolution demonstrated by acetonitrile. Optimization was initially carried out using 0.01 M H_3PO_4 and acetonitrile for the separation of the mixtures of standard compounds at the concentration ranges in **Table 1**.

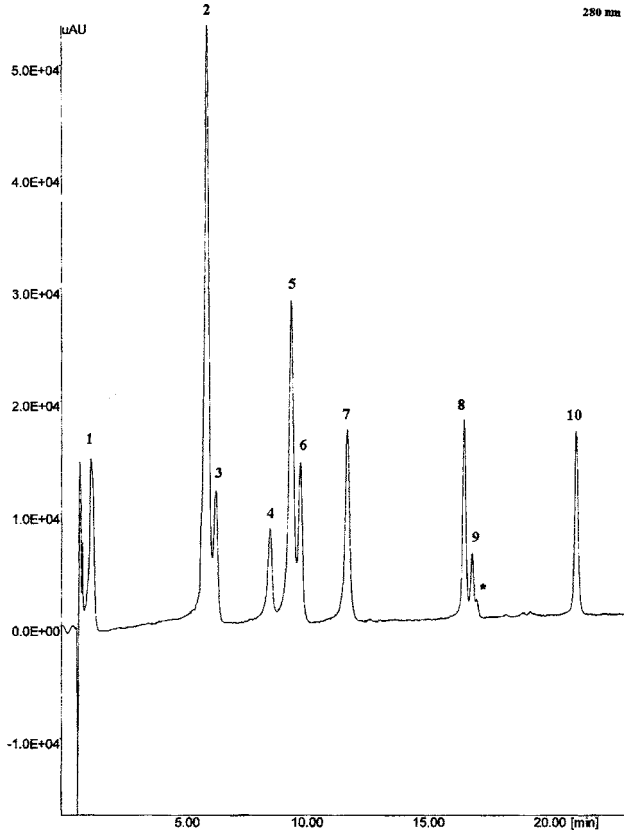

Figure 1. Chromatogram of standard compounds under optimized HPLC conditions. For peak identification see **Table 3**. For chromatographic conditions refer to Materials and Methods. (*) Standard of isoquercitrin in trace amount.

Table 2. Gradient Elution Conditions (A, 0.5% Methanol in 0.01 M H_3PO_4; B, Acetonitrile)

time (min)	A (%)	B (%)	elution
0	96.5	3.5	
11	90	10	linear gradient
22.5	80	20	linear gradient
23.5	96.5	3.5	linear gradient
24.5	96.5	3.5	isocratic

The optimized gradient was affected by the poor separation between (+)-catechin and chlorogenic acid. The resolution of these compounds is very important because their content in apples could be dramatically shifted to chlorogenic acid. A moderate addition (0.5%) of methanol to the 0.01 M H_3PO_4 solvent yielded a useful separation between (+)-catechin and chlorogenic acid. Moreover, the additional presence of this organic modifier induced a faster elution of phloridzin, thereby shortening the total run time. A solvent flow of 2.5 mL/min was used to obtain a suitable compromise between plate height and run time (*19*).

The final gradient, reported in **Table 2**, enabled the separation of the 10 standard compounds in <21 min (**Figure 1**) and showed good values for repeatability and reproducibility (**Table 3**).

LODs ranged from 0.2 μg/mL for coumaric acid to 1.5 μg/mL for procyanidin B2. These data are comparable with data

Table 3. Retention Times, Calibration Parameters, Method Robustness, and Recoveries for Standard Phenolic Compounds

	compound	t_R (min) ± SD ($n = 5$)	calibration curve			repeatability RSD% ($n = 5$)	reproducibility RSD% ($n = 5$)	LOD (μg/mL)	recovery (%) ± SD ($n = 5$)
			a	*b*	R^2				
1	gallic acid	1.18 ± 0.06	24351	–11060	0.9983	4.60	6.32	0.43	98.9 ± 0.3
2	(+)-catechin	5.92 ± 0.11	9275	876	0.9998	2.54	4.89	1.40	98.6 ± 0.2
3	chlorogenic acid	6.16 ± 0.17	25606	809	0.9996	1.82	1.78	0.50	98.7 ± 0.3
4	procyanidin B2	8.49 ± 0.13	6878	–3651	0.9992	4.51	4.80	1.50	90.4 ± 0.5
5	*p*- coumaric acid	9.37 ± 0.10	57205	17225	0.9999	2.12	1.73	0.21	99.0 ± 0.2
6	(–)-epicatechin	9.69 ± 0.15	4643	–1159	0.9991	3.98	4.26	1.19	93.0 ± 0.4
7	ferulic acid	11.62 ± 0.14	50990	12176	0.9999	2.45	2.33	0.25	97.1 ± 0.2
8	hyperin	16.57 ± 0.12	21512	1815	0.9999	1.47	1.25	0.33	95.1 ± 0.4
9	rutin	16.87 ± 0.19	14491	–2254	0.9998	1.20	0.95	0.35	105.0 ± 0.7
10	phloridzin	20.70 ± 0.10	20286	–173	0.9998	1.03	0.67	0.45	98.1 ± 0.3

reported by Escarpa and Gonzalez with the exception of flavanols, for which LODs were twice the values reported by those authors. Method recoveries, calculated by adding known amounts of standard compounds to actual samples, were >95% for almost all of the compounds; only procyanidin B2 and (−)-epicatechin showed lower recoveries, of 91 and 93%, respectively.

Peak Identification. The method was applied to the analysis of apple peel and pulp extracts (cv. Golden Delicious). The extraction of these matrixes was carried out using aqueous methanol after the method of Escarpa and Gonzalez (*9*, *15*) with minor modifications. These authors had recoveries near 100% for compounds representing all of the chemical classes under analysis. Note that methanolic extraction is poorly effective with highly polymerized procyanidins, the latter being exhaustively extracted only when aqueous acetone is used (*20*−*22*). Because of this, RP-HPLC analysis of methanol extracts is simple and without unresolved clumps due to the coelution of highly polymerized procyanidins (*21*).

Chromatograms of apple peel extracts are shown in **Figure 2**. With the exception of *p*-coumaric and ferulic acids, all of the compounds present in the standard mixture were identified in real samples (**Table 5**). Chromatograms at 280 nm were used to quantify flavanols, procyanidins, and dihydrochalcones (**Figure 2**). Flavanols other than (+)-catechin, (−)-epicatechin, and procyanidin B2 were also found. The spectra of these unknown peaks were very similar, and comparison of their spectral features enabled a tentative identification of some.

According to Bartolomè et al. (*23*) the maximum of the second-derivative spectrum (λ_1) for procyanidins is hypsochromically shifted while increasing the number of units forming the oligomer. For each peak, the "min−max distance", defined as the difference between the wavelength of the maximum of the band around 280 nm and λ_1 (both from the second-derivative spectrum), was hence calculated and is reported in **Table 4**. Monomers such as (+)-catechin and (−)-epicatechin showed min−max distances of 30 nm, whereas, for the dimeric procyanidin B2, this value was 28 nm. Consequently, unknown procyanidins showing min−max distances of 28 and 26 nm were tentatively identified as dimers and trimers, respectively.

Bartolomé and co-workers, who set the photodiode detector for a scanning resolution of 0.2 nm, suggested that oligomeric procyanidins of (+)-catechin and/or (−)-epicatechin can be identified by reference to the value of the min−max distance, decreasing by 1.3 nm for each unit. In our case, due to the software limits, a scanning resolution of 1 nm was used, larger differences thus being found after derivative calculation.

As shown in **Table 4**, only monomers, dimers, and trimers were identified in our samples. This is somewhat in accordance with previous findings for apple methanolic extracts, which indicated an average degree of polymerization near 3 (*21*, *22*, *24*).

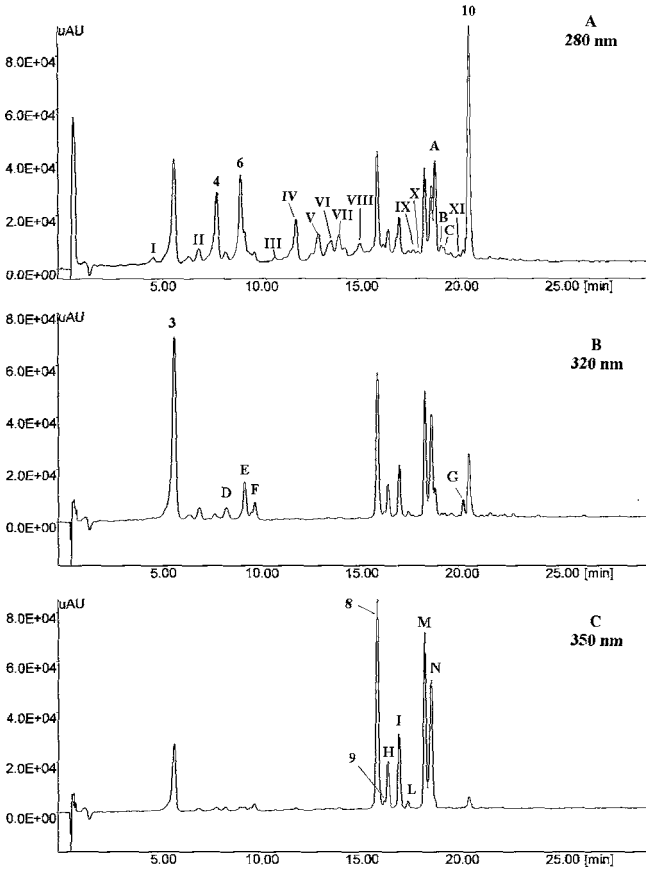

Figure 2. Chromatogram of apple peel extract. Compounds present in the standard mixture are numbered as in **Table 3**. Procyanidin oligomers are labeled with Roman numerals, and tentatively identified peaks are lettered: (**A**) detection at 280 nm [for procyanidin provisional identification (Roman numerals), see **Table 4**; other peaks: 4 = procyanidin B2, 6 = (–)-epicathechin, A = phloretin 2-xyloglucoside, B and C = unknown phloretin derivatives, 10 = phloridzin]; (**B**) detection at 320 nm (peaks: 3 = chlorogenic acid, D = cumaroyl derivative, E = *p*-coumaroylquinic acid, F = feruloyl derivative, G = coumaroyl derivative); (**C**) detection at 350 nm (peaks: 8 = hyperin, 9 = rutin, H = isoquercitrin, I = reynoutrin, L = unknown quercetin glycoside, M = avicularin, N = quercitrin).

Unknown monomers were also found (peaks II and X). They could correspond to (−)-epicatechin or (+)-catechin esterified with a nonphenolic acid.

It should be stressed, however, that data obtained in such a way (useful in the identification of unknown peaks correspond-

Table 4. Spectral Features and Provisional Identification of Unknown Flavanols of **Figure 2** (for Details See the Text)

peak	t_R (min)	λ_{max} (nm)	min–max distance (nm)	second derivative max (nm)	provisional identification
I	4.69	281	28	249	dimer
2	5.92	281	30	247	(+)-catechin
II	6.84	281	30	247	unknown monomer
4	8.49	281	28	249	procyanidin b2
6	9.69	281	30	247	(–)-epicatechin
III	10.87	281	26	251	trimer
IV	11.94	281	28	249	dimer
V	13.05	281	26	251	trimer
VI	13.46	281	26	251	trimer
VII	14.07	281	28	249	dimer
VIII	15.18	281	28	249	dimer
IX	17.56	281	26	251	trimer
X	17.76	281	30	247	unknown monomer
XI	19.98	281	28	249	dimer

ing to procyanidins that are commercially unavailable) need to be carefully evaluated (*23*), and a further LC-MS confirmation appears to be necessary.

Peak A showed a spectrum similar to that of phloridzin, with $\lambda_{max} = 279$ nm. Its retention behavior indicated the additional presence of a more polar moiety. This peak was hence identified as phloretin 2-xyloglucoside, a main phenol in apple and early proposed as a marker for apple products (*4*, *5*). Two other minor peaks (B and C) were identified as phloretin derivatives on the basis of their spectra. Because of the intermediate polarity, a (di)glucoside structure can be supposed. Recently, 3-hydroxyphloretin was identified in apple pomace extracts (*8*). Moreover, it has been reported that phloridzin can be partially replaced by phloretin 4′-glucoside and 3-hydroxyphloretin 4′-glucoside (*25*),

Figure 2B shows the chromatogram at 320 nm. With chlorogenic acid, other hydroxycinnamic derivatives were detected (peaks D−G). Only peak E could be assigned to coumaroylquinic acid, a compound often reported in apple pulps (*3*, *4*, *9*, *11*). Peak F, with λ_{max} at 320 nm, was tentatively identified as a feruloyl derivative on the basis of its spectrum.

Flavonol glycosides were detected at 350 nm (**Figure 2C**). Apart from hyperin and rutin (peaks 8 and 9), isoquercitrin and quercitrin (peaks H and N, respectively) were also identified by comparing their retention times with those of authentic standards. Unfortunately, for these two flavonol glycosides the standard amounts were too small to permit the construction of a suitable calibration curve. Quercitrin and isoquercitrin were thus quantified as hyperin. Peak M showed a λ_{max} of 350 nm, with a 4 nm bathochromic shift when compared with other quercetin glycosides. According to Mayr et al. (*26*), this feature could correspond to avicularin.

The flavonol glycoside elution order we obtained was in agreement with previous studies conducted using RP-HPLC under acetonitrile elution (*3*, *14*), and so the remaining peak I was provisionally assigned to reynoutrin.

An additional unknown flavonol glycoside was found (peak L). Very recently, Lommen et al. (*27*) reported a seventh quercetin glycoside. By coupling HPLC-NMR-MS, they narrowed its identity to quercetin 3-arabinopyranoside (guajeverin) or 3-apiofuranoside, the latter being an uncommon flavonol glycoside due to the presence of apiose, a sugar thought to be diagnostic for the hairy region of rhamnogalacturonan II (*28*).

Table 5. Contents of Phenolic Compounds in Peels and Pulps from Cv. Golden Delicious Apples

	content (mg/kg of fresh wt)					
	peels			pulps		
compound	min	max	av[a] ± SD	min	max	av ± RSD
gallic acid	nd[b]	5.1	1.54 ± 1.23	nd	<LOQ	
(+)-catechin	<LOQ	1.3	0.77 ± 0.38	<LOQ	0.80	0.54 ± 0.21
chlorogenic acid	86	110	101 ± 16.7	67.2	112	88.7 ± 19.5
procyanidin B2	129	164	142 ± 18.9	27.7	52.5	41.1 ± 10.5
(–)-epicatechin	197	255	231 ± 21.9	39.1	59.2	48.2 ± 8.46
p-coumaroylquinic acid[c]	4.2	7.8	5.65 ± 1.33	4.89	7.2	5.79 ± 0.91
hyperin	50	199	115 ± 51.0	0.6	1.6	0.95 ± 0.39
rutin	2.8	13.4	6.76 ± 3.97	nd	<LOQ	
isoquercitrin[d]	15	58	37.2 ± 14.5	nd	<LOQ	
reynoutrin[d]	28.1	71.1	48.9 ± 16.2	1.43	2.8	1.98 ± 0.50
avicularin[d]	70.0	157	110 ± 32.9	1.56	2.87	2.27 ± 0.46
quercitrin[d]	53	151	94.0 ± 36.0	5.06	10.0	7.76 ± 1.95
phloretin 2-xyloglucoside[e]	32	58	40.0 ± 9.01	6.7	10.7	8.62 ± 1.41
phloridzin	84	174	115 ± 35.9	9.2	13.2	11.8 ± 1.52

[a] Mean values of 10 samples. [b] Not detected. [c] Quantified as *p*-coumaric acid. [d] Quantified as hyperin. [e] Quantified as phloridzin.

An unknown flavonol glycoside, with an *m*/*z* of 433 (referable to a quercetin pentoside), was also reported by Schieber et al. (*14*).

Overall, when compared to the method proposed by Escarpa and Gonzalez (*9*, *15*), the present approach enabled a more suitable separation of the main apple phenols in virtually the same elution time. Because of the good column selectivity, procyanidins could be provisionally classified as to their polymerization degree. Moreover, whereas Escarpa and Gonzalez identified four or five glycosides of quercetin (respectively with acidified methanol or acetonitrile as eluent), all six quercetin glycosides reported in the literature as apple constituents were separated and quantified using our method.

Application. Table 5 lists the phenolic composition of the 10 samples of apple peel and pulp. In peels, (−)-epicatechins, procyanidin B2, phloridzin, and chlorogenic acid were the main phenols, with contents varying from 255 mg/kg for (−)-epicatechin to 86 mg/kg for chlorogenic acid. These values largely agree with previous findings for Golden Delicious (*9*, *26*) or other cultivar peels (*8*, *29*). Hyperin, avicularin, and quercitrin proved to be the principal quercetin glycosides, rutin being the least abundant. This latter finding is in accordance with data reported by Mayr et al. (*26*) and by Lu and Foo (*8*) but contrasts with Escarpa and Gonzalez's findings, who found a large predominance of rutin. As found in our trials, the problematic separation of rutin and hyperin and their possible coelution could have affected the quantification carried out by Escarpa and Gonzalez (who reported, in fact, only five quercetin glycosides), explaining these differences.

When compared with peels, apple pulps were poorer in phenolic compounds. Chlorogenic acid was the most abundant phenol, followed by (−)-epicatechin and procyanidin B2. Quercetin glycosides, represented almost exclusively by avicularin, quercitrin, and reynoutrin, were in very low amounts. Phloridzin and phloretin 2-xyloglucoside were identified in all pulp extracts, confirming their potential use as markers for apple juice (*14*).

Our data suggest, because of the beneficial effects of polyphenols on human health, that the consumption of unpeeled, pesticide-free apples should be encouraged.

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