JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitation of Polyphenols in Different Apple Varieties

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Forty-one apple samples, representing eight of the most widely cultivated varieties in western Europe, were collected in Trentino, Italy. Samples were extracted from fresh fruit with a mixture of acetone/water to achieve a good extraction of polyphenols, including proanthocyanidin oligomers which were analyzed by normal-phase HPLC. Up to 20 compounds including catechin, epicatechin, B_2 procyanidin, hydroxycinnamates, flavonols, anthocyanins, and dihydrochalcones were analyzed by reversed-phase HPLC and LC-MS. Total polyphenol content was independently measured with an optimized Folin−Ciocalteu assay. The mean content of total polyphenols lay between 66.2 and 211.9 mg/100 g of FW depending on the variety. With chromatographic analysis, it was possible to explain the whole amount of total polyphenols measured by the FC assay. Flavanols (catechin and proanthocyanidins) are the major class of apple polyphenols (71−90%), followed by hydroxycinnamates (4−18%), flavonols (1−11%), dihydrochalcones (2−6%), and in red apples anthocyanins (1−3%).

KEYWORDS: Apple; polyphenols; hydroxycinnamates; flavonols; dihydrochalcones; anthocyanidins

INTRODUCTION

Fruits and certain kinds of beverages and to a lesser extent vegetables are the most important sources of polyphenolic antioxidants in the human diet. It is possible to show evidence that the habit of regular consumption of certain kinds of food and beverages such as apples, berries, red wine, coffee, and tea may influence significantly the quantity of polyphenolic antioxidants in a diet. Regular daily consumption of these compounds is considered important for the assurance of daily protection, since they are relatively quickly metabolized in our organism. A daily intake of 1 g/day of polyphenols with the Western diet has been suggested in a pioneering paper by Kühnau (*1*), and such a value was also thought reliable by Scalbert and Williamson (*2*) who estimate that flavonoids contribute approximately two-thirds of the amount of polyphenols in the Western diet while phenolic acids contribute the remaining one-third.

Apples are an important source of polyphenolic antioxidants which are responsible for most of the antioxidant activities of the fruit, that is, far over the amount explained by the presence of ascorbic acid (*3*). It was confirmed that regular use of apples in a diet contributes in a significant way to the intake of polyphenols (*4*, *5*). Especially for the class of flavanols (*4*) and flavonol glycosides (*5*), apples turn out absolutely as one of the most important dietary sources. A recent study estimated that the major source of proanthocyanidins in the American diet are apples with 32.0% and reported that proanthocyanidins account for a major fraction of the total flavonoids ingested in the Western diet (*6*).

A recent paper reported on the polyphenolic profiles in eight apple cultivars, in which the amounts of polyphenols in apple skin and flesh obtained by spectophotometric and chromatographic methods were compared (*7*). However, there were not any data about oligomeric proanthocyanidins reported in the study. Most of the results concerning the dessert apples do not take into account proanthocyanidins which are the major polyphenolic compounds in dessert varieties (*8*). The quantification of oligomeric and polymeric proanthocyanidins is often underestimated because only dimers and trimers which can give resolved peaks are considered (*9*). It was reported that in immature apples about one-half of proanthocyanidins are monomeric flavan-3-ols (catechin, epicatechin), dimeric and trimeric proanthocyanidins, and the other half are proanthocyanidins of higher degree of polymerization (*10*). Guyot et al. (*8*) reported that in dessert apples the average degree of polymerization depends on the variety and tissue varying from 5.7 to 7.1.

The present study discusses the differences in the composition of polyphenols including 20 different compounds plus the oligomer proanthocyanidins and ascorbic acid which were thoroughly investigated in eight apple varieties sampled in Trentino, Italy, and provides a sound explanation for the total amount of polyphenols in fruits.

MATERIALS AND METHODS

Standards and Reagents. All chromatographic solvents (methanol, acetonitrile, acetone, formic acid) were HPLC grade and were purchased

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10.1021/jf049317z CCC: $27.50 © 2004 American Chemical Society
Published on Web 09/28/2004

from Carlo Erba (Rodano, Italy); metaphosphoric acid and Folin−Ciocalteu were purchased from Merck (Milano, Italy).

(−)-Epicatechin, (+)-catechin, phloridzin, and 5′-caffeoyl quinic acid were purchased from Roth (Germany), ascorbic acid from Carlo Erba (Italy). Quercetin 3-glucoside was purchased from Indofine Chemical Co (Hillsborough, NJ), cyanidin 3-galactoside, cyanidin 3-arabinoside, and cyandine 3-glucoside from Polyphenols Laboratories (Sandnes, Norway). β-glucosidase, β-xylosidase, β-hesperidinase, and β-galactosidase were purchased from Sigma (Steinheim, Germany).

The buffer solution at pH 7.0 ± 0.05 (20 °C) was prepared by diluting to 500 mL with water the potassium dihydrogen phosphate−sodium hydrogen phosphate Normex solution (final 17.34−27.2 mmol/L, respectively) from Carlo Erba (Italy).

Sampling. The study included 41 samples which represent eight of the most widely cultivated varieties: Braeburn (2), Fuji (3), Golden Delicious (9), Granny Smith (3), Morgenduft (4), Renetta (6), Royal Gala (7), and Red Delicious (7). The apples (≥3 kg for each lot) were sampled in Trentino, Italy, between August and November 2001. Samples of the cultivar Renetta were collected in six orchards covering the area included in the Protected Designation of Origin "Val di Non". All the other varieties were sampled to cover, in a representative manner, the whole cropping area of Trentino: Valsugana, Arco, Bleggio Superiore, Sarche, Vigolana, and Trento South and North. Apples of regular size (within 70−80 mm in diameter) were collected from 20 to 25 trees, chosen at random following an X-path in each orchard. Harvest time was decided on the ground of the ripening indices commonly used by the Organization of Producers in Trentino: flesh firmness, sugar content by refractometry, titratable acidity and starch, evaluated by colorimetry (ordinal scale from 1 to 5). The Thiault quality index, which is the sum of the concentration of sugars, expressed in g/L, plus the titratable acidity, expressed as g/L of malic acid and multiplied by 10, was in the range 160−200 for all the samples.

Extraction of Polyphenols. Polyphenols were extracted following the method of Mattivi et al. (*11*). To limit the enzymatic and chemical reactions (especially oxidation), both the apples and the extraction solution were cooled to 4 °C. The core of three fruits per sample was removed with a corer and each apple was cut into equal slices. Three slices (cortex + skin) from the opposite side of each fruit (total weight 90 g) were used for the preparation of aqueous acetone extracts. The sample was homogenized in a blender Osterizer mod. 847-86 at speed one in 375 mL of mixture acetone/water (70/30 w/w). The centrifuged extract was divided into two aliquots and stored at −20 °C for the analysis of polyphenols and antioxidant capacity (*12*).

Extraction of Ascorbic Acid. An aliquot of 100 g of the edible part of the frozen fruit sampled with the same technique as for polyphenols was homogenized in 1 L of a solution of metaphosphoric acid (6%) in water containing sodium meta-bisulfite (1 g/L) for the extraction of ascorbic acid.

The HPLC analyses were done immediately after the sample extraction according to Mattivi et al. (*11*).

Sample Preparation (Normal-Phase HPLC). An aliquot of 50 mL of the extract was evaporated to about one-third of the volume by rotary evaporation under reduced pressure and at 35 °C. The sample was neutralized at pH ≥7.00 with NaOH 1 M. To remove the organic acids, sugars, amino acids, proteins, and other hydrophilic compounds which could cause interference, a cleanup was performed with a Sep-Pak C-18 (Waters, Milford, MA, 1 g) previously conditioned with 5 mL MeOH followed by 20 mL of buffer solution, pH = 7.0. The neutralized extract was slowly loaded on the conditioned Sep-Pak, and the polar substances—including the 5′-caffeoyl quinic acid and the 4′-*p*-coumaroyl quinic acid—were removed with 20 mL of buffer solution, pH = 7.0, and 4 mL of water. The flavan-3-ols were eluted with 20 mL of MeOH, brought to dryness by rotary evaporation, and dissolved in 1 mL of methanol for HPLC analysis.

Sample Preparation (Flavonols, Dihydrochalcones, Flavan-3-ols, and Hydroxycinnamates). To remove acetone from the extract, 20 mL of extract was evaporated to about one-third of the volume in a 100-mL pear-shaped flask using rotary evaporation and reduced pressure at 35 °C. The sample was brought back to 20 mL with deionized water (for flavonols and dihydrochalcones) or with the initial HPLC solvent (for flavan-3-ols and hydroxycinnamates) and filtered through 0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA) into LC vials for HPLC analysis.

Sample Preparation (Anthocyanins). An aliquot of 10 mL of extract was evaporated to about one-third of the volume using rotary evaporation and reduced pressure at 35 °C. The sample was brought back to 10 mL with 0.5 M sulfuric acid. An aliquot of 5 mL of extract reconstituted in sulfuric acid was applied to a Sep-Pak C-18 cartridge (Waters, 0.5 g) previously activated with 2 mL MeOH, followed with 5 mL of 5 mM sulfuric acid. The cartridge was washed with 3 mL of 5 mM sulfuric acid and the anthocyanins were eluted with 2 mL of MeOH. The eluate was brought to dryness with a rotary evaporator and dissolved in 2 mL of initial HPLC solvent.

Spectrophotometric Analysis. The total amount of polyphenols was measured with an optimized Folin−Ciocalteu method (*13*) according to which interfering compounds such as sugars, amino acids, and ascorbic acid were removed by cleanup on a C-18 cartridge (0.5 g, Sep pak, Waters) from the sample reconstituted in water as described above. The results are expressed as equivalent of (+)-catechin, mg/100 g of FW.

Enzymatic Hydrolysis of Flavonol Glycosides. An aliquot of 10 mL of extract reconstituted in water was applied to a C18 SPE cartridge (0.5 g, Sep-Pak, Waters) previously activated with 3 mL MeOH, followed with 5 mL of H_2O. Flavonols were eluted with ca. 10 mL of ethyl acetate, and the eluate was brought to dryness with rotary evaporator and to the initial volume with citrate buffer at pH 5. Afterward, specific enzymes were added: β-glucosidase, β-galactosidase, β-xylosidase, and β-hesperidinase. The disappearance of single peaks in the chromatogram and the formation of the corresponding aglycon was observed using HPLC after 1-h incubation at 35 °C with a specific enzyme.

HPLC Analysis (Normal Phase). The HPLC analysis of oligomeric proanthocyanidins was performed on a Hewlett-Packard Series 1090 instrument equipped with DAD and HP ChemStation and using a normal-phase column Luna Silica-2 (Phenomenex, 5 μm, 250 × 4.6 mm). The solvents used were the following: A, 1% formic acid in methylene chloride, and B, 1% formic acid in methanol. The gradients were as follows: from 8% to 50% B in 60 min, back to 8% B in 5 min. The column was equilibrated for 15 min prior to each analysis. The flow rate was 1.0 mL/min, the injection volume was 10 μL, and the analysis was at room temperature (20 °C). The UV−vis spectra were recorded from 210 to 400 nm, with detection at 280 nm, and data expressed as epicatechin. A cacao bean proanthocyanidins extract was injected at the beginning and at the end of each sequence (of about eight samples) to compare the retention times and the baseline.

HPLC Analysis (Reversed Phase). The HPLC analysis of flavan-3-ols (catechin, epicatechin, procyanidin B_2) and hydroxycinnamates was performed on a Hewlett-Packard Series 1090 instrument equipped with diode array detector (DAD) and HP ChemStation using a reversed-phase column Hypersil ODS RP-18 (Agilent, 250 × 2.1 mm, 5 μm) and precolumn Hypersil ODS (20 × 2.1 mm, 5 μm). The following solvents were used: A, 0.5% formic acid in water, and B, 2% formic acid in methanol. The gradients were as follows: from 8% to 22% B in 14.3 min, from 22% to 27.8% B in 5.5 min, from 27.8% to 100% B in 1 min, 100% B for 2 min, back to 8% B in 2 min. The column was equilibrated for 7 min prior to each analysis. The flow rate was 0.4 mL/min, the injection volume 10 μL, and the oven temperature was 40 °C. The detection of flavan-3-ols was at 280 nm and that of hydroxycinnamates at 320 nm. Each compound was quantified as mg/100 g of FW by means of calibration with external standard. Procyanidin B_2 was expressed as equivalent of epicatechin, and 4′-*p*-coumaroyl quinic acid was expressed as 5′-caffeoyl quinic acid. The coefficients of variation (CV), obtained experimentally with six analyses of the same Renetta extract, were the following: (+)-catechin, 1.96%; procyanidin B_2, 0.58%; (−)-epicatechin, 2.34%; 5′-caffeoyl quinic acid, 0.50%; 4′-p-coumaroyl quinic acid, 0.58%; and p-coumaric acid, 0.14%.

The analyses of anthocyanidin glycosides, flavonol glycosides, and dihydrochalcone glycosides were carried out on a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters, Milford, MA), Micromass ZQ electrospray ionization mass spectrometry (ESI-MS) system (Micromass, Manchester, U.K.), and MassLynx Software version

3.5 (Micromass, Manchester, U.K.). The separation was performed using a column Xterra MS C18, 3.5 μm, 2.1 × 150 mm, precolumn Xterrra MS C18, 3.5 μm, 2.1 × 10 mm (Waters, Milford, MA).

The mobile phases for the HPLC analysis of anthocyanins consisted of 5% formic acid in H_2O (A) and 5% formic acid in methanol (B). The separation was carried out at 40 °C in 30 min under the following conditions: linear gradients starting at 10% B, to 30% B in 10 min, to 40% B in 7 min, to 51.2% B in 4 min, to 64% B in 5 min, to 90% B in 4 min. The column was equilibrated for 7 min prior to each analysis. The flow rate was 0.2 mL/min and the injection volume 5 μL. The UV−vis spectra were recorded from 230 to 600 nm with detection at 520 nm. The MS detector operated at capillary voltage 3000 V, extractor voltage 6 V, source temperature 105 °C, desolvation temperature 200 °C, cone gas flow (N_2) 30 L/h, desolvation gas flow (N_2) at 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of mass analyzer. ESI-mass spectra ranging from *m*/*z* 200 to 700 were taken in positive mode with a dwell time of 0.1 s. The cone voltage (CV) was set in scan mode at the values of 50 V for the quantification based on the aglycon peak and of 30 V for the identification based on both the fragment aglycon and the molecular ion. Samples were quantified by single ion monitoring in MS. The single ion (*m*/*z* 287.2, CV 50 V) was monitored for the quantification of all cyanidin derivatives (with the external standard method) and expressed as cyanidin 3-glucoside, mg/100 g of FW. Peaks were identified on the basis of comparison of retention times, UV−vis, and MS spectra with standards (cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-glucoside). The identity of cyanidin 3-xyloside was confirmed by enzymatic hydrolysis. After the preparation of sample as for flavonols and the addition of β-xylosidase, the selective disappearance of cyanidin 3-xyloside peak in the chromatogram and the formation of the corresponding aglycon were observed. Also, the MS spectra of this peak corresponded to cyanidin-pentose (*m*/*z* 419.2). The coefficients of variation (CV), obtained experimentally with six analyses of the same Morgenduft extract, were 2.52% for cyanidin 3-galactoside and slightly higher for the minor components: cyanidin 3-arabinoside, 5.12%; cyanidin 7-arabinoside, 3.25%; and cyanidin 3-xyloside, 2.53%.

The mobile phases for the HPLC analysis of flavonol glycosides and dihydrochalcone glycosides consisted of 1% formic acid in H_2O (A) and acetonitrile (B). The separation was carried out at 40 °C in 30 min, under the following conditions: linear gradients starting at 15% B, to 17.4% B in 16 min, to 100% B in 14 min. The column was equilibrated for 7 min prior to each analysis. The flow rate was 0.2 mL/min and the injection volume 10 μL. The UV−vis spectra were recorded from 230 to 500 nm with detection at 350 nm. The MS detector operated at capillary voltage 3000 V, extractor voltage 5 V, source temperature 110 °C, desolvation temperature 250 °C, cone gas flow (N_2) 30 L/h, and desolvation gas flow (N_2) at 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of mass analyzer. ESI-mass spectra ranging from *m*/*z* 200 to 700 were taken in positive mode with a dwell time of 0.1 s. The cone voltage (CV) was set in scan mode at the values of 50 V for the identification based on the aglycon peak and of 30 V for the identification based on both the fragment aglycon and the molecular ion. Quercetin derivatives were quantified by single ion monitoring (*m*/*z* 303.2, CV 45 V) in MS with the external standard method and expressed as quercetin 3-glucoside, mg/100 g of FW. The coefficients of variation (CV), obtained experimentally with six analyses of the same Renetta extract, were the following: quercetin 3-rutinoside, 3.27%; quercetin 3-galactoside, 2.32%; quercetin 3-glucoside 4.50%; quercetin 3-xyloside, 1.59%; quercetin 3-arabinoside, 2.29%; and quercetin 3-rhamnoside, 1.29%.

Dihydrochalcone glycosides were analyzed with the same method as flavonols with detection at 280 nm. The peaks were quantified by UV−vis and the peak identities were confirmed by MS detector in positive mode—operating as for flavonols—and expressed as phloridzin, mg/100 g of FW. The coefficients of variation (CV), obtained experimentally with six analyses of the same Renetta extract, were 0.96% for phloridzin, 1.07% for phloretin-xyloglucoside, and 2.36% for 3-hydroxyphloridzin.

Ascorbic Acid. The separation was performed on a Hewlett-Packard Series 1100 instrument equipped with DAD and HP ChemStation using a reversed-phase column Discovery, RP C18 (Supelco, 250 × 4 mm, 5 μm). The solvent used was 0.07% phosphoric acid in water. The isocratic separation was carried out at 30 °C, and the retention time of ascorbic acid was 7.3 min. After each run, the column was washed 5 min with 15% acetonitrile in 0.07% phosphoric acid in water and afterward equilibrated for 7 min prior to each analysis. The flow rate was 0.6 mL/min, the injection volume was 20 μL, and the UV−vis signal was set at 243 nm.

RESULTS AND DISCUSSION

Variety. The varieties chosen for the study included the four "old" cultivars Golden Delicious, Red Delicious, Granny Smith, and Morgenduft which together with Royal Gala are the five most widely cultivated in Italy according to Eurofel (Brussels) forecast data for the 2002 production (*14*). Fuji, Braeburn, and Royal Gala are the "new" varieties for which importance in western Europe has been growing most rapidly and for which production increased in Europe in 2002 by 67%, 49%, and 40%, respectively, with regards to the average production of the five previous years from 1997 to 2001 (*14*). Renetta belongs to a group of peculiar traditional varieties cultivated in western Europe, in particular in France and Italy. The varieties chosen for this survey represented 88.1% of the total production of the dessert apple in Italy with similar percentages for southern Europe (France, Spain, Greece, and Portugal). In the total of western Europe (EU-15), these eight varieties represented 70.1% of the 2002 production.

All the samples were collected in Trentino, Italy, but in the light of the importance of the varieties investigated it is expected that the results of this survey will allow us to draw a very realistic estimate of the intake of apple polyphenols in both Italy and western Europe.

All data appearing in this paper are given in milligram per 100 g FW while for the study of antioxidant activity the data on molar basis were found more convenient (*12*).

Total Polyphenols. The average content of total polyphenols in the apple evaluated by the FC assay was 110.2 mg/100 g of fresh fruit with significant differences depending on the apple variety (**Table 1**). Renetta has a much higher content of total polyphenols than any other variety in this study. The mean content of total polyphenols lies between 66.2 and 211.9 mg/100 g of FW according to the variety in the following increasing order: Fuji, Braeburn, Royal Gala, Golden Delicious, Morgenduft, Granny Smith, Red Delicious, and Renetta. These data confirm the fact that regular consumption of apples in a diet can contribute an important amount of polyphenolic antioxidants. A single serving of apple (150 g) can contribute from 100 mg (Fuji) to more than 300 mg (Renetta) of total polyphenols.

The aim of this work was to build a complete picture of apple polyphenols including oligomeric proanthocyanidins since their presence as a major component of the apple was already demonstrated by thioacidolysis (*8*), normal-phase fractionation (*10*), and conversion to anthocyanidins by the Bate−Smith reaction (*11*). The mixture acetone/water used for the extraction allowed to obtain stable extracts because of the denaturation of polyphenol oxidase; in addition, a good extraction of polyphenols, including proanthocyanidins, 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 are not extracted (*9*, *15*, *16*, *17*). For cider apple varieties, it was reported that the average degree of polymerization was around 3 for methanol extractable proanthocyanidins and around 11 for aqueous acetone extractable proanthocyanidins (*9*). Such oligomeric compounds cannot be resolved at molecular level but can be quantified with

Table 1. Average Concentration of Ascorbic Acid, Total Polyphenols (FC Assay), Flavanols, Hydroxycinnamates, Flavonols, Dihydrochalcones, and Anthocyanins (Milligrams per 100 g of FW) in Different Apple Varieties and Theoretical Percentage of the FC Assay Calculated from the Chromatographic Data[a]

	Renetta	Red Delicious	Granny Smith	Morgenduft	Golden Delicious	Royal Gala	Braeburn	Fuji
no. of samples	6	7	3	4	9	7	2	3
ascorbic acid	5.4	0.7	2.7	5.6	7.7	0.4	8.1	2.1
sd	2.66	0.51	0.31	2.73	2.83	0.32	0.28	0.85
total polyphenols	**211.9**	**131.1**	**121.0**	**105.8**	**86.3**	**83.9**	**75.4**	**66.2**
sd	*20.3*	*13.8*	*7.0*	*8.0*	*7.5*	*12.3*		*13.1*
fraction of polyphenols explained by HPLC (%)	113.6	99.4	96.9	114.6	100.6	98.7	97.3	100.8
5′-caffeoyl quinic acid	30.60	7.09	4.01	17.81	9.18	9.03	4.48	12.34
p-coumaroylquinic acid	7.10	2.46	0.34	0.81	1.35	1.99	1.67	0.76
p-coumaric acid	0.70	0.32	0.13	0.27	0.16	0.44	0.30	0.31
total hydroxycinnamates	**38.40**	**9.87**	**4.48**	**18.89**	**10.69**	**11.46**	**6.45**	**13.41**
sd	*8.30*	*1.20*	*0.86*	*3.20*	*1.90*	*1.30*		*4.60*
(+)-catechin	3.40	2.37	2.60	1.37	0.47	1.09	0.45	0.82
(–)-epicatechin	18.40	10.55	9.04	10.18	5.18	5.66	5.40	6.11
procyanidin B_2	19.30	7.91	10.66	10.69	6.76	5.56	5.77	6.50
proanthocyanidin oligomers	162.2 4	91.61	83.15	77.40	58.39	55.53	47.30	38.81
total flavanols	**203.3 4**	**112.44**	**105.45**	**99.64**	**70.80**	**67.84**	**58.92**	**52.24**
sd	*26.50*	*17.00*	*9.20*	*5.70*	*8.70*	*14.90*		*16.90*
cyanidin-3-galactoside	nd	1.94	nd	3.11	nd	0.82	0.41	0.33
cyanidin-3-arabinoside	nd	0.19	nd	0.14	nd	0.12	0.04	0.03
cyanidin-7-arabinoside (ti)	nd	0.19	nd	0.19	nd	0.05	0.03	0.02
cyanidin-3-xyloside	nd	0.20	nd	0.23	nd	0.02	0.04	0.02
total anthocyanins	**nd**	**2.52**	**nd**	**3.67**	**nd**	**1.01**	**0.52**	**0.40**
sd	*nd*	*1.25*	*nd*	*2.80*	*nd*	*0.40*		*0.10*
3-hydroxyphloridzin	0.23	0.28	nd	nd	0.04	0.06	0.23	0.02
phloridzin	9.11	3.23	0.64	0.85	1.49	0.76	1.01	1.31
phloretin-xyloglucoside	6.14	0.85	1.38	1.62	1.26	1.17	1.04	0.68
total dihydrochalcones	**15.48**	**4.36**	**2.02**	**2.47**	**2.79**	**1.99**	**2.28**	**2.01**
sd	*2.70*	*0.90*	*0.40*	*0.50*	*0.50*	*0.30*		*0.40*
quercetin-3-rutinoside	0.15	0.19	0.15	0.18	0.17	0.15	0.22	0.25
quercetin-3-galactoside	0.96	2.23	1.63	1.71	2.86	2.39	4.11	1.85
quercetin-3-glucoside	0.29	0.41	0.51	0.69	0.69	0.47	0.75	0.41
quercetin-3-xyloside	0.54	0.80	0.63	0.76	0.70	0.54	0.97	0.60
quercetin-3-arabinoside	0.78	1.42	0.98	1.17	1.17	1.12	1.30	1.02
quercetin-3-rhamnoside	0.56	0.59	0.91	0.80	1.39	0.67	0.78	0.54
quercetin	0.12	0.22	0.12	0.12	0.16	0.12	0.19	0.10
total flavonols	**3.40**	**5.86**	**4.93**	**5.43**	**7.14**	**5.46**	**8.32**	**4.77**
sd	*1.10*	*3.00*	*1.70*	*1.60*	*2.00*	*2.50*		*2.00*

[a] Legend: sd, standard deviation; nd, not detectable; ti, tentative identification.

precision by NP HPLC−UV as already proven for the evaluation of oligomers and polymers in wine (*18*). A good correlation between this method and spectrophotometric indexes for determination of low and high molecular mass proanthocyanidins in wine was reported (*19*).

A typical chromatogram is shown in **Figure 1**, top. The whole area under the broad band corresponding to the oligomer proanthocyanidins was integrated and corrected for the area of phloridzin (peak 4 in **Figure 1**) which elutes as a single, well-resolved peak and is the only interfering compound present in this area of the chromatogram. Monomer anthocyanins and flavonols do not cause intereference with the normal-phase method (*18*−*19*). The cleanup of apple proanthocyanidins was modified in respect of that of ref 19, so as to remove the hydroxycinnamates, which do absorb at 280 nm. The apple proanthocyanidins elute in the normal-phase chromatogram at similar retention times as the cacao beans proanthocyanidins (**Figure 1**, bottom). The linear gradient elution causes a moderate drift in the baseline, which is similar for both extracts, and must be taken into account for a correct integration (**Figure 1**).

Single Classes of Polyphenols. With chromatographic analysis, it was possible to quantify all the major polyphenols present in the extract. The use of various columns for the analysis was time-consuming but it was chosen since it allowed a baseline separation and a precise quantification of all the major compounds. The oligomeric proanthocyanidins were analyzed by NP HPLC−UV and catechin, epicatechin, B_2 procyanidin, and hydroxycinnamates by reversed-phase HPLC−UV (**Table 1**). Flavonol glycosides, anthocyanidin glycosides, and dihydrochalcone glycosides were analyzed by RP HPLC−UV as well as with MS in positive mode (**Table 1**). In this way, it was possible to build a detailed database containing the amount of up to 20 single compounds belonging to five different chemical classes and the absolute concentration of the oligomeric proanthocyanidins which were not resolved at the molecular level. The final results for the percentage distribution of the different classes of polyphenols in each variety are presented in **Table 2**.

1. Flavanols. Flavanols (catechins, dimers, and oligomeric proanthocyanidins), which are the major class of apple polyphenols (**Table 1**), represent between 71 and 90% of the polyphenolic compounds in apple (**Table 2**). These data are in agreement with other authors (*8*, *9*, *17*). The variety with the highest relative amount is Granny Smith (90% of all polyphenolic compounds). Oligomeric proanthocyanidins represent the major fraction inside this class. The average concentrations for single varieties range between min 38.8 mg/100 g in the variety Fuji up to 162.2 mg/100 g in Renetta (**Table 1**). Among monomers and dimers, the most important compounds are procyanidin B_2 (5.6−19.3 mg/100 g) and epicatechin (5.2−18.40 mg/100 g) while the presence of catechin is lower (0.45−3.40 mg/100 g). Our results showed

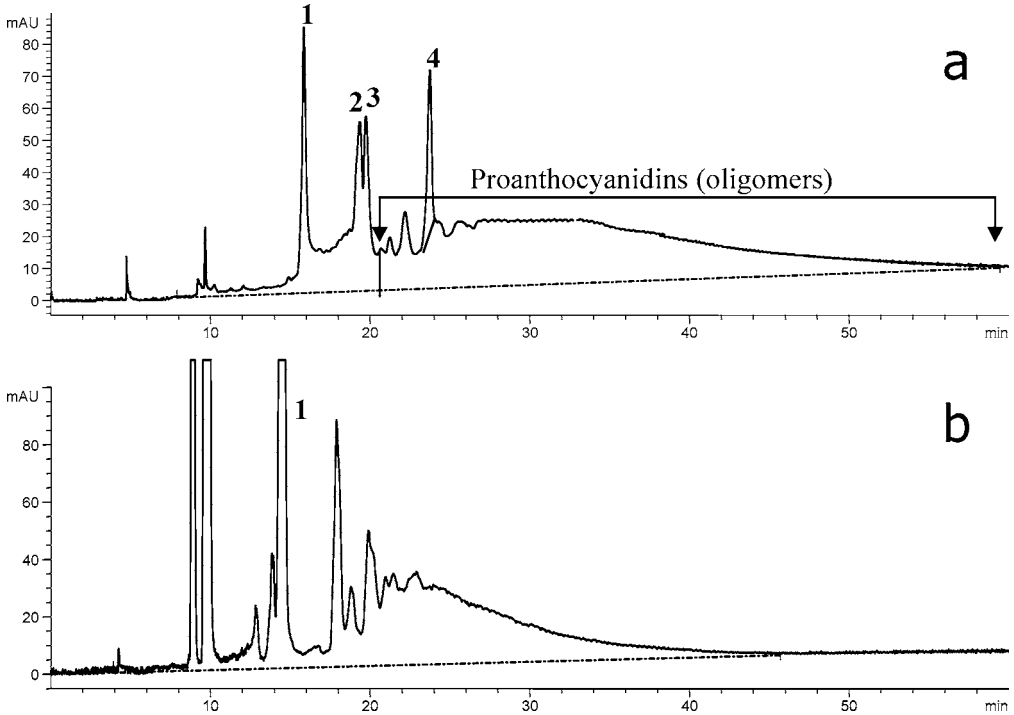

Figure 1. Normal-phase HPLC chromatogram monitored at 280 nm of (**a**) the purified apple extract, cv. Granny Smith (top), and of (**b**) a cacao bean proanthocyanidins extract (bottom). Legend: 1, epicatechin and catechin; 2, phloretin-2-xyloglucoside; 3, procyanidin B_2; 4, phloridzin.

Table 2. Average Relative Composition of Different Apple Varieties (Percent)

	Renetta	Red Delicious	Granny Smith	Morgenduft	Golden Delicious	Royal Gala	Braeburn	Fuji
hydroxycinnamates	15	7	4	15	12	13	8	18
flavanols	78	84	90	76	77	78	77	71
anthocyanins	0	2	0	3	0	1	1	1
dihydrochalcones	6	3	2	2	3	2	3	3
flavonols	1	4	4	4	8	6	11	7

that oligomeric polyphenols represent from 59% (Fuji) to 77% (Renetta) (**Table 1**) of the total polyphenols obtained by spectrophotometric method and from 53% (Fuji) to 71% (Granny Smith) of the sum of HPLC values of polyphenols.

2. Hydroxycinnamates. In apple, significant concentrations of hydroxycinnamates are present (**Table 1**) representing from 4 to 18% of apple polyphenols (**Table 2**). In the percentage of total polyphenols, the highest concentrations of hydroxycinnamates were found in Fuji (18%) (**Table 2**) while in the absolute amount the richest variety was Renetta which contains 38.4 mg/100 g on the average (**Table 1**). The concentrations in other varieties varied between 4.48 mg/100 g (Granny Smith) and 18.9 mg/100 g (Morgenduft). The main compound was always 5′-caffeoyl quinic acid (4.0−30.6 mg/100 g) followed by *p*-coumaroyl quinic acid (0.30−7.10 mg/100 g) while *p*-coumaric acid was present only in traces (0.13−0.70 mg/100 g). The results confirmed earlier reports on 5′-caffeoyl quinic acid as the main monomeric polyphenol in apple (*20*−*22*), with the exception of Red Delicious and Granny Smith, in which the content of epicatechin was higher (**Table 1**). In the variety Braeburn, the amounts of 5′-caffeoyl quinic acid and epicatechin were similar (**Table 1**).

3. Flavonols. Because of a strong bioactivity of quercetin, this class of compounds is surely the most studied class among all the flavonoids of apple and represents 1−11% polyphenols in this fruit (**Table 2**). Flavonols in apple are a mixture of six different quercetin glycosides with only traces of free quercetin. Among monosaccharides, 3-galactoside, 3-arabinoside, 3-rhamnoside, 3-xyloside, and 3-glucoside followed by minor amounts of 3-rutinoside appear. The average concentrations between the varieties are very variable in the following increasing order: Renetta, Fuji, Granny Smith, Morgenduft, Royal Gala, Red Delicious, Golden Delicious, and Braeburn (**Table 1**).

4. Dihydrochalcones. Dihydrochalcones (**Table 2**) also represent a significant amount of apple polyphenols (2−6%). The highest relative and also absolute amounts are found in Renetta (average concentrations 15.5 mg/100 g) while the varieties with the lowest concentrations are Royal Gala, Granny Smith, and Fuji (2.0 mg/100 g) (**Table 1**). The average concentrations in Red Delicious are 4.36 mg/100 g. The major two compounds in this group are phloridzin (the average concentration 9.1 mg/100 g in Renetta) and phloretin-xyloglucoside (the average concentration 6.1 mg/100 g in Renetta). The compounds were identified by the following molecular ions: phloridzin *m*/*z* 437.2, phloretin-xyloglucoside *m*/*z* 569.2, and 3-hydroxyphloridzin *m*/*z* 453.2. These results are in agreement with the previous studies (*7*, *23*). The identity of the last two compounds was further confirmed by typical fragmentation. Phloretin-xyloglucoside showed, besides the molecular ion, the fragment ions at *m*/*z* 421.2 and 275.2 which correspond to the sequential losses of xylose and glucose moieties, respectively. 3-hydroxyphloridzin showed, besides the molecular ion, the ion *m*/*z* 291.2 which corresponds to 3-hydroxyphloretin.

5. Anthocyanins. In red apples, the anthocyanins can represent from 1 to 3% of total polyphenols (**Table 2**). The concentration is very variable also inside each variety. In this study, the highest

amounts were found in the varieties Morgenduft (3.67 mg/100 g) and Red Delicious (2.52 mg/100 g) (**Table 2**). Anthocyanins in apple are a mix of four different cyanidin glycosides, of which cyanidin 3-galactoside (average concentration 3.11 mg/100 g in the variety Morgenduft) is the main one followed by traces of 3-arabinoside and 3-xyloside (**Table 1**). Besides these compounds, on the basis of mass spectra and retention time, cyanidin 7-arabinoside also was tentatively identified. For one part of the samples, it was possible to detect traces of cyanidin 3-glucoside but, because of a very low concentration, a reliable quantification was not possible. These results are in agreement with the previous studies which reported that the main cyanidin glycoside in apple skins was always the 3-galactoside while cyanidin 3-glucoside, 3-arabinoside, 3-xyloside, and 7-arabinoside were present in minor amounts in some red varieties (*24*, *25*).

Ascorbic Acid. Besides the polyphenolic antioxidants, the apples, especially the varieties Braeburn, Golden Delicious, Morgenduft, and Renetta, also contain a relatively high concentration of ascorbic acid (**Table 1**). The recommended daily doses of ascorbic acid for the Italian population range between 35 mg/day for infants up to 70−90 mg/day for pregnant and nursing women while 60 mg/day are recommended for the population older than 15 years (*26*). A serving of apple (150 g) contains 12.1 mg (Braeburn), 11.6 mg (Golden Delicious), 8.4 mg (Morgenduft), 8.1 mg (Renetta), 4.1 mg (Granny Smith), and 3.2 mg (Fuji) of ascorbic acid. A single portion of Braeburn or Golden Delicious can therefore contribute ca. 20% of the recommended daily dose, Morgenduft and Renetta 14%, and Granny Smith and Fuji 5−7%. As for polyphenols, apples can be an important source of ascorbic acid and can contribute significantly to the recommended daily dose.

HPLC versus FC Assay. The expected value of the total polyphenols obtained by the FC assay was calculated on the basis of the HPLC data. The reactivity at the FC assay of an equal weight of pure compounds belonging to the five main classes of apple polyphenols was compared to that of (+)-catechin (=1.00) and found to be, in decreasing order, (−)-epicatechin (1.04), quercetin 3-glucoside (0.91), *trans*-5′-caffeoyl quinic acid (0.53), cyanidin 3-glucoside chloride (0.48), and phloridzin (0.37). The expected value of total polyphenols was calculated assuming that the average reactivity at the FC assay of each chemical class of polyphenols was corresponding to the values reported above and that the contribution of all the polyphenols was simply additive. Our experimental value of the FC assay should be unbiased from the presence of undesired reacting compounds since a sample pretreatment was done to separate the potential interfering compounds. The expected value (**Table 1**) for the different apple varieties was in the range between 96.9% and 114.6% of the true experimental value, the expected values being only slightly higher on average than the experimental one (102.7%). Good agreement between the two sets of independent measures strongly supports the fact that the whole amount of total polyphenols measured by FC assay can be explained by chromatographic analysis.

Apple Contribution of Dietary Polyphenols. The average amount of antioxidants in a single standard serving (150 g) of apple was computed for Italy and western Europe. The contribution of each apple variety was weighted by the relative amount of that variety produced in the year 2002. The contribution of other varieties not included in the study (the remaining 11.9% of the total production for Italy and 29.9% for western Europe) was assumed equal to the average of seven major varieties, not considering Renetta because of its unusually high content.

Apples provide a clearly important dietary contribution of total polyphenols in Italy and western Europe estimated at 121.7 and 144.8 mg per serving, respectively. The mean composition of a single serving of apples in Italy (and western Europe) contains 101.9 mg (121.7 mg) of flavanols, 13.6 mg (16.2 mg) of hydroxycinnamates, 8.2 mg (8.9 mg) of flavonols, and 3.8 mg (4.4 mg) of dihydrochalcones while the content in anthocyanins at 0.9 mg (1.2 mg) is very low and possibly irrelevant for the average of the population, even if it might be higher for people preferentially consuming the red apples. Beside the polyphenols, a serving of apple also provides 7.4 (Italy) and 7.1 mg (western Europe) of ascorbic acid. These figures are valid for a consumption of the fruit with the skin, since it is well known that the major part of apple polyphenols, in particular all the flavonols and anthocyanins, as well as the major part of dihydrochalcones (*7*, *27*, *28*) and ascorbic acid, are located in the skin.

The values obtained are, to our knowledge, the most complete estimate of the average composition of apple presently consumed in Italy and western Europe considering all the main monomers and oligomer proanthocyanidins. The five "old" varieties, Renetta, Red Delicious, Granny Smith, Morgenduft, and Golden Delicious, whose production is decreasing, had a higher content of total polyphenols on the average (**Table 1**) and, in particular, of flavanols (catechins and proanthocyanidins) in respect of the "new" varieties Royal Gala, Braeburn, and Fuji which are appreciated by consumers and whose production is rapidly growing. This means that the average amount of polyphenols in apple consumed in western Europe will significantly decrease in the near future. This finding calls for the necessity of taking into account the content in these health-promoting compounds for the communication to consumers and for future breeding programs to counterbalance the loss in nutritional quality of this fruit fundamental to human diet. An effort in this direction is to be encouraged considering the fact that the choices of the producers and breeders can, in perspective, affect the health status of the consumers.

ACKNOWLEDGMENT

Special thanks to Mina Venturelli, Alessandro Dalpiaz, and Livio Fadanelli for their support to the design of the sampling plan and for helpful discussion, and to Luigi Capra, Marino Gobber, Michele Morten, Ferruccio Pellegrini, Umberto Piva, Alfeo Toniolli, Guido Trentini, and Ferruccio Zanotelli who realized the sampling. Thanks to Marjanka Panker-Cernos for her helpful contribution in the preparation of the manuscript.

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Received for review April 28, 2004. Revised manuscript received August 13, 2004. Accepted August 15, 2004. The characterization of the antioxidants was in part funded by Italian Ministry of Health (Progetto Ricerca Finalizzata 2001) and by Provincia Autonoma di Trento and National Council of Research (PAT-CNR, Project 2).

JF049317Z