EFFECT OF CHLORINE DIOXIDE AND ASCORBIC ACID ON ENZYMATIC BROWNING AND SHELF LIFE OF FRESH-CUT RED DELICIOUS AND GRANNY SMITH APPLES

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ABSTRACT

In this work, we tested the hypothesis that ascorbic acid (AA) reduces browning of fresh-cut apples (Red Delicious, RD, and Granny Smith, GS), and we investigated the impact of AA on phenylpropanoid metabolism of RD and GS. Apple slices were dipped in a solution of 100 mg/L of chlorine dioxide (ClO_2) and ClO_2 + 3% AA and stored at 4C for 96 h. Flesh firmness, solid soluble content and browning index, total phenols and flavonoids, and the activity of peroxidase and polyphenol oxidase were monitored upon storage (0, 48 and 96 h). Our results demonstrated that GS is less sensitive to browning and thus more suitable for minimally processed produce. Ascorbate reduces the browning index also in RD, a cultivar largely appreciated by consumers but more prone to browning. AA likely contrasts browning appearance by interacting with peroxidase and polyphenol oxidase and/or promoting the regeneration of phenols and flavonoids.

PRACTICAL APPLICATIONS

Browning of fresh-cut apple is one of the main problems that limit the shelf life of this type of produce. Given that this produce is highly appreciated by consumers, different antibrowning treatments have been tested to extend the shelf life of fresh-cut apple. We found that treatment with 100 mg/L of ClO_2 + 3% of ascorbic acid significantly reduces the browning appearance in apple slices. Browning was also reduced in Red Delicious cultivar that is more prone than Granny Smith to this phenomenon, but that is highly appreciated by consumers.

INTRODUCTION

Consumer attention is rapidly growing for ready-to-eat products, especially when they associate high nutraceutical value to their practicality (Landi *et al.* 2015). Among the minimally processed fruits, apple is one of the main sources of phenols that are responsible for the high antioxidant ability of this well-appreciated fruit (Boyer and Liu 2004; Wolfe *et al.* 2008; Carbone *et al.* 2011). In contrast, the contribution of ascorbic acid (AA) to the total antioxidant activity is less than 0.4% in apple (Varming *et al.* 2013).

The minimal operations that are necessary to prepare fresh-cut products induce rapid changes in organoleptic as well as nutritional characteristics of the raw material because the tissue integrity of fruits is more easily altered during processing. This makes fresh-cut commodities more perishable than the raw materials. In particular, minimally processed apples have a shorter shelf life than the whole fruits because they are more sensitive to enzymatic browning and tissue softening. Browning phenomenon represents indeed the main reason that discourages consumers from purchasing fresh-cut apples. It has long been known that enzymatic browning is caused by the activity of polyphenol

oxidase (PPO) and peroxidase (POD), enzymes that catalyze the oxidation of phenolic compounds into the corresponding *o*-quinone in wounded tissues (Degl'Innocenti *et al.* 2005; Toivonen and Brummel 2008; Supapvanich *et al.* 2012). *O*-quinones are relatively uncolored, but due to their chemical instability they tend to polymerize into brown pigments. This reaction is oxygen dependent and for this reason the exposition of fruit tissue to increased oxygen level enhances strongly the development of brown compounds. Color changes have been extensively reported in fresh-cut apple fruits (Valentines *et al.* 2005; Pristijono *et al.* 2006; Lunadei *et al.* 2011), and the browning phenomenon has been considered many times as the limiting factor for the shelf life of minimally processed apple (Martinez and Whitaker 1995; Rojas-Graü *et al.* 2009; Supapvanich *et al.* 2012). On the one hand, the richness in phenols represents a positive aspect for consumers' health; on the other hand, phenols increase the browning propensity of fresh-cut product given that those compounds are the preferential substrate of PPO and POD. Despite the fact that other research have investigated the browning phenomenon on minimally processed apple slices (Toivonen and Brummel 2008; Jang and Moon 2011), more progress can be made to reduce the loss of this widely merchandise produce.

A wide range of treatments have been applied in an attempt to reducing the browning phenomenon of fresh-cut produce, including the use of natural browning inhibitors (Rojas-Graü *et al.* 2006; De La Rosa *et al.* 2011; Loizzo *et al.* 2012; Cefola *et al.* 2014a; Pace *et al.* 2015), salt and chemical treatments (Cocci *et al.* 2006; Aguayo *et al.* 2010; Oms-Oliu *et al.* 2010; Barbagallo *et al.* 2012), edible coating agents (Rojas-Graü *et al.* 2007; Robles-Sánchez *et al.* 2013; Ojeda *et al.* 2014) and the combined effects of ultrasound and AA (Jang and Moon 2011). Physical means such as heat, controlled atmosphere and irradiation have also been utilized to inhibit browning (Lopez-Galvez *et al.* 1996; Peng and Jiang 2004; Zhang *et al.* 2006; Manzocco *et al.* 2009; Cefola *et al.* 2014b). However, physical treatments can potentially alter organoleptic characteristics and nutrient level of fresh-cut produce (Alothman *et al.* 2009) and, like many chemical antibrowning products, are generally much expensive. For this reason, fresh-cut industry needs an effective as well as a relatively cheap method that can substitute the current methods.

Chlorine dioxide is widely used to maintain nutritional and microbiological quality of food. It is well known that ClO_2 is not only a powerful oxidizing agent but also a sanitizing agent according to its broad-spectrum and strong biocidal activity (Fu *et al.* 2007). Moreover, this compound does not react with organic compounds to produce toxic chlorinated by-products (as do liquid chlorine and hypochlorites) and does not affect sensory attributes (Gomez-Lopez *et al.* 2009). Recently, Chen *et al.* (2010) reported that ClO_2 represents a promising approach to inhibit enzymatic browning and to prolong the shelf life of fresh-cut asparagus and lettuce, inhibiting the activity of both PPO and POD. In addition, the selection of cultivars that are less sensitive to the browning phenomenon can contribute to the reduction of loss of produce (Keenan *et al.* 2012).

Exogenous AA has been tested as an antibrowning agent in many fresh-cut produce, including apple (Chiabrando and Giacalone 2012; Gomez *et al.* 2012). However, its mechanism(s) of action has not been clarified yet. Three main mechanisms have been proposed: (1) AA may act as an antioxidant, promoting the regeneration of *o*-quinones and preserving them from polymerization into brown pigments (Walker 1995; Alscher *et al.* 1997); (2) AA can bind to histidine residues of PPO catalytic site, increasing the enzymatic Km of PPO and reducing the turnover of PPO-triggered oxidized phenols (Osuga *et al.* 1994); (3) as a weak acid, AA accumulation may lower cytosolic pH, thus downregulating the activity of browning promoting enzymes (PODs and PPOs) after cutting (Vamos-Vigyazo 1981; Landi *et al.* 2013). Among these three possible hypotheses, the latter appears less probable in lettuce as both PPOs and PODs maintain high activity under a wide range of pH (Landi *et al.* 2013).

Thus, the aim of this study was to compare the effects of ClO_2 combined to AA, largely utilized in Italy as antibrowning agent (Chiabrando and Giacalone 2012), on the shelf life of two cultivars of apple: the low browning-sensitive Granny Smith (GS) and the largely appreciated but more prone to browning Red Delicious (RD) stored as fresh-cut product for 96 h.

MATERIAL AND METHODS

Fruit Material and Slices Preparation

The experimental trials were carried out in autumn/winter of 2009 and 2010 on fruits provided by a commercial apple orchard located in Montopoli Val D'Arno (Pisa, Italy). RD and GS apples (*Malus domestica* Borkh) were harvested in the middle and at the end of September, respectively. Fruits were immediately stored at 0C. After 30 days of cold storage, fruits were transferred to 4C for 1 week prior to being processed as fresh-cut produce. Apples were prepared according to Tardelli *et al.* (2013) with few modifications. Twenty apples for each cultivar were washed with 3 L of water containing 100 mg/L ClO_2 (Sigma-Aldrich, Milan, Italy) for 2 min. After a 30 s rinse under running MilliQ water (Milli-Q® Advantage A10, Millipore, Merck, Darmstadt, Germany), each apple was cut into 16 slices with a sharp knife before removing the core portion of fruit. Slices were

rinsed in MilliQ water for 30 s, drained, dipped in a solution of 100 mg/L ClO_2 for 2 min, allowed to drain for 2 min and then randomly placed into 750 cm^3 rectangular packages of polyethylene terephthalate (Comital Cofresco, Volpiano, Turin, Italy). Other fruit slices previously dipped in a solution of 100 mg/L ClO_2 (as described earlier) were also dipped in a solution of 3% AA (Sigma-Aldrich), dried with sterile paper and randomly collected into containers. All containers were kept at 4C under light condition in order to simulate the commercial shelf conditions. Each package contained eight apple slices (~100 g). Six packages per treatment (ClO_2 and ClO_2 + AA) for each sampling time (0, 48, 96 h after cutting) were stored at 4C. On each sampling time, half of the slices were used for quality evaluations and half were rapidly frozen in liquid nitrogen and stored at −80C prior to biochemical analyses.

Flesh Firmness, Soluble Solid Content and Browning Index

The penetration test represents the measure of the strength necessary to insert a metal point of a dynamometer to an established distance into the flesh fruit. The measure was performed on the central zone of the slice (peeled portion) using a digital penetrometer installed on a driving column equipped with an 8 mm probe (model 53205, Forlì, Italy). Flesh firmness (FF) was estimated among the mean of six replicates of apple slices and expressed as Newton (N).

Soluble solid content (SSC) was estimated with a digital refractometer (model 53011 TR, Forlì, Italy) on apple juice obtained by squeezing an apple slice. Each value is the mean of six independent replicates. SSC measurements were carried out on the same fruit portion utilized for FF analyses and were expressed as °Brix.

The browning index (BI) indicates the proportion of phenols that are oxidized during the apple storage. For BI determination, the absorbance of an aliquot of the ethanol : acetone (7:3, v/v) extract was measured in triplicate with an Ultrospec 2100 Pro UV-vis spectrophotometer (GE Healthcare Ltd., Chalfont St. Giles, Buckinghamshire, UK) at 420 nm (Jeong *et al*. 2008). A blank was realized using the same amount of extraction solution. The final results were expressed as absorbance units per g fw (Viña and Chaves 2006).

Total Polyphenols and Total Flavonoids

The content of total phenol (TP) was determined using the Folin–Ciocalteu assay, according to Dewanto *et al*. (2002). The absorbance of an aliquot of extract (25 µL) was read at 760 nm, and TP concentration was expressed as gallic acid equivalents (µg GAE/100 g fw) using a calibration curve (50–600 µg/mL of gallic acid).

Total flavonoid (TFO) concentration was determined according to Du *et al*. (2009) with few modifications. In a 2 mL Eppendorf tube, 90 µL of apple extract were added to 1 mL ethanol 30% v/v, 45 µL of 50 mM $NaNO_2$, 45 µL of $AlCl_3 \times 6H_2O$ 0.3 M. After 5 min at room temperature, 300 µL of 1 M NaOH was added and the mixture absorbance was measured at 506 nm. TFO content was expressed as rutin equivalents (mg RE/100 g fw) using a calibration curve as standard (6.25–1,000 mg/mL of rutin).

Polyphenol Oxidase and Peroxidase Activity

Extraction of PPO and POD was carried out as described by Loiza-Velarde *et al*. (1997) with slight modifications. Apple tissue (8 g) was homogenized with purified sand and polyvinylpolypyrrolidone in 2.5 mL of 50 mM phosphate buffer (pH 6.8). Samples were centrifuged at 19,000× g for 20 min at 4C. The supernatant was then collected, kept at 4C and utilized for determinations.

Activity of PPO was determined spectrophotometrically according to Espin *et al*. (1997); POD was determined according to Rodriguez-Lopez *et al*. (2000) with some modifications. The reaction mixture for POD activity determination contained 50 mM phosphate buffer (pH 4.5), 2 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2 mM H_2O_2, 0.2 mM tropolone and 2.7 µg of protein of enzymatic extract in a final assay volume of 1 mL. One unit of POD was defined as the amount of enzyme that produces 1 mol of $ABTS^{+}$ per min.

High-Performance Liquid Chromatography Profile of Phenolic Compounds

Lyophilized apple tissue (0.25 g) was ground with 5 mL methanol/H_2O (70/30, v/v) solution by sonication at room temperature for 30 min. Subsequently, the mixture was centrifuged at 4,000× g for 10 min at 4C, and the supernatant was filtered with RC 0.45 µm pore filter and used for high-performance liquid chromatography (HPLC) analysis. Chromatographic separation was performed using a Shimadzu liquid chromatograph (SLC-10A VP Shimadzu-Dupont Co., Kyoto, Japan) equipped with a diode array detector setup at 280 and 325 nm. Polyphenol compounds were separated using a 250 × 4.6 mm ID (5 µm) Prodigy ODS3 100 Å column (Phenomenex, Torrance, CA). The eluants were: A water 0.2% formic acid; B acetonitrile/methanol (60:40, v/v). The gradient program was as follows: 20–30% B (6 min), 30–40% B (10 min), 40–50% B (8 min), 50–90% B (8 min), 90–90% B (3 min), 90–20% B (3 min) at a constant flow rate of 0.8 mL/min. Injection volume was 20 µL.

Mass spectrometry analyses of apple extracts were performed on an API 3000 mass spectrometer (Applied Biosystems, Ontario, Canada) equipped with a TurboIonSpray source using the following parameters: drying gas (air) heated to 400C, capillary voltage (IS) set to 4,000 V. Analyses were performed in multiple reaction monitoring working in the negative ion mode. Metabolites were identified by their molecular weight, fragmentation pattern and comparison of their retention time, UV absorption with those of commercial standards and literature data.

Statistical Analysis

Experiments were repeated twice (autumn/winter 2009 and 2010) with similar results. A representative data set is reported herein. Results represent the mean of six replicates ($n = 6$) ± standard deviation (SD) for each treatment and storage time after cutting. Two-way analysis of variance (ANOVA) was performed considering treatment (ClO_2 and ClO_2 + AA) and the storage time as variability factors. Results of HPLC phenolic profile at 96 h after cutting represent the mean of three replicates ($n = 3$) ± SD and two-way ANOVA was performed considering treatment (ClO_2 and ClO_2 + AA) and cultivar (RD and GS) as variability factors. Means keyed with different letters are significantly different after Fisher's least-significant difference test for $P = 0.05$. Constitutive levels of enzymatic activity, phenols and flavonoids were analyzed using Student's *t*-test. All the statistical analyses were performed using CoStat 6.4 (Cohort Software, Monterey, CA).

RESULTS AND DISCUSSION

Change of Firmness and Browning

Values of FF were constitutively higher in GS as compared with RD ($P < 0.05$) as already observed by Toivonen and Hampson (2009). Conversely, SSC and BI were significantly lower in GS than RD immediately after cutting (0 h). Thus, fruits belonging to the two cultivars are certainly constitutively different, even before any treatment (Fig. 1).

Both RD and GS slices treated with ClO_2 + AA had lower BI compared with the samples treated only with ClO_2 48 h after cutting (i.e., about −77% in RD and −79% in GS; see Fig. 1E and F). The ameliorative effect of AA on BI was consistent at 96 h as well. Treatment with AA induced an increment of SSC in RD at 48 h while in GS a significant increment was found only at 96 h (Fig. 1C and D). Our

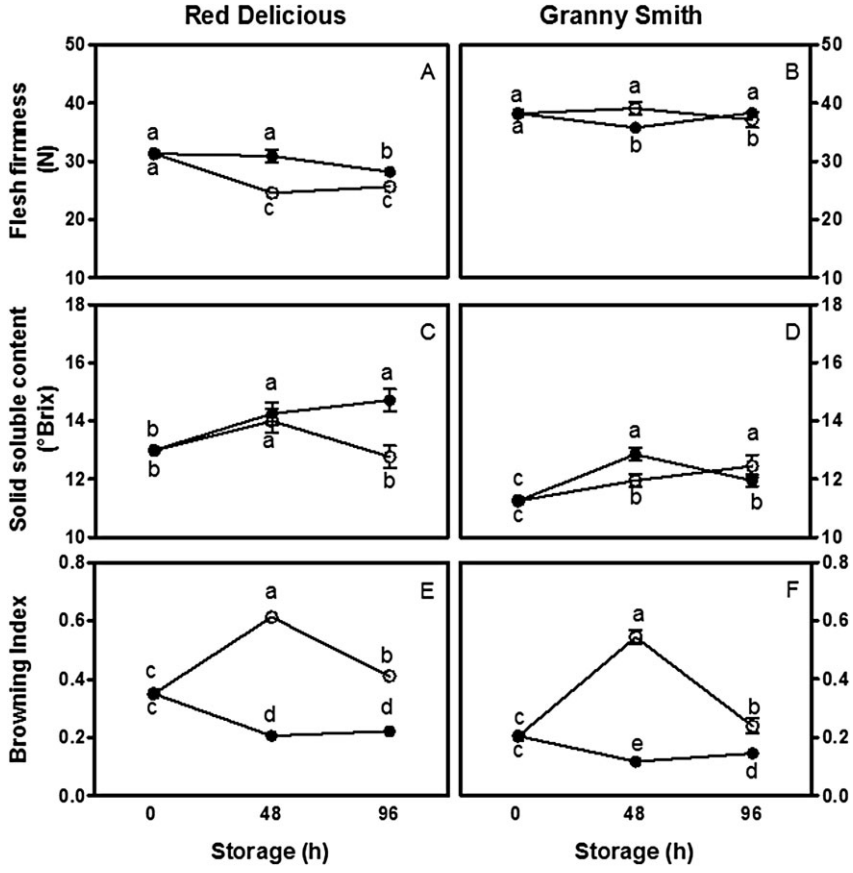

FIG. 1. (A–F) FLESH FIRMNESS, SOLID SOLUBLE CONTENT AND BROWNING INDEX IN FRESH-CUT RED DELICIOUS AND GRANNY SMITH APPLE SLICES STORED FOR 48 AND 96 H
The time 0 represents the moment of cutting. Slices are treated with ClO_2 (open circle) or ClO_2 + 3% ascorbic acid (closed circle). Means ($n = 6$; ± standard deviation) with the same letters are not significantly different for $P = 0.05$ following least-significant difference test.

TABLE 1. LIST OF COMPOUNDS IDENTIFIED IN THE CHROMATOGRAM INCLUDING CORRESPONDING RETENTION TIMES, MOLECULAR IONS AND FRAGMENT IONS

Peak	tr (min)	[M-H]- m/z	MS/MS ions m/z	Identification
1	6.3	577	451, 425, 407, 289	Procyanidin B1
2	7.8	353	191	Chlorogenic acid
3	8.1	577	451, 425, 407, 289	Procyanidin B2
4	9.3	865	847, 739, 695, 577, 451, 407, 289	Procyanidin trimer
5	9.4	289	245, 205, 203, 179, 137, 125	Epicatechin
6	9.5	865	847, 739, 695, 577, 451, 407, 289	Procyanidin trimer (isomer)
7	13.5	463	301	Isoquercitrin (quercetin-3-O-glucoside)
8	15.4	567	273	Phloretin-2-O-xyloglucoside
9	16.3	433	301	Quercetin-O-pentoside
10	16.4	447	301	Quercitrin (quercetin-3-O-rhamnoside)
11	18.4	435	273	Phloridzin (phloretin-2-O-glucoside)

results confirm that AA can reduce the browning development in apple slices in accordance with previous results reported by Gomez *et al.* (2012) in fresh-cut GS. Of note, Chiabrando and Giacalone (2012) reported that treatments with 1% of AA/$CaCl_2$ controlled enzymatic browning not only in GS but also in Golden Delicious and Scarlet Spur apples slices.

Effects of ClO_2 + AA on TFO and TP Quantitative and Qualitative Profile

The two apple cultivars differed also for TP and TFO content before cutting; RD has indeed higher level of phenols than GS (Fig. 2). After 48 h of storage fresh-cut slices of RD and GS treated with ClO_2 showed a significant increase in TP (about +33% and +54% in RD and GS, respectively) and TFO (+32% in RD and 50% in GS) (Fig. 2). The increment found at 48 h was not observed in GS nor RD slices treated with ClO_2 + AA where TP and TFO remained stable (or only slightly increased) upon storage until the end of the experiment. It is well known that TP biosynthesis is usually enhanced in fruit/vegetable tissues under stress (such as that induced by wounding) by the stimulation of phenylalanine ammonia lyase (PAL) pathway, in which PAL is the key enzyme (Degl'Innocenti *et al.* 2005). This process leads to incremented availability of phenols as substrate for enzymes involved in browning processes (PPO and POD), thus increasing the propensity to browning (Landi *et al.* 2013). We found that values of TP and TFO rapidly decreased at 96 h, likely in response to the higher activity of PPO and POD. However, the biosynthesis of phenols is not only a deleterious process for fruits, but rather a signal for tissues under stress (i.e., damages provoked by insects). For this reason, both the amount and the

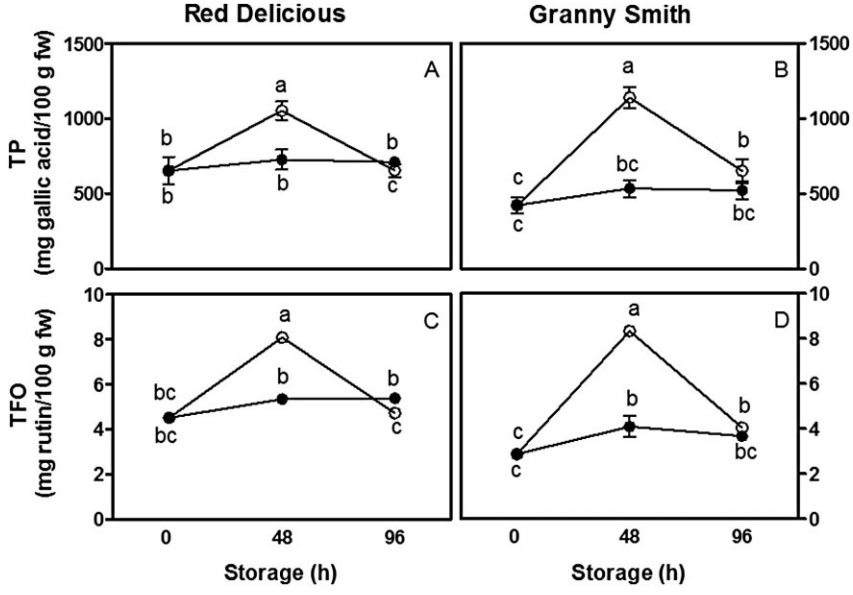

FIG. 2. (A–D) TOTAL PHENOL (TP) AND TOTAL FLAVONOIDS (TFO) IN FRESH-CUT RED DELICIOUS AND GRANNY SMITH APPLE SLICES STORED FOR 48 AND 96 H
The time 0 represents the moment of cutting. Slices are treated with ClO_2 (open circle) or ClO_2 and 3% ascorbic acid (closed circle). Means ($n = 6$; ± standard deviation) with the same letters are not significantly different for $P = 0.05$ following least-significant difference test.

TABLE 2. CONCENTRATION OF INDIVIDUAL AND TOTAL POLYPHENOLS DETERMINED BY HPLC

	Red Delicious		Granny Smith	
Concentrations polyphenolics	ClO_2	ClO_2 + AA	ClO_2	ClO_2 + AA
Procyanidin B1	114.8 ± 0.2d	121.9 ± 0.3c	126.8 ± 0.4a	124.8 ± 0.2b
Chlorogenic acid/procyanidin B2	84.8 ± 2.9a	61.8 ± 1.0b	22.0 ± 0.0c	21.9 ± 0.1c
Procyanidin trimer	13.0 ± 0.0d	15.0 ± 0.1a	14.3 ± 0.1b	13.4 ± 0.1c
Epicatechin	42.7 ± 0.3b	44.0 ± 0.1a	33.6 ± 0.1c	33.7 ± 0.0c
Procyanidin trimer (isomer)	45.1 ± 0.4a	42.8 ± 0.0b	21.2 ± 0.1d	37.8 ± 0.8c
Isoquercitrin (quercetin-3-O-galactoside)	2.1 ± 0.0c	10.8 ± 0.0a	4.9 ± 0.1b	2.1 ± 0.0c
Guajaverin (quercetin 3 O-arabinopyranoside)	7.5 ± 0.7d	11.6 ± 0.1c	13.1 ± 0.1b	15.7 ± 0.0a
Phloretin-2-O-xyloglucoside	5.5 ± 0.0c	10.8 ± 0.0b	9.8 ± 0.0c	13.8 ± 0.3a
Avicularin (quercetin 3 O-arabinofuranoside)	1.5 ± 0.1c	4.3 ± 0.1a	2.2 ± 0.0b	1.7 ± 0.0c
Quercitrin (quercetin-3-O-rhamnoside)	4.6 ± 0.1c	5.5 ± 0.2b	5.8 ± 0.1ab	6.0 ± 0.0a
Phloridzin (phloretin-2-O-glucoside)	16.9 ± 0.1a	14.4 ± 0.0b	6.3 ± 0.0c	6.4 ± 0.0c
Total polyphenols	338.7 ± 1.6b	343.2 ± 0.9a	260.1 ± 0.1d	277.5 ± 0.9c

Fresh-cut Red Delicious and Granny Smith apple slices stored for 96 h. Slices are treated with ClO_2 or ClO_2 + 3% AA. Each value is the mean of three replicates ($n = 3$) ± standard deviation. Means with the same letters are not significantly different for $P = 0.05$ following least-significant difference test. Values are expressed as mg/100 g dw.
AA, ascorbic acid; HPLC, high-performance liquid chromatography.

profile of TP and TFO in tissues are dynamic process, representative of the trade-off between their synthesis and oxidation (Reyes *et al*. 2006).

The identification of polyphenolics in apple fruits was performed using liquid chromatography-tandem mass spectrometry (LC/MS/MS) while quantitative analysis was performed by HPLC (Table 1 and Table 2). Flavan-*3*-ols (procyanidin B1, procyanidin B2, procyanidin trimer and epicatechin) and hydroxycinnamic acid (chlorogenic acid) were the main phenolic compounds in apple fruits (Table 2). This group contributed about 86% to the total phenolics (average of the two cultivar's content), whereas flavonols (isoquercitrin, guajaverin, avicularin and quercitrin) and dihydrochalcones (phloretinxyloglucoside and phloridzin) represented 7.6 and 6.4%, respectively, of total phenolics of ClO_2-treated slices. Proportions of phenolics were similar in ClO_2 + AA-treated slices in terms of average content of the two cultivars. Interestingly, chlorogenic acid, one of the phenolic acids with higher affinity for PPO, is about fourfold higher in ClO_2-treated RD (the cultivar characterized by the higher browning sensitivity) than GS cultivar. Similar profile of phenols and flavonoids has been already reported in apple (McGhie *et al*. 2005; Rossle *et al*. 2010).

The total phenolic content evaluated by HPLC in ClO_2 and ClO_2 + AA slices of RD at the end of storage was 338.7 versus 343.2 mg/100 g dw, respectively (Table 2). In GS, the content of polyphenols was increased in ClO_2 + AA slices (277.5 mg/100 g dw) as compared with those treated without AA (260.1 mg/100 g dw). The difference was significant ($P < 0.05$) and, as previously reported, this result is in agreement with values of TPs spectrophotometrically determined (Fig. 2A). Of note, at 96 h the concentration of chlorogenic acid in RD slices treated with ClO_2 + AA was lower than that found in ClO_2-treated slices. Conversely, procyanidin B1, procyanidin trimer and isoquercetin levels were higher in RD slices treated with ClO_2 + AA (Table 2). Phloridzin levels were retained better in GS slices treated with ClO_2 + AA, but procyanidin B1, procyanidin trimer and isoquercitrin were lower under that treatment (Table 2). These results suggest that 3% of AA influenced the phenolic content and profile of apple slices. Accordingly, Gil *et al*. (1998) showed that an antibrowning treatment of 2% AA prevented total phenolic content decrease during storage of Fuji apple slices. Also, Cocci *et al*. (2006) found that apple slices treated with 1% AA (without and with modified atmosphere) underwent a lower decrease of bioactive compounds upon storage. Notably, Aguayo *et al*. (2010) reported that exogenous calcium ascorbate had only a small significant effect on the polyphenolic content of apple slices, but overall the phenolic compounds were relatively stable with storage duration and atmosphere.

Effect of Treatments on PPO and POD Activity

According to Tardelli *et al*. (2010), the two cultivars showed constitutively different values of PPO activity that were higher in RD as compared with those of GS (Fig. 3), whereas no differences were found in constitutive values of POD in the two apple cultivars ($P < 0.05$).

The activity of PPO increased significantly in ClO_2-treated slices of RD at 48 h, whereas ClO_2 + AA-treated slice had values similar with those found immediately before cutting (Fig. 3A). No significant changes were observed in GS at 48 h (Fig. 3B). The rapid increase of PPO activity is a

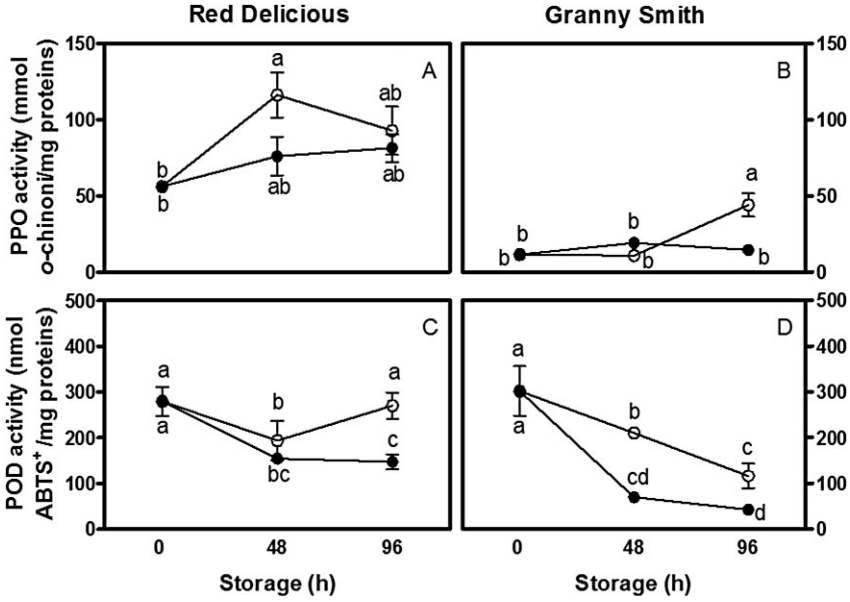

FIG. 3. (A–D) POLYPHENOL OXIDASE (PPO) AND PEROXIDASE (POD) ACTIVITY IN FRESH-CUT RED DELICIOUS AND GRANNY SMITH APPLE SLICES STORED FOR 48 AND 96 H

The time 0 represents the moment of cutting. Slices are treated with ClO_2 (open circle) or ClO_2 and 3% ascorbic acid (closed circle). Means ($n = 6$; ± standard deviation) with the same letters are not significantly different for $P = 0.05$ following least-significant difference test.

typical response induced by wounding; many preservatives, including AA, have demonstrated to effectively control the PPO activity and the browning appearance (Rocha and Morais 2005; Jeong *et al*. 2008; Soares and Fonseca 2008; Tardelli *et al*. 2010; Chiabrando and Giacalone 2012; Landi *et al*. 2013). However, at the end of the storage in RD slices no differences were found between ClO_2 and ClO_2 + AA treatments (Fig. 3A), while in GS slices the PPO activity increased, but not in ClO_2 + AA-treated slices (Fig. 3B). It indicates that in this cultivar, where the activity of PPO is constitutively lower than that of RD, ClO_2 is sufficient to control PPO only within a short period of storage (48 h), while the addition of AA is necessary to control the level of PPO activity for more prolonged period of storage.

On the contrary, POD activity decreased significantly in both the cultivars and treatments after 48 h of storage (Fig. 3C and D). Slices of RD treated with ClO_2 + AA maintained lower values of POD, even at 96 h after cutting, compared with slices treated only with ClO_2 (Fig. 3C). Differently, in GS the POD activity decreased significantly on storage and independently to the treatment. Notably, lower values of POD were found in slices treated with ClO_2 + AA (Fig. 3D).

The obtained results concerning the enzymatic activities of PPO and POD, the key enzymes involved in the oxidation of polyphenolic compounds, should be considered together with the patterns of phenolic compounds. It is well known that among all the phenolic compounds located in apple flesh, only a few of them have high affinity for the catalytic site of apple PPO, i.e., chlorogenic acid and catechins (Chow *et al*. 2011). In RD, TP levels are constitutively higher than those of GS. In addition, 48 h after cutting a further increase in TP and TFO was found in RD slices treated with ClO_2, whereas no changes were observed in slices treated with ClO_2 + AA. Concomitantly, 48 h after cutting PPO activity in RD slices treated with ClO_2 showed the typical increase induced by cutting while no changes were observed in samples treated with ClO_2 + AA. In addition, chlorogenic acid (the one of the high-affinity substrate for PPO) was lower in ClO_2 + AA-treated slices than in ClO_2-treated slices (84.8 versus 61.8 mg/100 g dw, respectively).

In synthesis, a large number of inherent constitutive differences were observed between RD and GS apple fruits. Lower levels of phenolics coupled with a higher FF might partially explain the constitutive less sensitivity of GS to the enzymatic browning when processed as fresh-cut produce. Indeed, if fewer phenols are available for PPO and POD, and at the same time, less oxygen is available due to the higher compactness of tissues, a reduced browning propensity is expected in GS. Furthermore, in RD a relevant percentage of phenol moiety was represented by chlorogenic acid (>25%). Differently, that compound is much more less abundant in GS (<10%).

Upon storage, the rise in phenolic compounds, which was evident during the first 48 h in apple slices of both the cultivars treated with ClO_2, can contribute to the incremented BI. The buildup on phenolic content is likely a direct consequence of PAL activation, enzyme that plays a key role in phenolic synthesis. That increase was not observed in ClO_2 + AA-treated slices suggesting a positive effect of AA on PAL activity. As a consequence of higher phenol level in slices of both the cultivars treated only with ClO_2 than those treated with ClO_2 + AA at 48 h, incremented levels in the

activity of browning-related enzymes (PPO and POD) were found (at 48 or 96 h).

Although we attribute the ameliorative effect to AA against browning appearance, the mechanism by which AA prevents that phenomenon is still unknown. We cannot exclude that AA directly inhibits the activity of enzymes related to phenol biosynthesis (PAL) and/or oxidation (PPO and POD) as already proposed by Landi *et al.* (2013), or that AA alternatively promotes the regeneration of *o*-quinones, hence preserving them from polymerization into brown pigments (Walker 1995; Alscher *et al.* 1997). However, what is remarkable for industrial application is the ability of AA to modulate the phenylpropanoid pathway, controlling the browning phenomena in many fresh-cut products, including apples. Curiously, the more clear evidence of a role for AA in browning prevention directly arises from the observation that fruits and vegetables with high AA (e.g., kiwi and rocket salad) exhibited a relatively less browning susceptibility after cutting than those with a reduced content of that antioxidant (e.g., apple and lettuce).

CONCLUSIONS

The aim of this study was to compare the effect of ClO_2 and ClO_2 + AA treatment on two cultivars of apple, RD and GS, prepared as ready-to-eat product. We clearly evidenced all the treatments and processes need to be developed specifically for a selected cultivar because the effectiveness of a treatment is strictly linked to fruit-specific characteristics. However, a relatively inexpensive and safe molecule as AA can significantly contribute to reduction of browning phenomenon also in RD, a cultivar largely appreciated by consumers, but usually characterized by higher loss of produce due to its higher browning sensitivity. Despite this, we consider dipping in AA a promising treatment to counteract the browning of ready-to-eat apple fruit; this field of research warrants further investigation to find other (combination of) preservatives that can further reduce the deleterious effect of browning on apple, hence extending the shelf life of this highly appreciated fresh-cut fruit.

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