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Effect of Reddening–Ripening on the Antioxidant Activity of Polyphenol Extracts from Cv. 'Annurca' Apple Fruits

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Apple is among the most consumed fruits worldwide, and several studies suggest that apple polyphenols could play a role in the prevention of degenerative diseases. 'Annurca' apple fruit undergoes, after harvest, a typical reddening treatment to turn the apples' skin red, and it is noted for its high firmness. This paper reports the effect of reddening–ripening treatment on polyphenol concentration and antioxidant activity of both peel and flesh extracts. The in vitro antioxidant properties have been compared with the protective effect against the cytotoxic effects of reactive oxygen species using Caco-2 cells as model system. Pretreatment of cells with different polyphenolic apple extracts provides a remarkable protection against oxidative damage. This effect seems to be associated with the antioxidant activity of 'Annurca' apple polyphenolic compounds. The flesh has antioxidant properties comparable to those possessed by the peel. Neither the reddening nor the fruit conservation causes changes in the antioxidant properties possessed by this apple variety. The data indicate that polyphenolic compounds in 'Annurca' apples are relatively stable in the peel and also in the flesh; therefore, the health benefits of polyphenols should be maintained during long-term storage. Finally, a diet rich in apple antioxidants could exert a beneficial effect in the prevention of intestinal pathologies related to the production of reactive oxygen species.

KEYWORDS: 'Annurca' apple; polyphenols; ripening; antioxidant activity; Caco-2 cells; oxidative stress

INTRODUCTION

The general perception that apples are good for health has encouraged many researchers to look for the "magic" ingredient in this fruit. Scientific studies have suggested that eating apples on a regular basis may help to prevent certain diseases and improve overall human health. Recent epidemiological studies have shown an inverse correlation between the consumption of apple and/or related products and many chronic human diseases. Most noticeably, eating apples has been associated with lowered risk of cardiovascular disease (*1*), lung dysfunctions, and liver, colon, lung, and prostate cancers (*2*).

Many studies have led to the hypothesis that the polyphenol compounds play an important role in the healthful properties of apples.

Apples provide approximately 22% of the total fruit phenols consumed per capita in the United States, and this fruit contains as much as 2 g of phenols/kg of wet weight, or about 400 mg of total phenols/apple (*3, 4*). It has been calculated that 100 g of fresh apple has an antioxidant activity equivalent to 1500 mg of pure vitamin C (*5*).

The main classes of polyphenols in apple are flavonoids, including quercetin, (–)-epicatechin and (+)-catechin, procyanidins, and anthocyanidins; dihydrochalcones such as phloretin and phloridzin; and other polyphenolic compounds such as chlorogenic acid. Apple polyphenols have shown high antioxidant capacity in vitro (*6*), and it has been reported that the consumption of apple juice increases the antioxidant status of blood (*7*). Furthermore, it has been shown that apple polyphenols inhibit the proliferation of cancer cells (*8*) and also exhibit antiarteriosclerosis activity by reducing low-density lipoprotein (LDL) oxidation (*9*). Dose-dependent antiproliferative activity on colon as well as liver cancer cells has been observed using extracts from fresh apples (*5*). It has been demonstrated that the polyphenol composition of apple juice possesses promising growth-inhibitory properties, affecting proliferation-associated signaling cascades in colon tumor cells (*10*).

It is possible to assert that the protection against oxidative damage is one of the most widely described attributes of polyphenols and one of the major selling points on which apples are marketed as dietary supplements. Probably, the main

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antioxidant activity that has been associated with polyphenols is the ability to scavenge free radicals. In fact, most polyphenols act as terminators of free radicals and may also chelate metal ions that are capable of catalyzing lipid peroxidation (*11*).

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen. There is an increasing interest in the possible role of ROS as mediators of cellular damage in several gastrointestinal diseases. In fact, ROS have been implicated in ischemia-induced permeability changes of the intestine, in Crohn's disease, and in ulcerative colitis (*12*).

In this paper we have studied some properties of polyphenol extracts obtained from 'Annurca' apple fruits. This apple variety is widely cultivated in southern Italy (Campania region) and is well-known for its crispness and white flesh. With an income of 100 ME/year, 'Annurca' apple represents 60% of the regional and 5% of the national apple production. This variety of apple undergoes peculiar postharvest storage: generally, the fruit is harvested in October and placed for 20–40 days in special boxes called "melai", a layer of straw or sawdust on the land, sprayed daily with water. When the sun-exposed side turns red, the apple is rotated in order to give its opposite side the chance to turn red (*13*). 'Annurca' apple extracts prevent exogenous damage to both human gastric epithelial cells in vitro and rat gastric mucosa in vivo; this effect seems to be associated with the antioxidant activity of the apple polyphenolic compounds (*14*). Whether apple polyphenols have a protective effect against oxidative stress related injury to intestinal epithelial cells is still unknown, and we decided to use Caco-2 epithelial intestinal cells as an experimental model (*15*). When this cell line, originally derived from a human colon carcinoma, is grown in culture, it undergoes enterocytic differentiation to form a polarized monolayer, closely resembling, both morphologically and functionally, the human small intestine epithelium (*16*). Therefore, differentiated Caco-2 cells are a suitable model for evaluating the physiological response of enterocytes to oxidative injury (*17*). This model cell system has also been selected because it has been already demonstrated that its proliferation is inhibited after exposure to apple extract concentrations above 20 mg mL^{-1} in a dose-dependent manner (*5*).

In this paper we have studied the antioxidant activity of polyphenols in the peel and flesh of 'Annurca' apple during fruit maturation and long-term storage. Moreover, we have investigated the possible protective effect of these polyphenolic components against ROS-mediated oxidative injuries in intestinal cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose, minimum essential medium (MEM), fetal calf serum (FCS), nonessential amino acids, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and PBS tablets were purchased from Gibco Life Science Technologies (S. Giuliano Milanase, Milan, Italy). Acetate buffer (CH_3COOH/CH_3COO^-), Folin–Ciocalteu reagent, H_2O_2 (30% aqueous solution), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), sodium carbonate, (2,2′-diphenyl-1-picrylhydrazyl (DPPH), and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO).

Fruit Collection. 'Annurca' apple fruits were collected in Sant'Agata dei Goti, near Caserta (Italy), in October 2003, right after the fruit had been harvested (green peel). Tissue samples from a group of these freshly harvested apples were submitted to extraction for analytical analysis. The remaining green fruits were reddened in specially constructed "melai", which consist of a raised bed of well-drained soil covered with a layer of straw, for about 30 days, and then stored in a climatic cell at 0 °C and 98% dampness. Moreover, a comparison between 'Annurca' and 'Golden Delicious' (GD) varieties has been taken into consideration.

Polyphenol Extraction. Forty grams of flesh (the edible portion of the apple without the peel) was homogenized for 5 min by a Tefal rondo 500 homogenizer using 40 mL of 80% methanol and 20% water plus 0.18 N HCl (15 mL 12 N of HCl/L). The slurry was centrifuged at 18000*g* for 25 min. The extracts were frozen at −80 °C until analysis. Apple peels were treated similarly using 30 g of sample. All experiments were carried out in triplicate.

Polyphenolic Content and HPLC Analysis. The total polyphenolic content of apple extracts was assessed approximately by using the Folin–Ciocalteu phenol reagent as described in ref *18*. The extracts (100 μL) were mixed with the Folin–Ciocalteu phenol reagent (0.5 mL), deionized water (0.9 mL) and Na_2CO_3 (7.5% w/v, 4 mL). The absorbance at 765 nm was measured 2 h after incubation at room temperature using a Cary ultraviolet–visible spectrophotometer (Varian). The measurement was compared to a standard curve of prepared catechin solutions and expressed as milligrams of catechin equivalent (CAEs) per 100 g ± SD apple component. The *o*-diphenolic content was determined colorimetrically using the Arnow reagent (*19*).

HPLC separation of polyphenols was performed by reversed-phase chromatography on a 150 × 4.6 mm C_{18} 5 mm column (Kromasil), using a Beckman Apparatus (Gold-126) equipped with an UV detector fixed at 278 nm. The column was eluted at a flow rate of 1.0 mL/min with 0.2% acetic acid, pH 3.1 (A)/methanol (B) as the mobile phase; the gradient was changed as follows: 95% A/5% B for 1 min, 85% A/15% B in 1 min, 75% A/25% B in 20 min, 0% A/100% B in 15 min, and 95% A/5% B in 3 min. Fifty microliters of sample was analyzed. The main *o*-diphenols were identified on the basis of the retention times of authentic standard references: (+)-catechin, (–)-epicatechin, chlorogenic acid, quercetin, and quercetin glysosides.

Stable Free Radical Scavenging Capacity. The free radical scavenging capacity of the extracts was analyzed with DPPH, according to the procedure described by Belinky et al. (*20*). The method is based on the reduction of methanolic 2,2′-diphenyl-1-picrylhydrazyl radical ($DPPH^•$) in the presence of a hydrogen-donating antioxidant. $DPPH^•$ solution shows an absorption band at 517 nm, appearing intensely violet-colored. Absorption and color were lowered when $DPPH^•$ was reduced by an antioxidant compound. The remaining $DPPH^•$ corresponds, inversely, to the radical scavenging capability of the antioxidant. Briefly, 10 mg of apple sample or 1 mM apple phenols were added to 1.4 mL of 0.1 M $DPPH^•$ (in methanol). The sample was mixed in a cuvette, and the absorbance at 517 nm was measured at 1, 5, and 10 min versus blank using a UV–vis DU 530 (Beckman) spectrophotometer. The results were expressed as a percentage of reduction of the initial $DPPH^•$ adsorption by test compounds. Percentage reduction of the initial $DPPH^•$ adsorption $= A_{DPPH(t)} - A_{sample(t)} / A_{DPPH(t)} \times 100$, where $A_{DPPH(t)}$ is the absorbance of $DPPH^•$ at time t and $A_{sample(t)}$ is the absorbance of sample at the same time t. All experiments were carried out in triplicate.

FRAP Assay. The total antioxidant power of the APEs was determined using the ferric reducing antioxidant power (FRAP) assay, a colorimetric method based on the reduction of a ferric–tripyridyltriazine complex to its ferrous form, according to the method of Benzie and Strain (*21*), with minor modifications. Twenty-five microliters of extract was mixed with 1 mL of working solution, prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl), and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$. The absorbance at 593 nm was read after 6 min at room temperature, and data were translated into the FRAP value (micromolar), using a methanolic solution of Fe^{II} in the range of 100–2000 μM ($FeSO_4 \cdot 7H_2O$) for calibration.

Cell Culture and Induction of Oxidative Stress. Human colon carcinoma (Caco-2) cells were routinely grown at 37 °C, in a humidified incubator with 5% CO_2/95% air atmosphere, in DMEM, supplemented with 10% FCS, glutamine (2 mM), penicillin (50 units/mL), streptomycin (50 units/mL), and 1% nonessential amino acids. For oxidative stress experiments, Caco-2 cells were seeded at a density of 90000 cells/cm^2 in multiwell dishes (*15*), and the medium was changed every 48 h, using an iron-free medium (MEM). Twelve to fourteen days after confluence, differentiated monolayers were incubated with 40 mM H_2O_2

Table 1. Influence of Postharvest on Total Polyphenolic and *o*-Diphenolic Contents in 'Annurca' Apple

			total polyphenolic content (CAEs mg/100 g of sample)		*o*-diphenolic content (CAEs mg/100 g of sample)	
apple variety	storage time	peel color	peel	flesh	peel	flesh
'Annurca' (GA)	harvest (unripe apple)	green	107.1 ± 6.1	109.1 ± 5.2	5.4 ± 0.1	2.9 ± 0.1
'Annurca' (GRA)	15 days in "*melaio*"	green-red	210.2 ± 4.1	124.2 ± 3.1	7.7 ± 1.1	3.2 ± 0.1
'Annurca' (RA1)	1 month in "*melaio*" (ripe apple)	red	245.1 ± 5.2	127.1 ± 6.2	10.1 ± 1.3	3.2 ± 0.6
'Annurca' (RA2)	1 month in "*melaio*" plus 2 months at 4 °C (ripe apple)	red	178.0 ± 6.1	116.1 ± 3.0	4.0 ± 0.2	3.0 ± 0.4
'Golden Delicious' (GD)[a]	2 months at 4 °C (ripe apple)	yellow	226.4 ± 5.2	63.1 ± 2.2	5.4 ± 0.3	1.4 ± 0.2

[a] We have considered 'Annurca' apple properties in comparison to 'Golden Delicious' variety.

for 20 h. At the end of oxidative stress induction, cell viability was evaluated by MTT assay (see above). After removal of the solvent, every sample was dissolved in PBS. To assay the APEs' protective effect on Caco-2 cells from H_2O_2-induced oxidative injury, the cells were pretreated at 37 °C for 18 h in the presence of different microliters of APEs, and the medium was changed to remove the extract before the addition of 40 mM H_2O_2 (*15*). At the end of the incubation, cell viability was evaluated as above-described.

Cell Viability. Caco-2 cell viability was determined using the MTT assay. The MTT assay depends on the extent to which viable cells convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide to an insoluble colored formazan product that can be determined spectrophotometrically. After oxidative stress, the medium was removed, cells were washed in PBS, and 200 μL of DMEM without phenol red, containing 5 mg/mL MTT, was added to each well. Three hours later, the DMEM–MTT solution was removed, and the converted dye was solubilized with acid isopropanol (0.1 N HCl in isopropanol). The resulting absorbance from each well was measured at a wavelength of 570 nm with background subtraction at 630 nm (*22*).

Statistical Analysis. Statistical analysis was performed by Student's *t* test. The results are presented as the mean ± SD. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Apple represents an important source of natural antioxidants, and the considerable antioxidant power of this fruit is thought to be related to the high polyphenolic content, particularly flavonoids. The 'Annurca' is an apple variety widely cultivated in southern Italy, which typically undergoes a reddening treatment after harvest. The polyphenol content and the antioxidant properties of 'Annurca' apple have been investigated, in both peel and flesh, during the different phases of its peculiar ripening process.

Effect of Redding Postharvest on Phenolic Content in 'Annurca' Apple Extracts. The concentrations of both the total polyphenols and *o*-diphenolic fraction extracted from the 'Annurca' apple, analyzed at different times after harvest, are listed in **Table 1**. It is important to emphasize that *o*-diphenols are the polyphenolic subclass with the major antioxidant capacity. At harvest time (GA 'Annurca'), no difference between flesh and peel polyphenol samples was observed, whereas a change of *o*-diphenolic content was measured (5.4 ± 0.1 in GA peel; 2.9 ± 0.1 in GA flesh, $p < 0.05$) (**Table 1**). With regard to the peel, the concentration of the extractable polyphenols increased during the reddening–ripening process (from 107.1 ± 6.1 in GA to 245.1 ± 6.1 in RA1, $p < 0.05$), whereas the rise observed in extractable flesh polyphenols was not statistically significant (from 109.1 ± 5.2 in GA to 127.1 ± 6.2 in RA1, $p > 0.05$) (**Table 1**). The same result was obtained by measuring the *o*-diphenolic content. The increase during ripening of peel polyphenols could be due to the ethylene action. In fact, this hormone stimulates the activity of phenylalanine ammonia-lyase, a key enzyme in biosynthesis of phenols with the consequent accumulation of polyphenolic constituents (*23*). The increase in peel *o*-diphenol concentration measured during reddening in RA1 peel sample coincides with the increase of quercetin glycosides and cyaniding 3-galactoside. Besides, the red color of the apple peels is due to the presence of the *o*-diphenol cyanidin 3-galactoside in the vacuoles of the cells of the same peel (*24*). Finally, we point out a significant decrease in peel polyphenol concentration during storage at 4 °C (from 245.1 ± 5.2 in RA1 to 178 ± 6.1 in RA2; $p < 0.05$), whereas no significant changes in apple flesh polyphenolic concentration were observed (from 127.1 ± 6.2 in RA1 to 116.1 ± 3.0 in RA2; $p > 0.05$) (**Table 1**). The same experimental data were obtained when the *o*-diphenol concentration was measured (**Table 1**).

In this study, we have compared the properties of 'Annurca' apple with those of a commercially important worldwide variety apple, 'Golden Delicious' (GD). With regard to GD apple, the peel polyphenol concentration is comparable to that measured in the 'Annurca' RA1 and RA2 apples. On the contrary, GD apple flesh possesses both total polyphenol and *o*-diphenol contents lower than values measured in all of the samples of 'Annurca' apple flesh (GA, GRA, RA1, and RA2) ($p < 0.05$).

An accurate analysis of the 'Annurca' apple polyphenolic compounds was performed by Cefarelli et al. (*25*), who described the structures of the main polyphenols (eight species) and the low molecular weight phenols (eight species) isolated from reddened 'Annurca' apple fruit (peel and flesh together). These authors have reported that (+)-catechin and (–)-epicathechin are the more abundant polyphenols in reddened 'Annurca' fruit. Napolitano et al. (*26*) analyzed the amount of phenolic compounds in the flesh of 'Annurca' apple at harvest and after different times of storage. Particularly, the concentrations of the major polyphenols, namely, (+)-catechin, (–)-epicatechin, and chlorogenic acid, did not change in a significant manner during the ripening (*26*).

In light of these data, we have analyzed the HPLC profile of the polyphenols in both flesh and peel of 'Annurca' apple during ripening; particularly, we have identified the main apple *o*-diphenols: (+)-catechin, (–)-epicathechin, chlorogenic acid, quercetin, and quercetin glycosides (considering that it is reported that the apple peels are rich in quercetin derivatives) (**Figure 1**).

In **Figure 2** is reported the HPLC analysis of flesh and peel polyphenol extracts of GA and RA1 fruits. We have identified the main *o*-diphenols from the methanolic extract of cv. 'Annurca' apple. The obtained data related to the flesh have confirmed the results obtained by Napolitano et al. (*26*). Moreover, the peel data proved the quercetin glysosides increased during ripening.

Radical Scavenging Capacity during Storage of 'Annurca' Apple Extracts. In the literature the relationship between

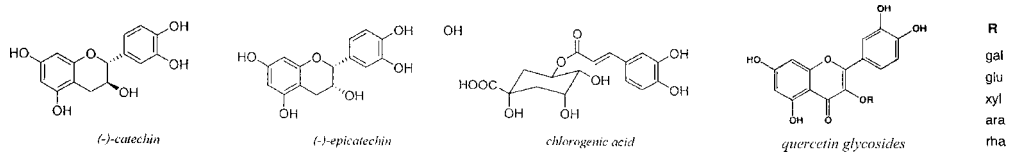

Figure 1. Structures of the main *o*-diphenols isolated from 'Annurca' apple.

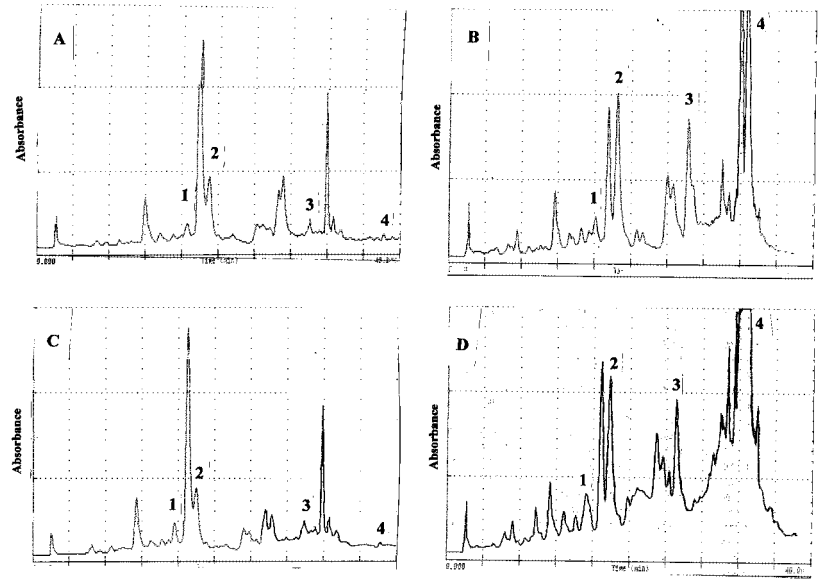

Figure 2. HPLC chromatograms of the flesh and peel extracts of the GA and RA1 'Annurca' apples. HPLC analysis was run as described under Materials and Methods. The analyzed *o*-diphenols were (1) catechin, (2) chlorogenic acid, (3) epicatechin, and (4) quercetin plus quercetin glycosides: (**A**) flesh of GA; (**B**) peel of GA; (**C**) flesh of RA1; (**D**) peel of RA1.

antioxidant activity and food concentration of polyphenolic compounds is highly disputed. Some studies have not found any correlation between the antioxidant activity and the concentration of polyphenolic constituents in apple extracts (*27*), whereas others have found a significant correlation between antioxidant activity and total phenols (*28*). This discrepancy is likely due to the different methodologies used to measure the antioxidant activity and the different extraction procedures adopted by various investigators. In this work, we have selected methanol (particularly, we have used 80% methanol and 20% water plus 0.18 N HCl) for the preparation of antioxidant extracts, because all of the polyphenols, which are mainly responsible for apple antioxidant activity, are well extractable in this solvent. Particularly, we have obtained a better extraction by using this solvent compared to those data obtained by other authors (*29*).

The radical scavenging of the APEs was determined by using two well-known spectrophotometric assays. The use of different methods is necessary in antioxidant activity assessment. First, it was tested by the colorimetric decrease in absorbance of the radical DPPH due to the chemical trapping of unpaired electron. DPPH is a stable free radical, and it is often used to evaluate the antioxidant activity of several natural compounds. Antioxidants interact with DPPH, by either transferring an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character. The absorbance of the DPPH at 517 nm has been measured at 1, 5, and 10 min versus a blank, by using either 10 mg of apple sample or 1 mM apple phenols. In **Figures 3** and **4** are reported the results related to the DPPH assay. All analyzed samples have shown a significant antioxidant power after only 5 min. When we compared the effect of 10 mg of sample on percent reduction of absorption DPPH, we observed an increase in peel antioxidant activity with respect to the flesh, in accordance with the increase in the polyphenol content (**Figure 3**). On the contrary,

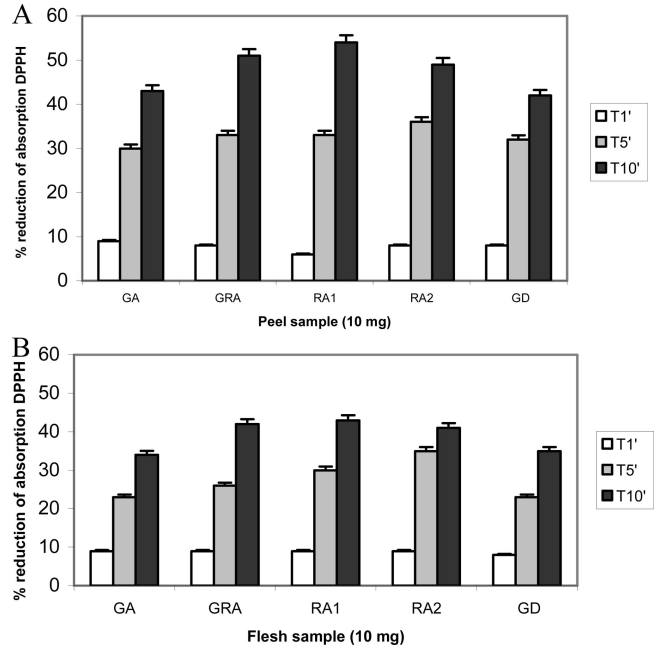

Figure 3. Percentage reduction of DPPH absorption in the presence of 10 mg of 'Annurca' apple samples obtained at different storage times: (**A**) peel samples; (**B**) flesh samples. Three different analysis times are indicated. Results are expressed as means ± SD of at least three independent experiments performed in triplicate.

we have not observed a significant difference when we have used the same polyphenol concentration (**Figure 4**). It is interesting to note that sample GRA at 1 mM phenols showed a high antioxidant capacity in both flesh and peel samples (**Figure 4**).

Besides, antioxidant capacity has been measured according to the FRAP method. This assay was initially developed to

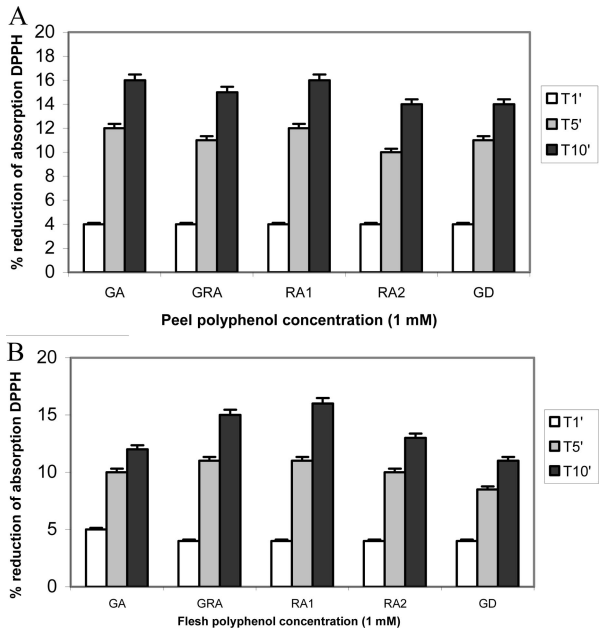

Figure 4. Percentage reduction of DPPH absorption in the presence of 1 mM polyphenol extracts obtained from 'Annurca' apple variety at different storage times: (**A**) polyphenol from peel samples; (**B**) polyphenols from flesh samples. Three different analysis times are indicated. Data are representative of at least three independent experiments performed in triplicate.

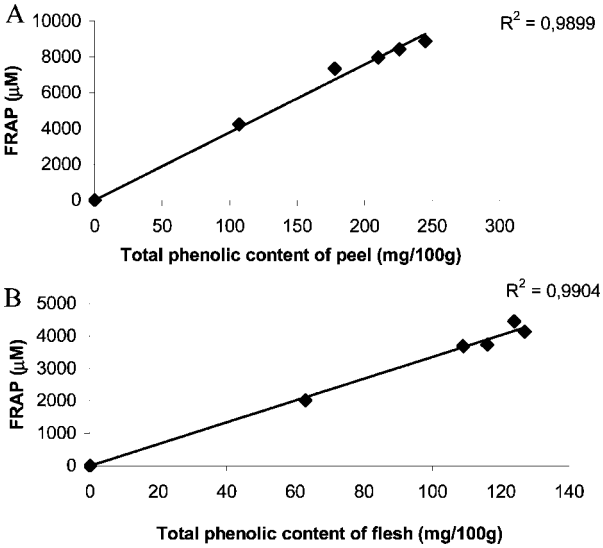

Figure 5. Correlation between FRAP values and polypehnol content for the APEs analyzed. FRAP values were calculated as indicated under Materials and Methods. The correlation coefficient (R^2) was calculated using Pearson's χ^2.

measure the plasma antioxidant capacity, but now it can also be used to analyze other samples (*29*). This method measures the ability of the antioxidants, contained in the analyzed sample, to reduce ferric–tripyridyltriazine (Fe^{3+}–TPTZ) to a ferrous form (Fe^{2+}), which absorbs light at 593 nm (*30*).

FRAP values of both peel and flesh apple extracts versus the respective amounts of polyphenols have been plotted; correlation coefficients of 0.98 (for peel) and 0.99 (for flesh) have been calculated (**Figure 5**). Therefore, it is possible to confirm that the APEs' antioxidant power strictly depends on their polyphenol content.

The concentration is quite variable among different apple varieties (*30*). Flavanols (catechins, dimers, and oligomeric and proanthocyanidins) are the most abundant phenolic compounds presented in apples, varying in percentage from 71 and 90%. Significant concentrations of hydroxycinnamates (4–18%) and dihydrochalcones (2–6%) are also present. In red apples, the anthocyanins may represent from 1 to 3% of the total polyphenols. Because of a strong bioactivity of quercetin, the flavonols are the most studied class of flavonoids in the apple. In general, the flavonoids occur in plants like glycosides (*31*), and flavonols in apple are a mixture of six different quercetin glycosides with only traces of free quercetin (**Figure 1**). Particularly, the apple peel is very rich in quercetin derivatives; the nature and distribution of these polyphenols between the flesh and the peel of the apple is also different. Some research groups have demonstrated, by studying different apple varieties, that all polyphenolic compounds possessed by the flesh are generally present in the peel, as well as additional flavonoids, as quercetin glycosides, not found in the flesh. Besides, the red color of the apple peel is due to the presence of *o*-diphenol cyanidin 3-galactoside (*24*).

Table 2. FRAP Value and DPPH Assay of the Main *o*-Diphenolic Apple Standards

group	compound	FRAP[a] (μM)	% reduction of absorption DPPH[b]
flavonoids			
flavonols	quercetin	4660	95
	quercetin-3-D-galactoside	1866	85
	quercetin-3-rhamnoside	820	80
	quercetin-3-β-D-glucoside	3698	90
flavanols	(+)-catechin	1840	50
	(–)-epicatechin	2252	48
phenolic acids			
hydroxycinnamates	chlorogenic acid	1942	88

[a] We used 1 mM standards solution. [b] We used 10 mM standard solution. Percent reduction of absorption DPPH was measured after 10 min of incubation.

The 'Annurca' apple flesh polyphenol extracts have been analyzed by Napolitano et al. (*26*). The HPLC pattern of phenolic compounds is similar to the other already examined varieties, including the 'Golden Delicious' apple. The main compounds are chlorogenic acid, (+)-catechin, (–)-epicatechin, and phloridzin. In that light, the antioxidant activity of seven different polyphenol standards (1 mM) has also been studied, and the relevant obtained results are shown in **Table 2**. Quercetin exhibits the highest FRAP, which was 2.5 times higher than that of (+)-catechin, whereas the FRAP of quercetin-3-galactoside, for example, a glycoside of quercetin, is equal to that of (+)-catechin. Quercetin possesses the highest antioxidant power, because its chemical structure determined a high molecular stability. Our result has suggested that the incorporation of a sugar moiety into quercetin affects its reducing capacity; the same result has been obtained with the DPPH assay (**Table 2**).

In conclusion, our results show an increase of the antioxidant activity during the reddening for peel 'Annurca' apple. On the contrary, we have not observed a variation in polyphenol concentration and, therefore, in antioxidant capacity of flesh samples.

Effects of 'Annurca' Apple Extracts on Caco-2 Human Cells. Numerous studies indicate that naturally occurring antioxidants exert protective biochemical effects in a number of biological experimental models. In particular, polyphenolic antioxidants, which are widely distributed in vegetable foods, are considered to play an important role in the prevention of oxidative damage in living systems. Grossé et al. (*32*) have demonstrated that apple polyphenol polymers such as procya-

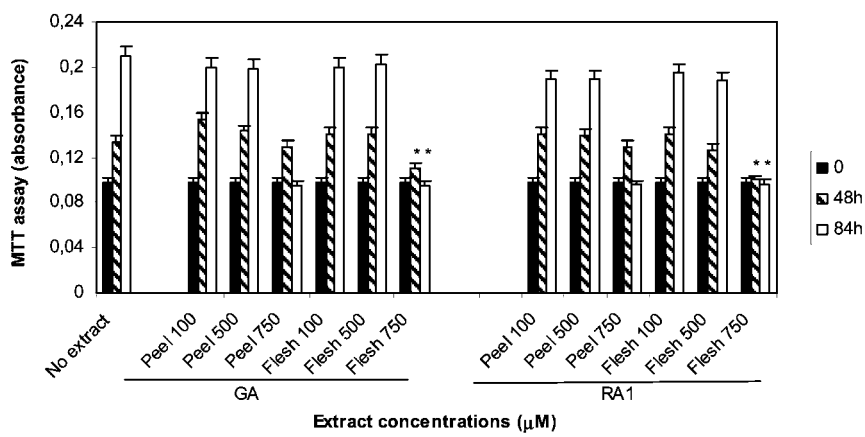

Figure 6. Effect of APEs on Caco-2 cell proliferation. Experiments were conduced as described under Materials and Methods. Cell proliferation was determined by using the MTT assay. Results are expressed as means ± SD of at least four independent experiments performed in triplicate; *, $p < 0.05$.

nidins alter intracellular signaling pathways and polyamine biosynthesis and trigger apoptosis in human metastatic colon carcinoma SW620 cells. Moreover, it has been found that the anthocyanin fraction of the apple extract decreased HT29 (colon carcinoma) cell proliferation (*33*). Particularly, the in vitro capacity of (+)-catechin in preventing plasma lipid oxidation has been demonstrated (*34*). Because the generation of ROS has been considered to be a possible mediator of cellular damage in several gastrointestinal syndromes and because ROS have been implicated in ischemia-induced permeability changes of the intestine, in Crohn's disease and in ulcerative colitis (*35*), we have decided to investigate whether APEs may prevent oxidative stress induced injury to cultured intestinal cells. The Caco-2 cell line is a well-established model for examining the cytotoxicity of various toxic stimuli. Moreover, this cell line represents the best in vitro model of normal intestinal mucosa. Therefore, Caco-2 cells are ideal for proving information on the uptake of apple polyphenols by the "gut". However, the responses of live cells are complicated by some variables, such as the membrane permeability and the intracellular metabolism of flavonoids (*36*). Experiments using live cells can provide more insight into the physiological importance of flavonoids. We used Caco-2 human cells because intestinal cells have the highest exposure to dietary polyphenols under physiological conditions. Particularly, the transport, metabolism, and biological activities of some polyphenols, the flavonoids, have been characterized in Caco-2 cells (*37*). In studies using single layers of Caco-2 cells as a model of intestinal absorption, it has been shown that dimers and trimers of catechins or epicatechins are able to cross the intestinal epithelium (*38*). Whether polyphenolic compounds exert exclusively antioxidant activity in cell systems is controversial. In fact, some studies have reported that polyphenolic compounds are pro-oxidants and cytotoxic (*39*). It is possible that pro-oxidant effects of flavonoids and phenolic compounds on cultured cells derive from disguised generation of oxidative stress. Polyphenolic compounds may indeed react with other components of the cell culture medium (such us metal ions), producing cytotoxic metabolites that have pro-oxidative effects (*40*).

In **Figure 6** we have reported the data related to the effect of APEs on Caco-2 proliferation. Caco-2 cells were incubated for 84 h with different concentrations of peel or flesh polyphenol extracts equivalent to 100, 500, and 750 μM polyphenols. The incubation of Caco-2 cells with <500 μM 'Annurca' apple polyphenolic extracts, either peel or flesh, apart from the reddening of the sample, has not slowed cell proliferation. At

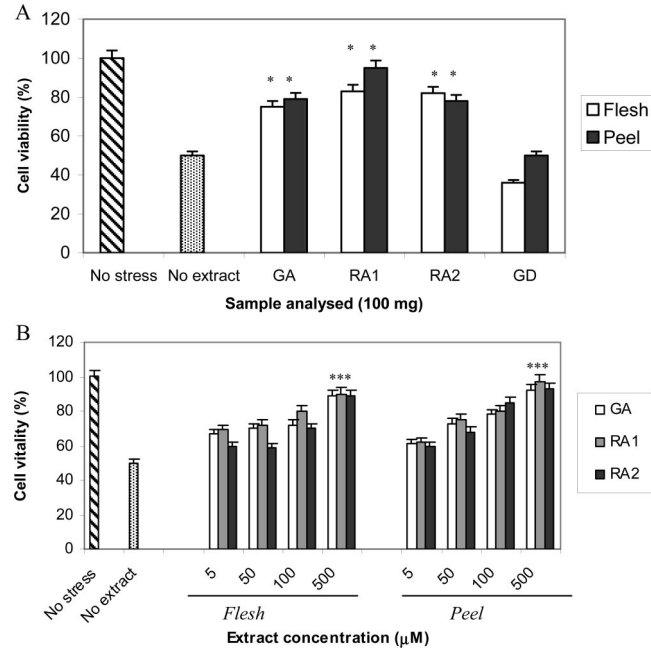

Figure 7. Effect of APEs on hydrogen peroxide-induced cytotoxicity on Caco-2 cells. The cells were preincubated for 18 h with 100 mg of sample (**A**) or different concentrations of the chosen APE (**B**) and then treated for 20 h with 40 mM H_2O_2. At the end of incubation, cell viability was measured by MTT assay, as described under Materials and Methods. Values are expressed as means ± SD of at least three independent experiments performed in triplicate ($n = 3$); *, $p < 0.05$.

a polyphenolic concentration >500 μM, we have observed a reduction in cell proliferation. No cytotoxicity of the apple extracts has been seen at any of the tested concentrations. These results are consistent with literature data related to the capacity of apple polyphenol extracts to inhibit Caco-2 cell proliferation in vitro (*5*).

Then, we tested the antioxidant efficiency of APEs in intestinal cells and, to the best of our knowledge, this is the first time that the total antioxidant activity of apple during ripening has been measured using a cell model system. Caco-2 cells have been preincubated for 4 h with 100 mg of apple sample (flesh or peel) (**Figure 7A**) or different concentration of polyphenols (**Figure 7B**) and then exposed to H_2O_2 to induce an oxidative stress, as indicated under Materials and Methods. As shown in **Figure 7**, the APEs decrease the ROS-induced cell injury, as assessed by checking cell viability with the MTT

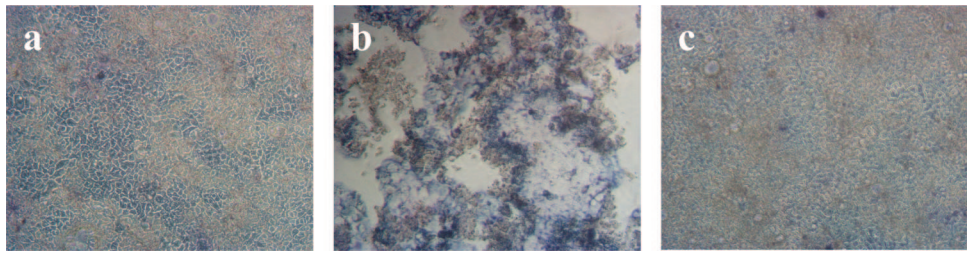

Figure 8. Phase contrast microscopic appearance of Caco-2 cell monolayer: (**a**) Caco-2 cell control; (**b**) Caco-2 cells incubated with 40 mM H_2O_2 for 20 h; (**c**) Caco-2 cells pretreated with 500 μM flesh-GA APE and then submitted to oxidative stress using 40 mM H_2O_2 for 20 h.

assay. This property is common to peel and flesh samples. Furthermore, the extent of cell protection varies in a dose-dependent manner. In particular, we have observed an almost complete protection (about 95–98%) in Caco-2 cells pretreated with 500 μM APEs, obtained by GA, RA1, and RA2, for both flesh and peel extracts (**Figures 7** and **8**). The antioxidant capacity shown by the analyzed extracts seems to be independent of the type of sample, peel or flesh, and of the degree of reddening of the fruit by which the extracts have been obtained.

We have shown that 'Annurca' apple peels possess a high content of polyphenolic compounds compared to other edible parts of the apple. These data are comparable to those previously reported and relative to different apple varieties (*30*). The 'Annurca' apple peel has been shown to possess a high content of polyphenolic compounds, but not a significantly higher antioxidant activity than flesh when oxidative stress is induced in Caco-2 cells. The content of total polyphenols or flavonoids in fruits often does not directly reflect the total antioxidant capacity (*41*). The measurement of the antioxidant capacity of each bioactive compound in vitro should be compared with a result obtained by in vivo experiment. For example, Yi et al. (*42*) have demonstrated that the bioavailability of some sugar moieties, particularly the transport through the Caco-2 cell monolayer, although the transport/absorption was efficient, was relatively low compared to other aglycone polyphenols. Particularly, more free hydroxyl groups and fewer OCH_3 groups can decrease the bioavailability of polyphenols.

In general, during storage in air at 0 °C, the concentration of total extractable peel polyphenols in various cultivars increased during the first few months, remaining relatively constant over a normal air storage period of 6 months (*43*). We have observed, with regard to the peel, that the polyphenol content decreased only 30% after 2 months when conserved at 4 °C. In the flesh, instead, polyphenolic content remained relatively constant during maturation and storage. Our data show that the flesh, which represents 80% of the wet weight of fruits (edible portion), possesses a high polyphenolic content, already present in the flesh obtained from the green fruit, just harvested, even before the ripening process in the *melaio*.

Other research groups have also noted that apple peel has higher polyphenolic content than flesh (*44*). Quercetin, the major representative of the flavonol subclass of flavonoids (polyphenolic compounds), is a common dietary component and one of the most potent natural antioxidants (*45*) (**Table 1**). In apple peel, flavonoids are a mixture of different quercetin glycosides, with only traces of free quercetin (*30*), and indeed it is often stated that flavonoids present in foods cannot be absorbed from the intestine because they are bound to sugar as glycosides. A commonly accepted concept is that the polyphenols are absorbed by passive diffusion. For such diffusion to occur, the glycosylated polyphenols need to be converted to the aglycone form by the glycosidases present in the food or in the gastrointestinal mucosa or in the colon microflora. For example, polyphenol glucosides are hydrolyzed by human β-glucosidase in the intestine, whereas polyphenol rhamnosides need to be hydrolyzed by microflora α-rhamnosidases in the colon (*46*). Recent studies have suggested that a glucose transport system may be involved in the absorption of quercetin-3-*O*-glucoside. The bioavailability of quercetin glucoside is much higher than that of quercetin rutinoside in humans, suggesting that the glucoside is actively absorbed in the small intestine. In any case, a carrier-mediated transport process may be a common process in the absorption of polyphenols (*46*).

The glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (*47*). Therefore, it is possible to think that our results concerning the antioxidant power on the cellular model could be underestimated, as the presence of the digestive enzymes removes the sugar and the total antioxidant power of the absorbed polyphenols could be increased at intestinal level. Upon absorption of quercetin glycosides, the compounds are hydrolyzed to quercetin and subsequently converted to quercetin glucuronides and sulfates in the human body (*48*). Moreover, the amount of polyphenols derived from apple juice, as well as from other fruits and vegetables, is important for the ability to achieve protective effects in the colon. However, to date there is still a lack of information about the colonic availability of food-derived polyphenols (*49*).

The antioxidant power of polyphenols, extracted from peel and flesh, increases with the concentration (**Figure 7**). Our data indicate that polyphenolic compounds in 'Annurca' apple are relatively stable in both peel and flesh, and their health benefits should be maintained during long-term storage.

Our data confirm that eating apple peels may have health benefits for consumers. Peels are often discarded in the production of processed apple products, but clearly they possess high levels of antioxidant and bioactive compounds.

In conclusion, Caco-2 cells are a suitable in vitro system for screening vegetable compounds with antioxidant properties; APEs prevent ROS-induced injury to cells by permeating cell membranes, increasing intracellular antioxidant activity; the flesh has antioxidant properties comparable to those shown by the peel; the reddening does not cause a change in the antioxidant properties possessed by this apple variety. These results suggest that a diet rich in apples might exert a beneficial effect in the prevention of intestinal diseases related to the generation of ROS.

Finally, considering the important nutritive value of the flesh from unreddened apples, we suggest the use of the just harvested 'Annurca' apple for the preparation of the apple manufacture, as applesauce, puree, etc., to reduce the prime cost by avoiding the reddening process in *melaio*. Moreover, because the flesh of the just harvested apple is not very sweet, but has an average amount of aroma and good flavor characteristics, it can be recommended, for example, in a low-sugar diet.

ABBREVIATIONS USED

APE, apple polyphenol extract; FRAP, ferric reducing antioxidant power; ROS, reactive oxygen species.

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