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Influence of Variety and Storage on the Polyphenol Composition of Apple Flesh

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Apple is among the most consumed fruits worldwide. It is available on the market for the whole year being a major source of dietary polyphenols. Several studies suggested that apple polyphenols could play a role in prevention of degenarative diseases. The action of these compounds has been partially ascribed to their antioxidative ability, and fruit antioxidants profile is influenced by apple variety and by the postharvest storage. In this work, the polyphenols composition of the flesh of four apple varieties cultivated in southern Italy were investigated by HPLC, and a flow injection MS/MS procedure to quantify cholorogenic acid and catechins was set up. Phenolic composition and the radical scavenging activity were monitored during a postharvest storage of four months. The quantification by flow injection procedure gives results comparable to those obtained by HPLC, and the increase of the antioxidant activity during storage correlated with an increase of the concentration of catechin and phloridzin. This trend is particularly evident for the variety "Annurca" which is a typical product cultivated in the area around Naples. The genetic characteristics of the Annurca variety together with the anticipated harvest time and the peculiar postharvest conditions are likely responsible for this increase of the antioxidant activity.

KEYWORDS: Flavonoid; Annurca apple; storage; antioxidant activity

INTRODUCTION

Many studies have shown that food has a fundamental role in the prevention of cancer and coronary heart diseases, the main reasons of mortality in Western countries. Epidemiological investigations have shown that apple intake is negatively associated with lung cancer (*1*, *2*) and coronary heart disease (*3*−*5*). Molecular studies performed both on animal model and in vitro system suggested that the beneficial properties are partially due to the antioxidant capacity of apple polyphenols (*6*, *7*).

Polyphenols are one of the most important dietary antioxidants, and apple is a major source of phenol compounds, since its consumption is widespread in many countries and it is available on the market for the whole year. It has been reported that 22% of the fruit phenolics consumed in the United States is from apples (*1*).

The phenolic composition of apples, and thus their antioxidant activity, is deeply influenced by their variety and can be modified by postharvest factors, including storage and processing (*8*). The concentration of total phenol compounds in the flesh of different apples varies in a large interval between 100 and 1000 mg/kg of fresh weight (*9*). This variability is mainly determined by the apple variety but is also influenced by the analytical methods used. Moreover, in some studies, the value is given as catechin equivalent, or total phenolic compounds, while in others single polyphenols are quantified separately by HPLC (*9*−*10*).

The main phenolic compounds of apple flesh are chlorogenic acid, catechin, and epicatechin; therefore, their rapid quantification without HPLC separation would be useful. This task can be achieved by flow injection MS/MS—an analytical tool which was scarcely applied to food matrixes up to now (*11*).

Apple cultivars having higher content of phenol compounds can be selected to promote their use for healthy reasons (*9*, *12*). Several recent studies regarding the composition of apple polyphenols during storage in cold chambers or in controlled atmosphere have been carried out (*12*−*14*). From these studies, it has been concluded that polyphenols present in apples are usually stable during postharvest storage (*15*). In other fruits, such as berries, an increase of phenolic compounds has been observed, (*16*) although no examples of increase in polyphenols concentration in apple flesh has been reported up to now.

Annurca is an apple variety, typical of the Naples area, which is well known for its crispness and its white flesh. With an income of 100 ME/year, Annurca represents 60% of regional apple production and 5% of the national one. These apples

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Table 1. Apple Samples Used in This Study, Data on Harvest Period, Storage Time, and Physicochemical Parameters at Harvest Time and after 4 Months of Storage

apple variety	harvest period	time of storage	average weight (g)		flesh firmness (kg/cm^2)		soluble solids (°Brix)		acidity (meq/100 mL)	
			harvest	stored	harvest	stored	harvest	stored	harvest	stored
Annurca Traditional	Oct 1–10	4 months	99.9	91.5	5.74	4.65	11.7	13.1	6.23	5.88
Red Delicious	Sept 10–20	4 months	172.8	165.7	4.15	2.93	13.3	12.9	3.57	3.65
Golden Delicious	Oct 1–10	4 months	170.6	163.2	3.65	2.84	15.0	14.5	3.50	3.42
Empire	Oct 1–10	4 months	188.2	180.4	4.37	3.01	11.6	11.6	3.07	3.32

undergo a peculiar postharvest storage: fruits are harvested before the complete maturity and are stored for about 1 month in specially constructed boxes called "melai".

The aim of this study is to develop a flow-injection ESI-MS-MS procedure to quantify chlorogenic acid and catechin in food extracts and to evaluate the influence of variety and postharvest storage conditions on the polyphenols composition of the apple flesh. In particular, we have considered Annurca variety in comparison to other apple varieties such as Golden Delicious (GD), Red Delicious (RD), and Empire (E).

MATERIALS AND METHODS

Chemicals. Chlorogenic acid, phloridzin, (+)-catechin, and (−)-epicatechin were purchased from Sigma. Eluents were obtained from Merck. 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and Trolox were from Aldrich, while BHT, DMPD, and $FeCl_3$ were from Fluka. L-(+)-Ascorbic acid was obtained from Sigma.

Fruit Sampling. The samples used in this study are listed in **Table 1**, together with the main information regarding their cultivation conditions.

Apple samples have been provided from fruits harvested from commercial orchards in Naples' area (Italy), from trees in full production, trained to free palmette, grafted on M26, and planted at 3.0 m within rows and 4.0 m between rows. For Red Delicious (RD) variety, fruits were harvested in the second decade of September, while for other varieties (Empire and Golden Delicious) during the first decade of October. According to the standard production protocol, Annurca fruits were harvested before the "stage of complete maturity" as shown by the variation of parameters shown in **Table 1**. The apple fruits of the cultivar "Annurca traditional" are then laid on the ground in specially constructed "melai" which consist of a raised bed of well-drained soil covered with a layer of straw. Then, every 4 or 5 days each apple is individually turned by hand so that different parts of the skin are exposed to the ripening effects of the sun. Then, the apple fruits of all cultivars were stored up to 4 months in the conditions usually adopted for marketing by means of cold storage at 2 °C.

Sample Preparation. From 5 kg samples, a sampling of 15 fruits was performed for each variety. Apples were peeled with a potato knife and cut in quarters eliminating seeds and core. The flesh was divided into three aliquots, lyophilized, and stored at −20 °C until analyses.

Apple poliphenols extracts, obtained according to Escarpa et al. (*9*), were used for the HPLC analysis and the determination of antioxidant activity (M-AA) of methanol extracts. Lyophilized sample (1 g) was extracted with 5 mL of methanol containing 1% of BHT and sonicated 1 h in an ice bath, in the dark. After sonication, the suspension was centrifuged at 4000 rpm for 5 min at 4 °C. The surnatant was filtered through a nylon 0.20-μm filter (Millipore Millex-GN). This procedure was repeated twice pooling the extracts.

Sample preparation for the measure of the water antioxidant activity (W-AA) was performed adding 7 mL of deionized water to 100 mg of lyophilized apple flesh. The suspension was homogenized 3 min by an Ultra-Turrax and centrifuged at 4000 rpm at 4 °C for 5 min. The surnatant was filtered through a nylon 0.20-μm filter as above. Extracts were always stored at 4 °C, in the dark, and analyses were carried out immediately after extraction to avoid any degradation of the extracted compounds.

HPLC Analysis of Polyphenols. Chromatographic analysis was performed by HPLC (Shimadzu, Japan) equipped with two LC10 pumps and a diode-array detector, using a Luna 10m Phenyl-Hexyl (250 × 4.6 mm) column (Phenomenex). Eluents were (A) acidified water (0.01 M phosphoric acid) and (B) methanol. Solvent gradient was 5% B as initial condition, 50% B for 10 min, 70% B for 5 min, 80% B for 5 min, and finally 100% B for 5 min. The flow rate was 1 mL/min. Peak identification was obtained comparing the retention time and the UV spectra of the apple flavonoids chromatogram with those of pure standards.

Table 2. Compound-Specific ESI MS/MS Parameters for Catechin and Chlorogenic Acid

compound	precursor ion (*m*/*z*)	monitor ion (*m*/*z*)	CE[a]	CXP[b]	ion mode (ESI)
chlorogenic acid	353 ($M + H^-$)	191	–21	–5	–
catechin	291 ($M - H^+$)	123.1	21	6	+
catechin	291 ($M - H^+$)	139.1	18	10	+

[a] Collision energy. [b] Collision cell exit potential.

Polyphenols concentrations were calculated by calibration curves obtained for each standard compound available. Chlorogenic acid and caffeic acid were detected at a wavelength of 325 nm and phloridzin and the catechins at 280 nm.

The recovery efficiency of the procedure was determined by adding to the flesh before extraction known amounts of pure standards (chlorogenic acid, (+)-catechin, (−)-epicatechin) and running the HPLC analysis as above-described. The recovery ranged between 91.4% for epicatechin and 98.0% for chlorogenic acid. Values reported in the result tables were corrected accordingly.

Flow-Injection ESI MS/MS. MS/MS analysis was performed by an API 3000 triple-quadrupole mass spectrometer (Applied Biosystem, Toronto, Canada). The flow injection analysis has been performed using a 5-μL loop and the acquisition has been carried out in MRM (multiple reaction monitoring) in the negative and positive ion mode for chlorogenic acid and catechin, respectively; 0.2 mL min^{-1} methanol and 450 °C temperature were selected as carrier liquid and desolvatation temperature, to obtain a better ionization of the precursor ion.

Chlorogenic acid (*m*/*z* 354) and catechin (*m*/*z* 290) were selected as they are the major polyphenols in the apple extract. For catechin, the specific transition ions selected were *m*/*z* 139.1 and *m*/*z* 123.1 while for the molecule of chlorogenic acid the precursor ion was selected at *m*/*z* 191 (see **Table 2**).

The performance of the system was optimized by evaluating the effect of sample injection parameters on the mass spectral ion intensity. The parameters were optimized for each compound analyzed, the voltage and the declustering potential applied were, respectively, 5500 V and 55 for catechine and −4500 V and −50 for chlorogenic acid.

The collision energy (CE) and collision cell exit potential (CXP) were optimized for each transition ions, as show in **Table 2**. The collision-induced dissociation was performed using nitrogen as collision gas in the collision cell.

Determination of Antioxidant Activity. The antioxidant activity of the apple extracts was determined using two well-known spectrophotometric assays. For the methanol extract antioxidant activity (M-AA), the ABTS method as described by Pellegrini et al. (*17*) was used while for the water antioxidant activity (W-AA) the method described by Fogliano et al. (*18*) was used. The M-AA was determined on the same extracts prepared for HPLC analysis, the extract was diluted 1:80 in methanol, and 100 μL was added to the ABTS colored solution. For the determination of W-AA, a sample of 20 μL was tested.

Table 3. Amount of Chlorogenic Acid and Catechin Plus Epicatechin (mg/kg of Fresh Weight) in Four Apple Varieties at Harvest Determined by Flow-Injection MRM Procedure

apple variety	chlorogenic acid	catechin + epicathecin
Annurca	130.6 ± 10.3	108.5 ± 8.2
Red Delicious	85.4 ± 23.5	141.3 ± 11.7
Golden Delicious	78.4 ± 15.6	111 ± 13.6
Empire	125.4 ± 15.2	99 ± 8.7

Statistical Analysis. Statistical analysis of data was performed on the original data by one- or two-way analysis of variance, and Tukey's test was used to value differences between couples of averages. The relations between variables were analyzed using linear simple correlation. It was considered a significance level at $p < 0.05$.

RESULTS AND DISCUSSION

Polyphenol Composition of Apple Flesh. Apple flesh polyphenol extracts were analyzed by HPLC and ESI-MS-MS. The HPLC pattern of phenolic compounds is similar in all the examined varieties and in accordance to literature data (*9*, *10*, *19*). The main compounds are chlorogenic acid, catechin, epicatechin, and phoridzine. Also, phloretin xiloglucoside was found in accordance with Burda et al. (*10*) who found this compound in the flesh of three apple cultivars (Golden Delicius, Empire, and Rhode Island Greening).

The concentration of each compound (see **Table 4**, harvest times) is similar to that reported by Escarpa and Gonzales (*9*); catechin and chlorogenic acid are the main phenolic compounds. In agreement with these authors, who compared Golden Delicious, Red Delicious, Granny Smith, and Green Reineta, we have found the lowest concentration of polyphenols in Golden Delicious flesh. Also, van der Sluis et al., (*20*) who compared Golden Delicious with Jungold, Cox's Orange, and Elstar, reported that this variety has the lowest antioxidant activity. Phloridzin was presented in a concentration similar to that reported in other studies and it is markedly higher in Red Delicious (*9*, *20*, *21*). No significant concentration of quercetin derivatives were detectable as already reported by several other authors (*9*, *21*), while in other studies (*20*) and in the USDA database a concentration of quercetin of 1.5 mg per 100 g of fresh weight for "raw apple without skin" is reported. This discrepancy is likely due to the sample preparation, considering that apple peels are rich in quercetin derivatives

As chlorogenic acid, catechin, and epicatechin are by far the most abundant phenolic compounds in apple flesh, a flow-injection ESI MS/MS procedure to get their rapid quantification was developed. The procedure quantifies the sum of catechin and epicatechin, the mass and the transition ions of these two compounds being the same. In **Figure 1**, the MRM profiles obtained under flow-injection parameters reported in **Table 2** are shown. For chlorogenic acid only, one transition (353.0/191.0) corresponding to the formation of quinic acid with loss of caffeoil moiety was observed as described in the literature (*22*). On the other hand, for catechin/epicatechin it was decided to operate in positive ion mode to enhance the sensitivity. Two transitions were detectable (291.0/139.1 and 291.0/123.1) because of the different possible fragmentations of ring B. In a product ion study on cocoa phenolic compounds (*22*), the detection of catechin/epicatechin was performed in negative ion mode with the formation of one fragment at 245 amu corresponding to the loss of a CH_2-CHOH moiety.

The procedure has a limit of detection of 1 μg mL^{-1} and a linearity range between 5 μg and 1 mg mL^{-1} for both compounds. Quantitative data obtained with flow-injection MRM procedure can be affected by the matrix composition, particularly when the analyte is a minor compound of the mixture. In the apple polyphenol extracts, chlorogenic acid and catechin are two major compounds; therefore, the flow injection procedure was used to quantify the amount of chlorogenic acid and catechin/epicatechin in the four apple varieties used in this work. Data, summarized in **Table 3**, are in agreement with those obtained by HPLC (see **Table 4** harvest time) confirming the reliability of this MS/MS analytical procedure. While for chlorogenic acid MS data are slightly higher than that obtained by HPLC, data about catechin epicatechin, although similar, are significantly lower. This discrepancy can be due to partial overlap in the HPLC chromatogram of catechin peak to that of other unidentified compounds.

Polyphenol Composition and Antioxidant Activity along Storage. The concentration of the main polyphenols present in apple flesh of all varieties at the different times of sampling is listed in **Table 4**. Data show that the chlorogenic acid concentration is significantly different ($p < 0.005$) among varieties and, in some cases, also in the same variety along the storage time. The lowest values, both at harvest time and after storage, were found in GD and RD samples. Annurca variety shows a marked increase of chlorogenic acid and catechin along storage, while an increase of the concentration of epicatechin after cold storage was detected in the variety GD, RD, and E.

Results obtained for methanol antioxidant activity (M-AA, ABTS assay) and water antioxidant activity (W-AA, DMPD assay) are shown in **Figures 2** and **3**, respectively. These data confirm an influence on these parameters both of variety and of storage time. As far as the M-AA, at harvest time Red Delicious have the highest value while Golden Delicious the lowest. There is a significant M-AA increase during storage ($p < 0.05$) for the Annurca variety, in accordance to the increase of the polyphenol composition shown above.

A clear effect of storage was detectable for W-AA (DMPD method) as shown in **Figure 3**. At harvest time, there were not statistical differences among varieties, and Golden Delicious and Empire showed lower values than others. At the end of cold storage, there was a decrease for all samples (from −47.3% for the Golden Delicious to −27.5.1% for Empire) except for Red Delicious (−8.2%). The lowest final values were for Golden Delicious and Empire, the highest value was for Red Delicious.

Polyphenols present in the flesh extracts are mainly responsible for the antioxidant activity (particularly of the M-AA) of the apple flesh. The correlation analysis showed that M-AA was significatly connected ($p < 0.05$) to catechin and phloridzin concentration but not to the chlorogenic acid. There was a statistical relationship ($r = 0.338$, $p < 0.05$) also between M-AA and W-AA.

In the literature, the relationship between antioxidant activity and food concentration of phenolic compounds is highly disputed. Some studies did not find any correlation between the antioxidant activity and the concentration of phenol constituents in apple extracts (*20*, *23*, *24*), while others found a strong correlation between antioxidant capacity and total phenols (*25*, *26*). This discrepancy is likely due to the different methodology used to measure the antioxidant activity and to the different extraction procedures adopted by various investigators. In this work, we have selected methanol for the preparation of antioxidant extracts because flavonoids, which are mainly responsible for apple antioxidant activity, are well extractable in this solvent. ABTS assay, which is the method of choice for

Table 4. Amount of Phenolic Compounds (Milligrams per Kilogram of Fresh Weight) in Apple Flesh at Harvest and at Different Times of Storage

sample	chlorogenic acid	catechin	epicatechin	phoridzin	phloretin xyloglucoside	caffeic acid	total phenols
Annurca, harvest	101.4 ± 12.4	75.5 ± 7.7	41.0 ± 0.4	12.92 ± 4.0	20.1 ± 4.4	1.9 ± 1.8	252.8 ± 30.7
Annurca, 3 months	130.9 ± 17.4	117.6 ± 11.7	62.2 ± 5.8	12.1 ± 0.5	25.0 ± 0.5	4.4 ± 1.8	352.2 ± 37.7
Annurca, 4 months	143.6 ± 21.7	88.1 ± 10.2	42.1 ± 6.1	12.7 ± 3.7	21.2 ± 1.2	3.0 ± 1.7	222.6 ± 44.6
Red Delicious, harvest	77.3 ± 20.5	137.3 ± 17.4	37.7 ± 21.1	20.2 ± 3.7	9.1 ± 1.0	2.7 ± 1.2	284.3 ± 64.9
Red Delicious, 3 months	68.7 ± 13.1	98.7 ± 6.6	56.1 ± 5.6	16.9 ± 0.2	7.5 ± 0.2	3.1 ± 0.4	251.0 ± 26.1
Red Delicious, 4 months	58.3 ± 16.5	124.3 ± 52.2	62.3 ± 10.2	25.3 ± 3.8	7.2 ± 0.4	2.5 ± 0.3	279.9 ± 83.4
Golden Delicious, harvest	61.1 ± 17.9	94.1 ± 21.9	26.9 ± 2.8	9.2 ± 3.7	6.1 ± 1.6	2.9 ± 1.1	200.3 ± 49.0
Golden Delicious, 3 months	64.9 ± 1.1	96.2 ± 8.7	56.5 ± 1.1	12.4 ± 2.8	8.1 ± 0.2	1.9 ± 0.1	240.0 ± 14.0
Golden Delicious, 4 months	65.0 ± 4.9	97.0 ± 9.4	47.1 ± 16.3	12.4 ± 1.7	8.2 ± 0.3	1.8 ± 0.3	223.3 ± 32.60
Empire, harvest	112.8 ± 13.5	81.6 ± 19.8	38.5 ± 10.1	11.2 ± 0.9	13.0 ± 3.4	1.8 ± 0.5	258.9 ± 48.2
Empire, 3 months	137.9 ± 7.5	123.4 ± 3.2	91.4 ± 36.1	12.9 ± 8.4	20.8 ± 6.8	2.0 ± 0.4	388.4 ± 62.4
Empire, 4 months	107.5 ± 7.5	88.6 ± 33.9	59.2 ± 21.3	9.9 ± 1.9	14.1 ± 3.4	2.3 ± 1.1	281.6 ± 69.1

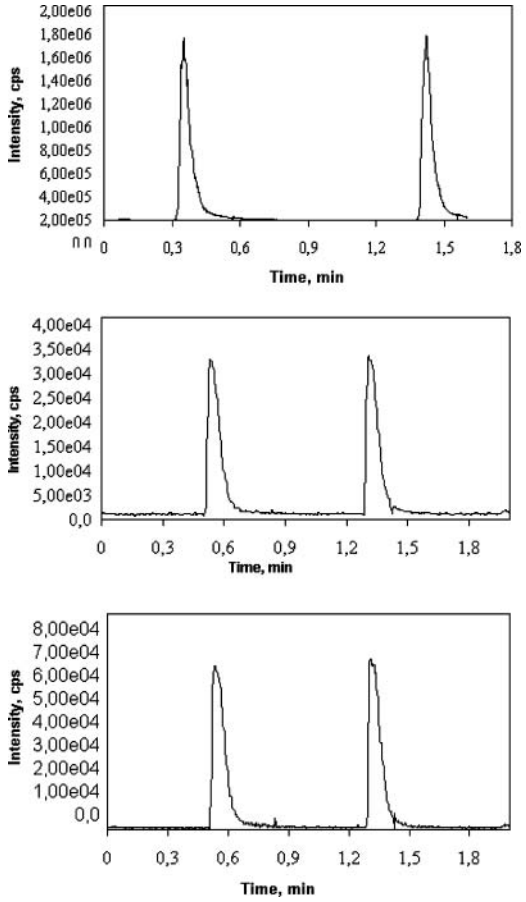

Figure 1. MRM profiles of the chlorogenic acid and catechin under flow injection ESI MS/MS conditions. Panel A: chlorogenic acid, transition 353.0/191.0; Panel B: catechin, transition 291.0/123.1; Panel C: catechin, transition 291.0/139.1.

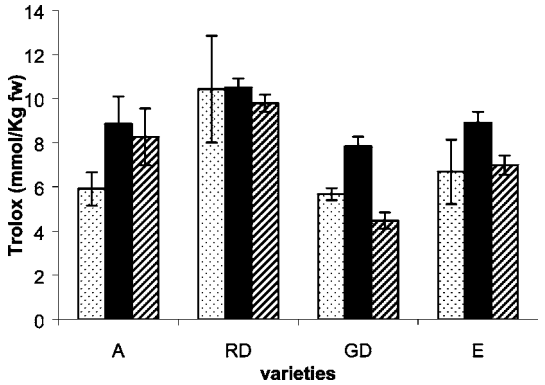

Figure 2. Antioxidant activity of the methanol extract (M-AA) of the apple flesh of different varieties. Dotted bars, harvest time; black bars, after 3 months of storage; shaded bars, after 4 mounths of storage.

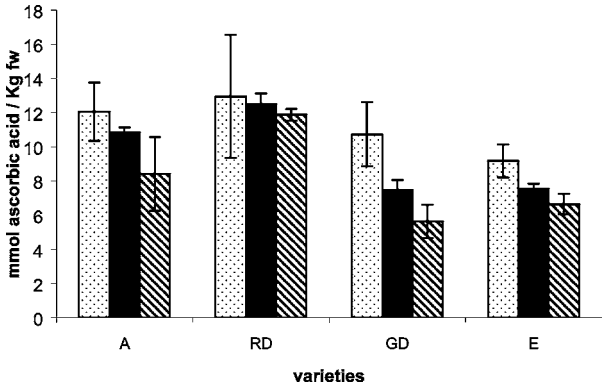

Figure 3. Antioxidant activity of the water extract (W-AA) of the apple flesh of different varieties. Dotted bars, harvest time; black bars, after 3 months of storage; shaded bars, after 4 months of storage.

the measure of the flavonoid antioxidant activity (*27*), was used for the M-AA. Moreover, the separation between M-AA and W-AA allows to distinguish the different contributions to the whole antioxidant activity and it makes it easier to find out the correlations.

The statistical analysis of the variation of the different parameters measured during storage clearly shows that single flavonoid concentration, as well as M-AA, significantly increases for Annurca variety. For Empire variety, the increase detectable after 3 months was counterbalanced by a decrease at 4 months of storage. Red Delicious and Golden Delicious parameters remained roughly constant during storage with a slight increase at 4 months for Golden Delicious. The decrease of W-AA, even if variable among varieties (from −8.2% to −47.3%), can be related to the ascorbic acid degradation that may happen during cold storage as described by De Ancos et al. (*28*) for four raspberry cultivars. Also, this finding can be due to the polymerization of free phenols into less water-soluble polymeric compounds (*29*).

The studies available on the polyphenols composition during storage were mainly focused on the peels. Golding et al. (*12*) showed that a storage up to 9 months, in air at 0 °C, of three varieties (Granny Smith, Lady Williams, and Crofton) induced few significant changes in peel phenolic concentrations. Similar results were also obtained on the peels by Burda et al. (*10*) and most recently by van der Sluis et al. (*20*) on the whole fruit (stored in cold chambers for 4 months). On the contrary, some studies also reported an increase of the antioxidant activity of

total polyphenol concentration during storage. Leja and coworkers (*30*) observed after 4 months of storage a 30% increase of total phenol compounds while the total antioxidant activity was roughly doubled. In another study, Lattanzio et al. (*21*) reported an increase in polyphenol concentration during the first 60 days of cold storage in Golden Delicious peels.

The increase during cold storage of peel total phenols could be due to the ethylene action. In fact, this hormone stimulates activity of phenylalanine ammonia lyase, a key enzyme in biosynthesis of phenolic compounds with the consequent accumulation of phenolic constituents (*31*, *32*). The increase of phenolic compounds observed in this study for Annurca variety can be related to the peculiar ripening process occurring during the postharvest treatment. At ambient temperature, ethylene production is higher, thus stimulating the biosynthetic pathway of phenol compounds.

In conclusion, our results showed a marked increase of the antioxidant activity during storage, particularly for apples of Annurca varieties. This increase is correlated with the concentration of catechin and phloridzin. The genetic characteristics of the Annurca variety together with the anticipated harvest time and the peculiar postharvest conditions are likely responsible for this effect.

Moreover, in this paper a new flow injection procedure for the detection of chlorogenic acid and catechin/epicatechin in food matrixes is reported. The analytical procedure will be particularly useful for the rapid quantification of food extracts where these compounds are major components.

ABBREVIATIONS USED

GD, Golden Delicious; E, Empire; RD, Red Delicious; M-AA, antioxidant activity; W-AA, water antioxidant activity

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