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High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties

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Abstract

Quantitative analysis of phenolic compounds from four apple varieties (Golden and Red Delicious, Granny Smith and Green Reineta) using high-performance liquid chromatography with diode-array detection was carried out. For each variety, both peel and pulp were analysed. The identification of phenolic compounds was made by comparing their retention times and UV spectra with those of standards. The results revealed differences between pulp and peel in all cases studied. The highest levels of phenolic compounds were found in the peel. High levels of catechins and flavonol glycosides, especially rutin, were found in apple peels. Chlorogenic acid was the major peak in the pulp for all apple varieties studied except for Granny Smith. Significant quantitative differences between the apple varieties were also found, the Golden Delicious variety showing the lowest content of phenolic compounds and Green Reineta variety the highest. The recovery of phenolic compounds from both peel and pulp was measured in all apple varieties. The values ranged between 95 and 105%, indicating close to quantitative recovery for the method used.

Keywords: Apples; Fruits; Food analysis; Phenolic compounds; Flavonoids

1. Introduction

Phenolic compounds are found in plants and thus are part of the human diet [1]. Dietary phenolic compounds, and especially the flavonoids, consist mainly of anthocyanidins, flavonols and catechins [2]. They are absorbed from the gastrointestinal tracts of humans and are excreted in faeces and urine. Accurate data on population-wide intakes of flavonoids are not available but a recent review [1] points out that the most important dietary sources are vegetables, fruits and beverages. It is estimated that tea, onions and apples are the main dietary sources of flavonoids [3]. However, current estimates of daily consumption of flavonoids differ considerably [4] and, consequently, more quantitative data are necessary [1].

The phenolic composition of fruits has been studied by HPLC–diode array detection (DAD) in pulps and juices with regard to their contribution to the color and flavor [5], their qualitative and quantitative differences appearing as a function of the species, degree of ripening and storage [6,7] as well as of their presence in commercial juices and jams [8,9].

However, few studies of extracts from fresh peel matrices have been reported [10,11], and comparative studies between peel and pulp are especially limited [12,13]. This comparison is important since apples are consumed both peeled and unpeeled. Taking into account all these considerations, more

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quantitative data of phenolic compounds from apples (peel and pulp) are needed.

The aim of this work was to carry out a comparative systematic study on the quantitative composition of phenolic compounds in fresh pulp and peel from the apple varieties, Golden Delicious, Red Delicious, Granny Smith and Reineta Green (Spanish apple). These varieties are usually consumed as fresh fruits in the Mediterranean diet [14].

2. Experimental

2.1. Reagents and standards

The standards (+)-catechin and (−)-epicatechin as well as gallic, caffeic and chlorogenic acids, phlorizdin and rutin were acquired from Sigma (St. Louis, MO, USA). Methanol of HPLC grade was acquired from Sharlau and all other chemicals of analytical-reagent grade were purchased from Merck. In all cases, the water used was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). All the samples (solutions and extracts) were filtered through 0.45-μm membranes (Millipore) and degassed by an ultrasonic bath before use.

2.2. Materials

Ten apples of four varieties (Golden and Red Delicious, Granny Smith and Reineta Green) were acquired in a local supermarket in Alcalá de Henares (Madrid, Spain). The apples were purchased from the same packet (October 1997). The peel was separated from the pulp. The peel fraction was carefully homogenized and the pulp was cut into little pieces. The sample tissues were extracted immediately and the extracts were stored at −20°C until analysis.

2.3. Apparatus for HPLC

A Varian model HPLC system consisting of a ternary solvent delivery system (9012), an autosampler (9100), and a photodiode array detector (9065) coupled with an analytical workstation was used. The column used was a Nucleosil 120 C_{18} (25 cm×0.46 cm I.D.) with 5 μm packing.

2.4. Extraction procedure

Samples of 5 and 10 g of peel and fresh pulp, respectively, were extracted at room temperature and in the absence of light with methanol containing 1% 2,6-di-*tert*.-butyl-4-methylphenol (BHT) using an ultrasonic bath. The extraction was carried out according to a method previously optimized to obtain a quantitative extraction. The sample was extracted with 10 ml of solvent for 1 h, 10 ml for 30 min, and then 5 ml for 30 min. The three extracts were combined to a final volume of 25 ml. Solutions to be analysed by HPLC were filtered through a membrane filter (0.5 μm pore size) prior to injection.

2.5. Isolation of procyanidins

Procyanidins were isolated from Granny Smith apples by using Sephadex LH-20. A 2-g amount of Sephadex was swelled in water and introduced into the column (0.7×30 cm). The bed was washed with 20% aqueous methanol and 3 ml of apple extracts were carefully applied to the column. Phenolic acids was eluted with 20% aqueous methanol and separated from the procyanidin fraction. These compounds were eluted from the column with methanol.

2.6. HPLC conditions

Detection was performed at 280 nm and the absorption spectra of compounds were recorded between 210 and 350 nm. The elution solvents used were A (aqueous 0.01 *M* phosphoric acid) and B (100% methanol). The samples were eluted according to the following gradient: 5% B as initial condition; 50% B for 10 min; 70% B for 5 min, 80% B for 5 min and finally 100% in B for 5 min. The chromatographic data on the peaks were integrated up to 25 min. The flow-rate was 1 ml/min with a column head pressure of 1500–2000 p.s.i. (1 p.s.i.= 6894.76 Pa) The column was operated at room temperature. The sample injection volume was 20 μl. Identification of compounds was achieved by comparing their retention time values and UV spectra with those of standards stored in a data bank. Concentrations of the phenolic compounds were calculated from integrated areas of the sample and the corresponding standards.

2.7. Recovery studies

The recovery efficiency was determined by adding measured amounts of pure standards (gallic acid, (+)-catechin, (−)-epicatechin, chlorogenic acid and rutin) to the samples prior to extraction of peel and pulp tissues. The samples were prepared as described above and 20 μl of the filtrate were injected into the HPLC column. Controls from both peels and pulps of apple varieties studied were prepared and subjected to the same extraction procedure. The recoveries were determined by subtracting the values obtained for the control matrix preparation from those of the samples prepared with the added standards. The recovery experiment was performed with four replicates. Mean values with standard deviations are reported.

3. Results and discussion

3.1. Separation and identification

Fig. 1 shows the chromatograms of the extracts from peel and pulp. Table 1 lists the retention times including standard deviation (S.D.) for six replicates of each variety studied, the selectivity factor (α), as well as UV absorption maxima of each peak obtained by DAD.

The gradient elution method used allowed a good separation of the phenolic compounds present with values of α above 1.00 in all cases. This method enabled the identification of the phenolic compounds in all samples studied.

(+)-Catechin and (−)-epicatechin (flavanols), chlorogenic and caffeic acids (hydroxicinnamic acids), phloridzin (chalcone) and rutin (a flavonol) were identified in both peel and pulp extracts. Peaks 1–9 shown in Fig. 1 were found in both peel and pulp. Peaks 1, 2, and 4 exhibited spectral characteristics identical with those of catechins, and were tentatively identified as procyanidins. In order to improve the identification of these compounds, procyanidins were isolated from Granny Smith apples by using of Sephadex as described in Section 2.5 [15]. Aqueous 20% methanol allowed the total elimination of phenolic acids and the elution of procyanidins with methanol was achieved by spectral

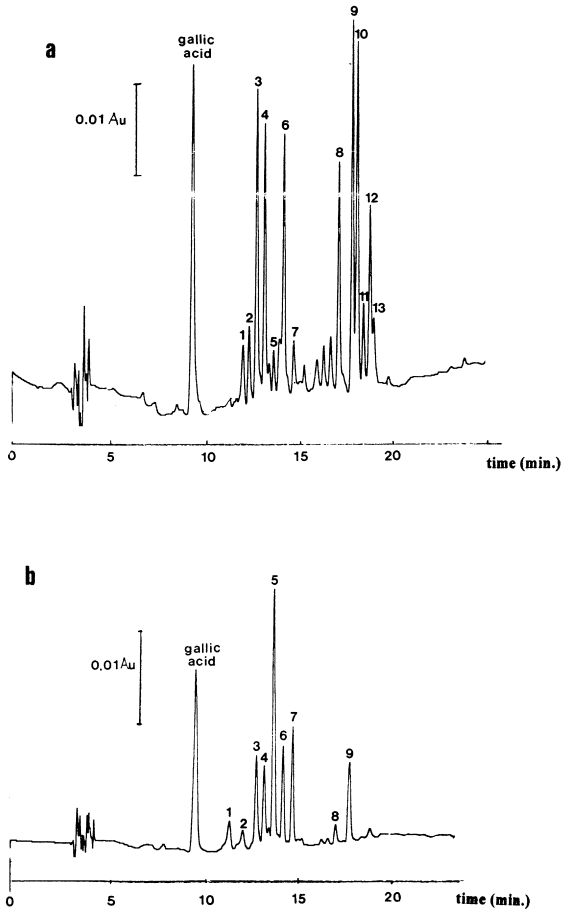

Fig. 1. HPLC chromatogram of (a) Golden peel and (b) Golden pulp extracts at 280 nm. Peaks: 1=procyanidin B3, 2=procyanidin B1, 3=(+)-catechin, 4=procyanin B2, 5=chlorogenic acid, 6=(−)-epicatechin, 7=caffeic acid, 8=phloretin derivative, 9=phloridzin 10=rutin, 11, 12 and 13=flavonol glycosides.

and chromatographic data. Peaks 1 and 2 eluted before (+)-catechin and were assigned to procyanidin B3 and B1 [15]. Peak 4 eluted between (+)-catechin and (−)-epicatechin and was identified as procyanidin dimer B2 [15,16]. Recently, a way has been proposed to establish differences between procyanidins by photodiode-array detection [17]. However, the procyanidins found in these samples showed an absorption maximum near 277.4 (±0.3) nm and procyanidins such as epigallocatechin which is easy to recognize (absortion maxima near 270.6 nm) were not identified. Beside phloridzin (peak 9)

Table 1
Retention times (t_R), selectivity factors (α) and UV absorbance maxima of the phenolic compounds from apple extracts (peel and pulp)

Peak no.	Analyte	λ_{max} (nm)	Apple peel		Apple pulp	
			$t_R \pm$S.D.[a]	α	$t_R \pm$S.D.[a]	α
1	Procyanidin B3	277.7	12.11±0.02	1.08	12.10±0.02	1.07
2	Procyanidin B1	277.7	12.84±0.02	1.03	12.78±0.02	1.02
3	(+)-Catechin	279.7	13.05±0.02	1.04	13.04±0.02	1.04
4	Procyanidin B2	278.1	13.48±0.03	1.05	13.47±0.02	1.04
5	Chlorogenic acid	235.0; 325.4	14.11±0.03	1.06	13.98±0.02	1.01
6	(−)-Epicatechin	279.7	14.42±0.04	1.09	14.43±0.02	1.09
7	Caffeic acid	220.0; 325.3	15.43±0.03	1.17	15.50±0.02	1.17
8	Phloretin derivative	282.4	17.41±0.02	1.05	17.57±0.02	1.04
9	Phloridzin	282.8	18.13±0.02	1.01	18.07±0.03	N.D.
10	Rutin	255.3; 357.6	18.41±0.03	1.02	N.D.	N.D.
11	Flavonol glycoside	255.3; 357.6	18.75±0.02	1.02	N.D.	N.D.
12	Flavonol glycoside	255.3; 357.6	19.08±0.03	1.02	N.D.	N.D.
13	Flavonol glycoside	255.3; 357.6	19.45±0.03	1.02	N.D.	

[a] Values are average of twelve determinations±standard deviation (S.D.) obtained between varieties.
N.D.=not detected.

another phloretin derivative was characterized in all varieties (peak 8). The spectrum indicated that the aglycone was phloretin, and the retention time indicated a more polar compound than phloridzin (phloretin glucoside). This compound has been identified as phloretin xyloglucoside [11,13,16]. In addition, apple peel showed the presence of four flavonol glycosides based on their spectra characteristics. These compounds eluted near the end of the chromatogram, after phlorizin. Peaks 10–13 shown in Fig. 1 were found only in apple peel. Peak 10 was identified as rutin and the next three compounds (peaks 11, 12 and 13) were tentatively attributed to other quercetin glycosides [10–12].

3.2. Quantitative analyses

In order to check the linearity of the relationship between peak area and concentration, six standard solutions were prepared and suitably diluted with methanol. Aliquots of six different concentrations (10, 20, 30, 50, 100 and 120 ppm) of each standard solutions were injected in the HPLC column and the peak areas were determined at 280 nm. The relationship between the concentration and the peak area is shown by the *a*, *b* and *r* values in Table 2, where *a* and *b* are the coefficients of the regression equation $y=ax+b$, *x* being the concentration of the phenolic compound (ppm), *y* the peak area, and *r* the correlation coefficient of the equation. All the phenolic compounds showed good linearity ($r=0.9999$) and obeyed Beer's law in the investigated concentration range of 10–120 ppm.

The limits of detection and quantitation were calculated as amounts exhibiting three and ten times, respectively, the area of the largest noise peak. The

Table 2
Analytical characteristics of the calibration graphs

Analyte	Linear range (μg/ml)	Slope ($\cdot 10^{-4}$)	Intercept ($\cdot 10^{-4}$)	r[a]	Detection limit (μg/ml)	Determination limit (μg/ml)
(+)-Catechin	2.0–120.0	1.5	−2.3	0.9999	0.37	1.23
Chlorogenic acid	1.9–110.0	2.4	−2.9	0.9999	0.40	1.27
(−)-Epicatechin	3.2–100.0	1.4	50.0	0.9999	0.63	2.00
Caffeic acid	2.0–108.0	2.7	−2.7	0.9999	0.33	1.30
Phloridzin	0.7–100.0	4.1	−2.0	0.9999	0.21	0.71
Rutin	2.9–110.0	2.1	−50.0	0.9999	0.42	1.41

[a] Correlation coefficients of the regression equation $y=a+bx$ where *x* is the phenolic compound concentration (ppm) and *y* is the peak area.

values obtained are summarized in Table 2. The detection limits ranged from 0.21 for phloridzin to 0.63 ppm for (−)-epicatechin. The quantitation limits ranged from 0.71 ppm for phloridzin to 2.00 ppm for (−)-epicatechin. These results agree with the experimental values and reveal that the quantitation limit is a true measure of the ability of the method to determine the smallest concentrations of these compounds. The results obtained for the quantitation limits also suggest that the proposed HPLC method is sufficiently sensitive for the determination of the phenolic compounds in this type of sample.

3.3. Recovery of phenolic compounds from peel and pulp of apples

Known amounts (at two different concentrations) of four phenolic compounds found in both apple peels and pulps were added to the fresh tissue prior to the extraction procedure in order to test the accuracy of the overall method. (The recovery of gallic acid — a hydroxybenzoic acid — which was not a component in these samples, was also measured in order to find the recovery of this type of compound). The samples were extracted and prepared as described in Section 2.4. The recoveries of the added phenolic compounds given in Table 3 ranged from 95 to 105% with similar S.D.s (within 5%). These values indicate good accuracy of the method for all matrices studied.

3.4. Application

3.4.1. Quantitative analysis in apple peels

Table 4 lists the phenolic compound contents obtained in peels from Golden, Reineta, Red and Granny apple varieties. The results are given as concentration intervals due to the sizable variability between different apples from the same packet. In contrast, when the extraction procedure was carried out on the same apple, the results obtained showed good precision. If the peel content of different varieties are compared, we observe that the Golden Delicious variety has the lowest phenolic content for all analytes studied, whereas Reineta and Red Delicious varieties contained the highest levels.

Levels of catechins in Reineta, Red and Granny apples were similar. In these samples the major catechins obtained were the monomers (+)-catechin and (−)-epicatechin, as well as procyanidin B2. Chlorogenic acid content was very variable between varieties of apple peel and the Reineta variety showed the highest levels. The major peak observed for red apple peel (peak 7) was attributed to the presence of an anthocyanidin pigment, based on its detection at 525 nm. Anthocyanidins were not found, however, in other varieties studied.

Golden and Red Delicious peel compounds have been quantified by other workers [12,13]; the results obtained in the present work largely agree with the reported values. However, the levels of (+)-catechin, (−)-epicatechin, and rutin obtained in our work were higher than the quantities reported by other researches, whereas the phlorizin levels were lower.

3.4.2. Quantitative analysis in apple pulps

Table 5 lists the phenolic compound content of apple pulps. In agreement with the literature, chlorogenic acid was the major peak identified in all varieties except for Granny Smith variety. High

Table 3
Recoveries of phenolic compounds from peel and pulp samples of Golden, Reineta, Red and Granny Smith apple varieties

Analyte	Mean recovery (%)±S.D.[a]							
	Apple peel				Apple pulp			
	Golden	Reineta	Red	Granny	Golden	Reineta	Red	Granny
Gallic acid	100.7±2.4	104.6±3.1	103.7±2.5	104.2±3.1	98.3±4.2	101.7±2.5	98.9±3.0	105.3±3.0
(+)-Catechin	99.2±2.3	102.4±3.4	104.9±2.3	96.3±4.0	98.7±3.8	100.1±3.1	102.4±3.1	94.4±2.9
Chlorogenic	100.4±4.3	103.3±3.2	104.8±1.6	100.2±3.4	104.5±3.8	102.3±4.0	92.0±2.8	101.0±3.5
(−)-Epicatechin	91.4±1.0	103.8±2.1	104.7±3.2	104.2±3.7	100.0±4.1	97.8±2.5	100.0±4.1	97.1±2.8
Rutin	105.8±2.1	110.3±3.8	107.2±2.4	95.1±2.4	100.3±3.2	98.1±2.9	99.3±2.5	100.1±2.5

[a] Mean values and standard deviation for four replicates.

Table 4
Contents of phenolic compounds in peels from Golden, Reineta, Red and Granny Smith apples

Peak no.	Analyte	Content[a] (mg per kg fresh sample)			
		Golden	Reineta	Red	Granny
1	Procyanidin B3[b]	25–66	125–158	11–14	70–124
2	Procyanidin B1[b]	32–53	103–242	127–172	173–241
3	(+)-Catechin	66–164	229–460	297–445	374–486
4	Procyanidin B2[b]	69–166	388–581	433–659	558–574
5	Chlorogenic acid	17–37	100–440	113–157	6–60
6	(−)-Epicatechin	82–168	238–439	248–481	246–312
7	Caffeic acid	N.D.	8–38	N.D.	10–14
7	Anthocyanidin[b]	N.D.	N.D.	585–1037	N.D.
8	Phloretin derivative	58–71	53–100	61–122	29–31
9	Phloridzin	31–71	83–418	104–159	12
10	Rutin	136–237	327–671	136–504	390–414
11	Flavonol glycoside[c]	35–57	19–146	67–146	164–179
12	Flavonol glycoside[c]	67–180	37–403	190–369	N.D.
13	Flavonol glycoside[c]	N.D.	N.D.	N.D.	N.D.

[a] Mean values for six apples. Each apple was analyzed in triplicate.
[b] Quantified as catechin.
[c] Quantified as rutin.
N.D.=not determined.

Table 5
Contents of phenolic compounds in pulps from Golden, Reineta, Red and Granny Smith apples

Peak no.	Analyte	Content[a] (mg per kg fresh sample)			
		Golden	Reineta	Red	Granny
1	Procyanidin B3[b]	21–27	33–41	20–28	61–100
2	ProcyanidinB1[b]	10–11	57–67	11–21	62–84
3	(+)-Catechin	28–49	113–136	44–70	136–182
4	Procyanidin B2[b]	23–32	82–94	34–54	97–105
5	Chlorogenic acid	29–57	266–357	63–106	28–71
6	(−)-Epicatechin	19–34	91–111	36–59	71–97
7	Caffeic acid	6–9	9–11	2.0	4–5
8	Phloretin derivative	5–8	19–21	2–4	7.0
9	Phloridzin	4–8	16–20	13–14	5.0

[a] Mean values for six apples. Each apple was analyzed in triplicate.
[b] Quantified as catechin.
N.D., not determined.

levels of (+)-catechin and (−)-epicatechin were also observed. The lowest content of phenolic compounds was found in Golden pulps, whereas Reineta apple pulp exhibited the highest levels for all analytes studied.

Values for chlorogenic acid, phloridzin and catechins in apple pulps have been reported. Chlorogenic acid and catechins contents in Reineta apple was very high with respect to values from others varieties reported in the literature [12,13,15,16,18,19].

4. Conclusion

The methodology developed in this work enabled the quantitative determination of the phenolic compounds present in peel and pulp from different varieties of apple. Significant quantitatively differences were observed between the varieties studied. Golden Delicious was the variety with the lowest phenolic content whereas Reineta was the variety with the highest levels of phenolic compounds. In all

cases, apple peels showed higher phenolic content than pulp extracts. These quantitatives differences were due to the flavonol glycosides present, as well as to the high levels of catechins and chlorogenic acid. Since the phenolic compounds content is a function of the matrix studied (pulp and peel), the particular apple, and the variety, more quantitative data are needed to determine the relative percentage of these compounds in the Mediterranean diet.

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