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Phenolic Compounds and Related Enzymes Are Not Rate-Limiting in Browning Development of Fresh-Cut Potatoes

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The effect of minimal processing on polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia-lyase (PAL), and phenolic compounds was studied in five potato cultivars (Agria, Cara, Liseta, Monalisa, and Spunta). Minimal processing caused an overall increase in PPO, POD, and PAL activities. The isoform pattern of PPO was the same for all of the cultivars before and after processing. No latent PPO was detected. The isoperoxidase pattern was approximately the same among cultivars. An increase in POD activity was related to the specific induction of an acidic isoperoxidase. PAL showed an induction pattern characterized by the presence of a maximum peak of activity after 4 days of processing for all of the cultivars. The sequence of browning susceptibility of potato cultivars was as follows: Monalisa > Spunta > Liseta > Cara > Agria. Browning development was only partially correlated to PAL activity (only during the first 4 days after wounding). However, this correlation could not explain the above sequence of browning susceptibility. Minimal processing caused an increase of chlorogenic acid, whereas tyrosine content remained unchanged. In summary, no significant correlation was found between either rate or degree of browning and any other biochemical and physiological attribute investigated (PPO, POD, hydrogen peroxide, ascorbic acid content, and initial phenolics content as well as total and individual phenolics accumulation).

KEYWORDS: Enzymatic browning; fresh-cut potato; isoelectric focusing; minimal processing; phenylalanine ammonia-lyase; PAL; peroxidase; POD; polyphenol oxidase; PPO

INTRODUCTION

Consumption of fresh-cut fruits and vegetables has increased as a response to the demand for quality and the modern way of life of consumers. However, the shelf life of minimally processed products is usually limited by enzymatic browning, which causes a decrease in food quality because it implies spoilage (*1*). Therefore, the prevention of this browning has been a challenge for food scientists (*2*). Minimal processing involves tissue injury because of some mechanical processes (such as handling, peeling, and cutting). Such wounding induces many physiological responses related to wound healing (*3*) with a common secondary side reaction: browning development.

Phenylalanine ammonia-lyase (EC 4.3.1.5; PAL) is the first committed enzyme in phenyl propanoid metabolism (*4*). PAL is a wound-induced enzyme; its activity increases due to cell injury provoked during minimal processing (*3*). An increase in PAL activity provokes an increase in the concentration of phenolic compounds, which are substrates for oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POD).

The key enzyme in the melanogenesis pathway is polyphenol oxidase (EC 1.14.18.1) (*5*). This copper-containing enzyme catalyzes two reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). These *o*-quinones are highly reactive compounds that nonenzymatically evolve to give rise to brown, black, or red pigments commonly called melanins, which are responsible for less attractive appearance and loss in nutritional quality (*1*).

Another important oxidative enzyme in the plant kingdom is peroxidase (EC 1.11.1.7). This heme-containing enzyme is usually associated with wound-healing processes such as lignification (*6*). POD performs single-electron oxidation of phenolic compounds in the presence of hydrogen peroxide (*7*). The possible role of POD in melanin formation has been questioned due to the low hydrogen peroxide content of vegetable tissues. However, the generation of hydrogen peroxide in the oxidation of some phenolics catalyzed by PPO could indicate a possible synergistic action between both PPO and POD, which suggests the involvement of POD in browning processes (*8*).

Previous studies tried to correlate or explain the browning susceptibility of potato tubers and different biochemical or physiological attributes (*9*). However, this approach has not been carried out in fresh-cut potatoes. In principle, the limiting factor

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or step involved in enzymatic browning must not be necessarily the same for both potato tubers (no wounding, long storage) and minimally processed potatoes (wounding, short storage). Research has been focused on the prevention of browning of fresh-cut potatoes by using different approaches: modified atmosphere packaging (*10*), use of inhibitors such as SH-containing compounds and citric and ascorbic acids (*10*, *11*), or combined methods using chemical reagents and some physical treatments (*12*, *13*). It is a common concern for many food scientists to avoid the use of sulfites, antibrowning agents that are (unfortunately) widely used to prevent potato browning. Sulfites can provoke bronchial asthma (*14*) and undesirable off-flavors as well as a significant reduction of the nutritional value of fresh-cut potatoes (*15*). Therefore, it is the purpose of many governments to prohibit sulfites as food additives. However, there is a lack of suitable (both efficient and economic) antibrowning agents so that the use of sulfites has not been fully avoided so far. The identification of the most critical step in the development of browning reaction in minimally processed potatoes could be useful to design alternative strategies to avoid enzymatic browning.

The aim of the present work was to study the effect of minimal processing on the phenolic-related enzymes PPO, POD, and PAL as well as other factors (ascorbic acid, hydrogen peroxide, and phenolic compounds) possibly related to browning susceptibility of fresh-cut potatoes.

MATERIALS AND METHODS

Reagents. Ascorbic acid (AA), mushroom PPO (EC 1.14.18.1; 50000 units), chlorogenic acid, L-tyrosine, 3-methyl-2-benzothiazolinone hydrazone (MBTH), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide (H_2O_2) (30%, v/v), benzamidine chloride, phenylmethanesulfonyl fluoride (PMSF), catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6), 3,4-dihydroxyphenylpropionic acid (DHPPA), tropolone, *N*,*N*′-dimethylformamide (DMF), sodium dodecyl sulfate (SDS), and Triton X-114 (TX-114) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade and supplied by Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Plant Material and Processing. Agria, Cara, Liseta, Monalisa, and Spunta potato cultivars were purchased from a local supplier in Murcia (Spain) and transported by car to the laboratory (5 km), where those with defects (cuttings and bruisings) were discarded. Sound tubers were kept at 4 °C and 70% relative humidity (RH) in darkness during 2 days prior to processing.

Potatoes were hand-peeled and cut in 8 × 8 mm strips with a manual potato cutter (Sammic CF-4, Azpeitia, Spain) at room temperature. Then, potatoes were washed with running water. Uniform strips were selected, and broken pieces discarded. No postharvest chemical washing treatment was applied in order to achieve the complete browning potential for each cultivar. Three replicates were used for each treatment and sampling date. One hundred grams of strips was selected at random from the whole bunch of potato strips and placed in 250 mL jars as one replicate. A continuous flow of humidified air at a rate of 10 mL min^{-1} was obtained by using flow boards and capillary tubing to maintain CO_2 levels below 0.15% (*16*). Samples were stored in darkness at 4 °C and analyzed immediately after cutting and after 1, 2, 3, 4, 5, and 6 days of storage.

Extraction of Phenolic Compounds. Frozen potato strips (5 g) were homogenized in a Polytron (30 s on ice) with 5 mL of an extraction solution of methanol/water (70:30). The water fraction contained 4 mM NaF and 5% formic acid. Homogenates were filtered through cheesecloth, and the solid fraction was collected for color measurements. The extract was then centrifuged at 11300*g* for 10 min. The supernatant was recovered carefully to prevent contamination with the pellet, filtered through a 0.45 μm membrane, and directly analyzed by HPLC after a period not exceeding 2 h.

Color Measurement. The protocol for color measurement was adapted from that of Tomás-Barberán et al. (*17*) with some modifications. The solid fraction above obtained was placed into 16 cm diameter polystyrene wells (Cell Wells, Corning, NY) and pressed slightly with a glass rod to make the depth of the solid fraction 14 mm; the color was measured at the well bottom. CIELAB color parameters (*L**, *a**, *b** values) were determined with a spectrophotometer (Minolta, CR-300) at three points through the bottom of the well.

HPLC Analysis of Phenolic Compounds. Samples of 50 μL of the above filtered supernatants were analyzed using an HPLC-DAD system (Merck-Hitachi, Darmstadt, Germany) equipped with a pump (model L-7100), a UV−vis detector (model L-7455), and an autosampler (model L-7200). A reversed phase C_{18} LiChroCART column (25 × 0.4 cm; LiChrospher 100, particle size = 5 μm) was utilized. Elution was performed using water/formic acid (19:1, v/v) (A) and HPLC grade methanol (B) as the mobile phases, on a gradient starting with 3% B in A to reach 35% B in A at 25 min and 90% B in A at 35 min. The flow rate was 1 mL min^{-1}, and chromatograms were recorded at 270 and 320 nm. Hydroxycinnamic acids were quantified by comparisons with an external standard of chlorogenic acid at 320 nm and tyrosine at 270 nm. The concentrations were expressed as milligrams per 100 mg of frozen weight. HPLC analyses were performed in triplicate.

Ascorbic Acid Determination. AA was determined by HPLC (*18*) with the equipment above-described but using a reversed phase Kromasil 100 C-18 column (25 × 0.4 cm; 5 μm particles size; Tecnokroma, Barcelona, Spain) with an ODS guard C-18 precolumn.

Sensory Evaluation. Browning of fresh-cut potatoes was evaluated immediately after cutting and after 1, 2, 3, 4, 5, and 6 day intervals by a panel of three judges. Evaluation was scored on a 10-point scale (0 = no browning; 5 = moderate browning; 10 = severe browning).

Enzyme Extraction. PPO and POD were extracted using the method of Cantos et al. (*19*) with minor modifications. Briefly, 20 g of fresh potato strips was homogenized by using a blender in 20 mL of cold 0.1 M sodium phosphate buffer (PB) (pH 7) containing 3% TX-114, 1 mM PMSF, 5 mM benzamidine, and 20 mM AA. The homogenate was incubated at 35 °C for 15 min, filtrated through three gauze layers, and then centrifuged at 12000*g* for 20 min at 25 °C. The pigment-free supernatant was used as enzymatic extract with both PPO and POD activities.

PAL was extracted from 6 g of frozen potato strips in 15 mL of 50 mM borate buffer (pH 8.5) according to the method of Ke and Saltveit (*20*).

The extraction protocols were repeated three times, and then the supernatants were mixed. The final supernatants were used as enzyme sources to perform kinetic assays.

Spectrophotometric Assays. PPO activity was determined according to the method of Espín et al. (*21*, *22*). This assay method measures the accumulation of the adduct formed between the enzymatically generated *o*-quinones and the nucleophile MBTH. This adduct (reddish in color) is stable and has high molar absorptivity (ε). Adduct accumulation was followed at 467 nm (ε = 22300 M^{-1} cm^{-1}, at pH 5.5). Two percent DMF (v/v) was added to improve adduct solubility with no effect on PPO activity as previously described (*21*, *22*) Unless otherwise stated, the standard reaction mixture for determining PPO activity contained 50 mM sodium acetate buffer (AB) (pH 5.5), 2% DMF, 3 mM MBTH, 2 mM DHPPA, and 2.7 μg of protein of enzymatic extract (5 μL of extract). When PPO was assayed, 10 μg/mL bovine catalase was routinely added to the medium to remove possible traces of hydrogen peroxide so that PPO activity was accurately measured without the possible interference of POD activity. To discriminate between latent and active PPO, 0.1% SDS was added to the reaction medium (*23*). One unit of PPO was defined as the amount of the enzyme that produces 1 μmol of MBTH−DHPPA−*o*-quinone adduct per minute (*21*). The final assay volume was 1 mL.

POD was determined according to the method of Rodríguez-López et al. (*24*) with some modifications by measuring the accumulation of the ABTS radical cation ($ABTS^{\bullet+}$) at 414 nm (ε = 31300 M^{-1} cm^{-1}). Unless otherwise stated, the standard reaction mixture for determining POD activity contained 50 mM AB (pH 4.5), 2 mM ABTS, 2 mM H_2O_2, 0.2 mM tropolone, and 2.7 μg of protein of enzymatic extract. No POD activity could be measured in the absence of H_2O_2 in the

assay mixture. The specific PPO inhibitor tropolone (*25*) was included in the medium to determine specifically POD activity. One unit of POD was defined as the amount of the enzyme that produces 1 μmol of $ABTS^{\bullet+}$ per minute. The final assay volume was 1 mL.

PAL activity was determined by following the accumulation of cinnamic acid at 290 nm, using 10 mM phenylalanine as PAL substrate (*20*). One unit of PAL was defined as the amount of the enzyme that produces 1 μmol of cinnamic acid per minute. The final assay volume was 3 mL.

The spectrophotometric assays for determining enzyme activities were recorded in a spectrophotometer (UV-1603 Shimadzu, Tokyo, Japan). Temperature was controlled at 25 °C for both PPO and POD activities and at 40 °C for PAL activity with a temperature controller (CPS 240 Shimadzu), checked using a precision of ±0.1 °C. The figures show the mean of three separate kinetic assays. The coefficient of variation was always <10%.

Data Analysis. Enzyme activity was determined by linear regression fitting of the spectrophotometric recordings. The fitting was carried out by using a Gauss−Newton algorithm (*26*) implemented in the Sigma Plot 6.0 program for Windows (SPSS Science, Chicago, IL). Correlation coefficient (*R*) and *P* values were calculated between the degree of browning estimated by a sensory panel and the different possible factors involved in browning development: PPO, POD, PAL (and the respective parameters that characterize their activities), AA, initial phenolics content, and phenolic compounds accumulation. *L** values were not used in these calculations because both *R* and *P* values were less statistically significant. Both *R* and *P* values were calculated using the same Sigma Plot program.

Polyphenol Oxidase and Peroxidase Activities in Fresh Tissue. Both PPO- and POD-catalyzed reactions were differentially detected on fresh potato tissue. To develop enzymatic reactions, the same assay mixtures previously described under Spectrophotometric Assays were used with some modifications. In this case, the compounds were sequentially added to discriminate the relative importance of both endogenous enzymes and endogenous substrates in the development of the reactions. To ensure a difference between PPO and POD activities, tropolone (to inhibit PPO) and catalase (to prevent POD activity) were used. Catechol (1 mM) was used as exogenous substrate for both enzymes. SDS (0.1%) was added to detect the presence of latent PPO. ABTS was not used to develop POD activity because there was no good color contrast between the green color of the ABTS radical cation (the product) and the white-yellow color of the potato strip (the matrix). MBTH (3 mM) was added to accelerate and to color both PPO and POD reactions.

Isoelectric Focusing (IEF) Experiments. Enzymatic extracts were obtained as above-described but with the ratio 20 g of potato/15 mL of buffer. The extract (pigment-free supernatant) was then brought to 20% saturation with solid ammonium sulfate. After 15 min, the mixture was centrifuged at 15000*g* for 15 min and the pellet discarded. The ammonium sulfate concentration was supplemented to reach a final 80% saturation. Again, after 15 min, the mixture was centrifuged at 15000*g* for 15 min and the supernatant discarded. The pellet obtained was dissolved in 250 μL of 0.1 M PB (pH 7) containing 5 mM AA and washed three times. The volume collected (750 μL) was pooled and centrifuged at 11300*g* in a Sigma 1-13 microcentrifuge (Braun Biotechnology International). The supernatant was dialyzed overnight at 5 °C against ultrapure water. The dyalized sample was concentrated in a Microcon YM-10 filter membrane (Millipore Corp.), and 4 μL of the concentrated sample was applied to the gel. Isoelectric points (IEP) of both PPO and POD were determined in the electrophoresis unit Phast System (Pharmacia, Uppsala, Sweden). IEF gels (PhastGel from Pharmacia) with an IEP range of 3−9 were used. Conditions for running IEF experiments were those suggested by the manufacturer. Broad IEP (3−10) kit markers from Pharmacia were used. After focusing, the gels were rinsed either in 50 mM AB (pH 5.5) (for PPO) or in 50 mM AB (pH 4.5) (for POD) and then transferred to a Petri dish for activity staining. Solutions to develop PPO activity contained 10 mM catechol, 5 mM MBTH, 50 mM AB (pH 5.5), and 10 μg/mL catalase. To develop POD activity, the solution contained 10 mM catechol, 5 mM MBTH, 50 mM AB (pH 4.5), 1 mM tropolone, and 2 mM H_2O_2. ABTS was not chosen as POD substrate because the reaction product ($ABTS^{\bullet+}$) is very soluble. For this reason, catechol was selected as POD substrate (*23*). For developing both PPO and POD activities, DMF was not added in order to facilitate precipitation of MBTH adducts in the gel (red-pink in color). In both cases, washing the gel in water and drying it stopped staining. Isoelectric experiments were repeated five times.

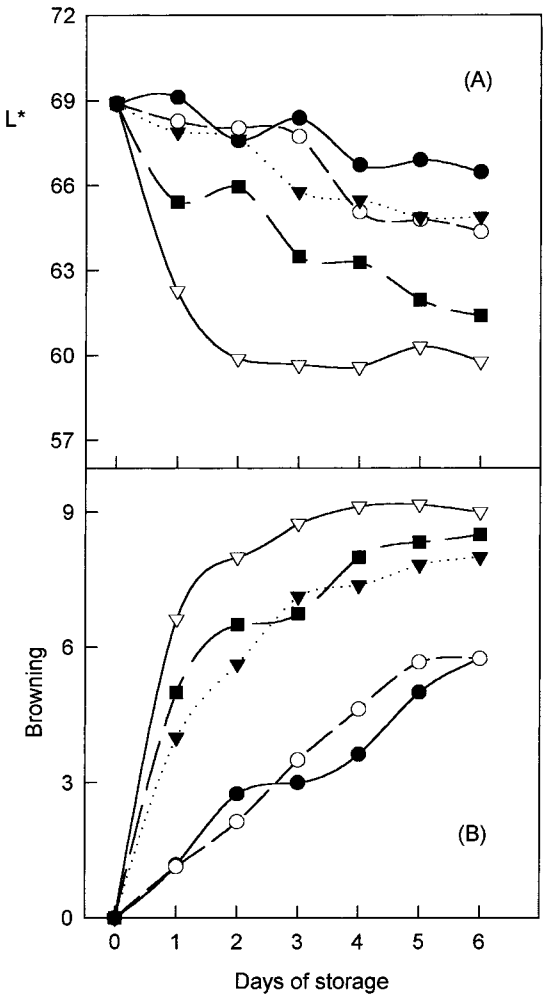

Figure 1. (A) Evolution of *L** values in fresh-cut potatoes upon storage: (●) Agria; (○) Cara; (▼) Liseta; (▽) Monalisa; (■) Spunta. (B) Degree of browning determined by a sensory panel of trained people. The scale ranged from 0 (no discoloration) to 10 (maximum discoloration observed). Coefficient of variation was <10%.

Protein Determination. Protein content was determined by using the method of Bradford (*27*) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Browning Susceptibility of Fresh-Cut Potato Cultivars. To assess the real browning potential for each cultivar, two variables were selected to be involved in minimal processing: cutting (which promotes browning) and refrigerated storage (which slows browning appearance and mimics the commercial shelf life of the fresh product). Among color parameters (*L**, *a**, *b**) and color indexes (hue angle, chroma, ΔE, etc.), the best indicator of browning appearance in fresh-cut potato strips was the *L** parameter (brightness factor) (**Figure 1A**). Browning susceptibility of potato cultivars was evaluated every day according to both *L** values and results obtained from the sensorial evaluation panel (**Figure 1B**). The order of browning susceptibility according to *L* values was (the lower the *L* value is, the more browning occurs) Monalisa ≫ Spunta > Liseta ≈ Cara > Agria. The other criterion to estimate browning susceptibility was based on visual assessment by a panel of

trained people. In this case, the order of browning development was similar to that indicated by *L* values: Monalisa > Spunta > Liseta ≫ Cara > Agria (**Figure 1B**). However, the panel of trained people observed two different groups of potato cultivars according to their browning susceptibility. One group of cultivars was highly susceptible to browning: Monalisa, Spunta, and Liseta. The other potato group less susceptible to browning involved both Cara and Agria cultivars. Monalisa was the potato cultivar most highly susceptible to browning because the maximum degree of browning was reached only 2 days after of the initial cutting (**Figure 1**). According to this browning potential Monalisa potatoes should not be used for minimal processing. On the other hand, the cv. Agria was the least susceptible to browning (**Figure 1**), which, in principle, could suggest its use for minimal processing.

In addition, it is of note that better correlation coefficients were found when the degree of browning estimated in the sensory panel was used. Therefore, this criterion will be used along the present study.

Polyphenol Oxidase. The involvement of PPO as a possible critical factor in the development of browning in potato has been widely studied. However, these studies were carried out either in tubers (blackspot susceptibility; *9*, *28*, *29*) or in model solutions (*30*, *31*). To our knowledge, this is the first study that reports the effect of minimal processing on PPO in potato.

Potato PPO showed the same broad optimum pH with a plateau from 5.25 to 5.75 for all of the cultivars (results not shown). PPO from the five cultivars studied here was soluble and fully active. To discriminate between latent and active PPO, 0.1% SDS was added to the assay medium (*23*). No increase in PPO activity (activation process) was observed with different SDS concentrations (0.01−0.3%), which demonstrated that PPO from these cultivars was fully active. In the presence of SDS, PPO activity either remained unaltered (0.01−0.08% SDS) or decreased because of denaturation (0.09% SDS or higher). To discriminate between soluble and membrane-bound PPO, the detergent TX-114 was used in the extraction protocol. In the presence of this detergent the entire PPO activity can be measured (*19*). However, in the present study, the same PPO activity was measured after extraction with different TX-114 concentrations (0.3−5%). This result indicated that PPO from these potato cultivars was fully soluble. Therefore, browning susceptibility was not related to a possible activation of latent PPO (it is fully active) or to a possible release of the enzyme from membranes (it is fully soluble).

PPO activity decreased the first day after cutting for all of the cultivars (**Figure 2**). However, PPO activity increased to reach an apparent linear accumulation after some storage time, which depended on the cultivar (**Figure 2**; **Table 1**). The pattern of PPO activity versus storage time after minimal processing was characterized by different parameters (**Table 1**): an initial velocity (V_0) (day 0, immediately after cutting), a slope that described the velocity of PPO increase versus storage time, the "burst" of activity (elapsed number of days to achieve the linear increase in PPO activity), and finally the variation of PPO activity after 6 days of storage of fresh-cut potatoes (ΔV). None of these parameters, which describe the pattern of PPO activity of minimally processed potato cultivars upon storage, could fully explain the different browning susceptibilities (**Figure 1**). In fact, ΔV for cv. Monalisa (the most susceptible to browning) was negative (**Table 1**). The slope for the increase of PPO activity was higher for cv. Spunta and Cara (cultivars with significantly different browning susceptibilities) and approximately the same for the rest of cultivars (**Table 1**). Finally, the

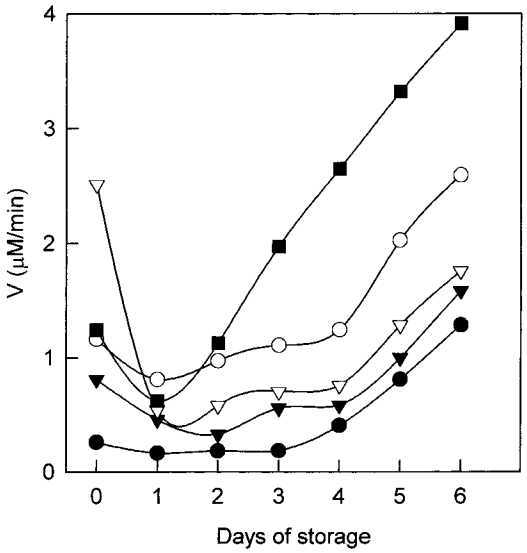

Figure 2. Effect of minimal processing on potato PPO: (●) Agria; (○) Cara; (▼) Liseta; (▽) Monalisa; (■) Spunta. Assay mixture (1 mL) included 50 mM AB (pH 5.5), 2% DMF, 3 mM MBTH, 2 mM DHPPA, 10 μg/mL bovine catalase, and 2.7 μg of protein of enzymatic extract (5 μL of extract). The mean of three separate experiments is shown. Coefficient of variation was <10%.

Table 1. Induction Parameters of Polyphenol Oxidase from Five Fresh-Cut Potato Cultivars[a]

cultivar	initial velocity V_0[b] (μM/min)	slope[c] (μM/min/day)	burst (no. of days)	ΔV[d] (μM/min)
Agria	0.26 ± 0.03	0.16 ± 0.01	4	1.04 ± 0.05
Cara	1.16 ± 0.05	0.24 ± 0.01	4	1.44 ± 0.08
Liseta	0.81 ± 0.03	0.18 ± 0.01	4	0.79 ± 0.05
Monalisa	2.51 ± 0.10	0.18 ± 0.01	4	–0.76 ± 0.08
Spunta	1.24 ± 0.05	0.24 ± 0.01	1	2.67 ± 0.13

[a] Assay conditions: 50 mM AB (pH 5.5), 2% DMF, 3 mM MBTH, 2 mM DHPPA, 10 μg/mL bovine catalase, and 2.7 μg of protein of enzymatic extract (5 μL of extract). [b] V_0 is the initial enzymatic activity (enzyme activity before minimal processing). [c] Slope was determined by linear regression fitting of experimental data from day 4 to day 6 (from day 1 to day 6 in the case of cv. Spunta). [d] ΔV = variation of PPO activity after 6 days ($V_6 - V_0 = \Delta V$).

apparent linear accumulation of PPO activity took place at the fourth day for all cultivars except for Spunta (first day) (**Table 1**). *R* and *P* values were calculated for PPO activity and degree of browning. The most significant correlations were found for cv. Agria, Liseta, Spunta, and Cara ($R = 0.98$, 0.92, 0.92, and 0.88, respectively) when the degree of browning and PPO activity during the last 4 days of storage were correlated. However, *P* values were >0.05, which indicated that the level of significance was low. It is also of note that there was no correlation at all between cv. Monalisa (the most susceptible to browning; **Figure 1**) and PPO. Maybe, the high initial PPO activity (V_0) in cv. Monalisa could be also partially related to the high browning development observed in this potato (**Figure 2**; **Table 1**). Therefore, in general, the apparent lack of correlation between PPO activity and browning susceptibility in minimally processed potato agrees with previous studies, which reported the same poor correlation in potato tubers (*28*, *29*, *32*, *33*). The same lack of correlation between PPO activity and browning susceptibility in minimally processed lettuce has been reported in previous studies (*19*, *34*).

The development of PPO activity on fresh potato tissue was also carried out in order to shed more light on the possible

different contributions of both endogenous PPO and phenolic compounds. This could be a more in vivo approach because it does not involve extraction procedures. The sequential addition of the reagents to develop PPO activity could be helpful to ascertain the different contributions of PPO and phenolics in browning development before and after minimal processing. First, the addition of buffer at pH 5.5 favored endogenous PPO activity at its optimum pH. Second, catalase (to inhibit any possible POD interference) was added. Third, MBTH was added to couple PPO-generated quinones to form more visible adducts and thus to amplify the reaction. In these conditions, the PPO-catalyzed reaction started involving both endogenous potato PPO and phenolic compounds (no exogenous addition of these reagents). When this reaction was developed on the potato surface at day 0, immediately after cutting, the reaction was very slow for every cultivar except for both Spunta and Monalisa. The same reaction was more evident for every cultivar when the reaction was developed in fresh potato stored for 6 days. This higher activity at day 6 could indicate the synergistic effect of both the induction of phenolic compounds and the increase in PPO activity after 6 days of storage (**Figure 2**). When catechol was added (as exogenous PPO substrate), no difference was found in the development of the reaction at both days 0 and 6 for every cultivar. In these conditions (the only limiting factor is endogenous PPO activity) the different endogenous PPO activity of each cultivar was not significant enough to justify browning susceptibility. In fact, residual PPO activity was enough to provoke significant browning. Exogenous PPO (commercial mushroom PPO) was added (without addition of exogenous PPO substrate) to ascertain the contribution of endogenous phenolic compounds in browning development (in these conditions the endogenous phenolic compounds were rate-limiting). At day 0, browning development was almost equal for all of the cultivars. At day 6, both Spunta and Monalisa cultivars developed the reaction more rapidly than the rest of the cultivars, which could be related to their higher final chlorogenic acid concentration. However, the difference in activity was not enough to justify the browning differences observed after minimal processing and subsequent storage for 6 days (**Figure 1**). Therefore, it can be concluded that both endogenous PPO and phenolic compounds were not rate-limiting in browning development in fresh tissue. Finally, no latent endogenous PPO was detected on fresh tissue because addition of SDS (as activator) did not increase PPO activity. Therefore, this approach was also useful to demonstrate that the presence of fully active PPO in the model solution was not due to a possible activation of latent PPO during the extraction protocol.

IEF experiments demonstrated the increase in PPO activity as well as the presence of six PPO isoforms in every cultivar before and after minimal processing with isoelectric points of 7.56, 7.27, 6.70, 6.55, 6.12, and 5.69 (results not shown). Therefore, minimal processing except for the presence of more evident bands after 6 days of storage due to the increase in PPO activity did not alter the pattern of PPO isoforms. All of these results indicated that, apparently, there was a de novo synthesis of PPO due to minimal processing, although there was no specific isoform induction associated with this processing. These results agree with those previously described by Thypyapong et al. (*35*), who reported the systemic wound induction of PPO in potato leaves. This de novo synthesis, apparently, did not occur in minimally processed lettuce (*19*), in which posttranslational processes (activation from latent to fully active PPO) were responsible for the increase of PPO activity as well as for the appearance of specific isoforms after minimal processing.

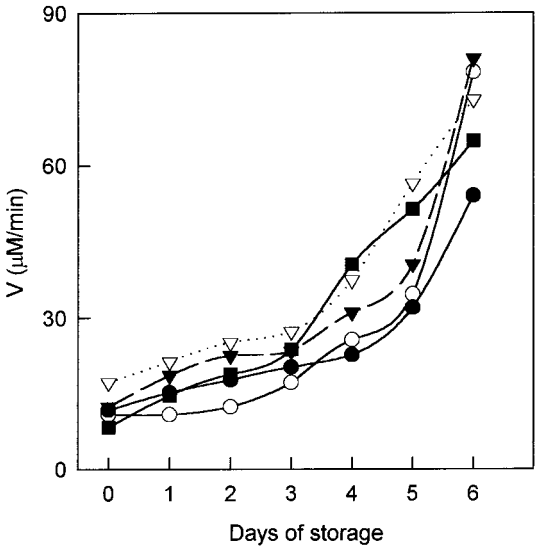

Figure 3. Peroxidase induction in fresh-cut potatoes upon storage: (●) Agria; (○) Cara; (▼) Liseta; (▽) Monalisa; (■) Spunta. Assay conditions: 50 mM AB (pH 4.5), 2 mM ABTS, 2 mM H_2O_2, 0.2 mM tropolone, and 2.7 μg of protein of enzymatic extract (final volume = 1 mL). The mean of three separate experiments is shown. Coefficient of variation was <10%.

Therefore, it seems that the effect of minimal processing on PPO depends on the processed product (lettuce, potato, etc.) and cannot be standardized by general rules.

Peroxidase. The role of POD in enzymatic browning has been previously questioned because of the low hydrogen peroxide in fruits and vegetables. However, there is increasing evidence that POD could be involved in this reaction due to the generation of hydrogen peroxide in PPO-catalyzed reactions (*8*, *36*), which could suggest a synergistic action of both PPO and POD in enzymatic browning.

Enzymatic extracts performed in the presence and in the absence of either 1 M NaCl or TX-114 revealed that almost the same POD activity was measured, which meant that the potato POD was found to be mainly soluble, or at least the possible presence of either covalent or ionically membrane-bound POD was not significant in the five potato cultivars studied here.

Potato POD showed an optimum pH of 4.5 for all of the cultivars (results not shown). POD activity presented a slow apparent linear increase during the first 3−4 days after cutting, depending on the cultivar. After this storage time, a sudden increase in POD activity was detected in every cultivar (**Figure 3**). The pattern of POD activity versus storage time after wounding was characterized by different parameters (**Table 2**): an initial velocity (V_0) (day 0, immediately after cutting), an initial slope (POD activity per day), a second slope that described the increase of POD activity after 3−4 days of storage, the burst of activity (elapsed number of days to reach the second slope), and finally the variation of POD activity after 6 days of storage of fresh-cut potatoes (ΔV). The correlation coefficient (R) was calculated between all of these parameters and browning degree (sensory panel). No statistically significant correlation was obtained between browning degree and POD activity pattern during 6 days of storage after initial wounding (results not shown). However, good correlation between browning degree and POD activity was found when the first slope was considered (**Figure 3**; **Table 2**). R values were 0.99 (Liseta), 0.97 (Agria), 0.93 (Monalisa), 0.93 (Spunta), and 0.90 (Cara) with $P < 0.01$ in every case. No correlation was found when the second slope

Table 2. Induction Parameters of Peroxidase from Five Fresh-Cut Potato Cultivars[a]

cultivar	initial velocity V_0[b] (μM/min)	slope 1[c] (μM/min/day)	slope 2[d] (μM/min/day)	burst (elapsed no. of days)	ΔV[e] (μM/min)
Agria	11.77 ± 0.09	1.4 ± 0.1	15.0 ± 0.3	4	15.6 ± 0.3
Cara	10.86 ± 0.10	1.0 ± 0.1	7.0 ± 0.1	5	20.6 ± 0.5
Liseta	12.42 ± 0.08	1.3 ± 0.1	16.1 ± 0.3	5	25.4 ± 0.6
Monalisa	17.25 ± 0.11	1.3 ± 0.1	6.6 ± 0.1	4	20.6 ± 0.4
Spunta	8.35 ± 0.06	1.9 ± 0.1	3.6 ± 0.1	4	20.9 ± 0.5

[a] Assay conditions: 50 mM AB (pH 4.5), 2 mM ABTS, 2 mM H_2O_2, 0.2 mM tropolone, and 2.7 μg of protein of enzymatic extract. [b] V_0 is the initial enzymatic activity (enzyme activity before minimal processing). [c] Slope 1 was determined by linear regression fitting of experimental data before burst occurred, i.e., from day 0 to day 3 for cv. Agria, Monalisa, and Spunta or from day 0 to day 4 in the case of cv. Liseta and Cara. [d] Slope 2 was determined as described for slope 1 but after "burst" occurred, i.e., from day 4 to day 6 in the case of cv. Agria, Monalisa, and Spunta or from day 5 to day 6 in the case of cv. Liseta and Cara. [e] ΔV = variation of peroxidase activity after 6 days ($V_6 - V_0 = \Delta V$).

Table 3. Peroxidase IEPs of Five Fresh-Cut Potato Cultivars[a]

Agria	Cara	Liseta	Monalisa	Spunta
3.08	3.08	3.08	3.08	3.08
3.67	3.67	3.67	3.67	3.67
5.98	5.98		5.98	5.98
6.45	6.45	6.45	6.45	6.45
		6.55	6.55	6.55
	6.63	6.63	6.63	6.63
6.70				
6.79	6.79	6.79	6.79	
6.85	6.85	6.85		
			6.89	
				6.96
7.15	7.15		7.15	
				7.24
	7.42	7.42		
				8.20
				8.72
8.90	8.90	8.90	8.90	8.90

[a] After focusing according to manufacturer specifications (Pharmacia), gels were developed for POD activity with a solution containing 10 mM catechol, 5 mM MBTH, 50 mM AB (pH 4.5), 1 mM tropolone, and 2 mM H_2O_2.

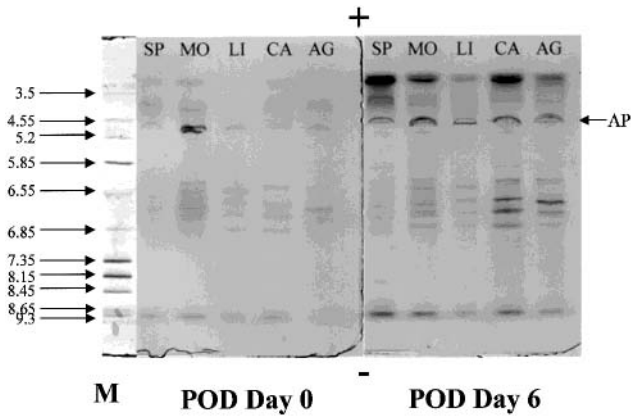

Figure 4. Isolectric pattern of isoperoxidases from fresh-cut potatoes at days 0 and 6 (AP, application point; M, markers; SP, Spunta; MO, Monalisa; LI, Liseta; CA, Cara; AG, Agria). See **Table 3** for the isoelectric point values; some of them are not visible in the image-processed gels. Staining solution for developing bands with POD activity included 50 mM AB (pH 4.5), 10 mM catechol, 5 mM MBTH, 1 mM tropolone, and 2 mM H_2O_2. Four microliters of concentrated enzymatic extract from each cultivar was applied to the gel. Day 0 amount of protein applied was 0.45 μg for each cultivar. The protein amount slightly varied depending on the cultivar at day 6: 0.5 μg for SP and CA, 0.4 μg for MO and AG; and 0.32 μg for LI. A representative gel from five replicates is shown.

was taken into account (results not shown). This preliminary apparent good correlation should be viewed with caution. First, it seems to be contradictory that the sudden increase of POD activity 3−4 days of initial wounding (**Figure 3**) is not correlated, whereas the slight initial increase is correlated. Second, the values of this first slope cannot explain browning susceptibility because cultivars with very different browning susceptibilities (Agria and Monalisa) share approximately the same values (**Table 2**). Third, the availability of hydrogen peroxide in potato tissue should be checked because potato peroxidase activity was absolutely dependent on hydrogen peroxide presence.

The development of POD reaction on fresh potato tissue demonstrated that neither endogenous POD nor phenolic compounds were rate-limiting in the reaction. The only possible limiting substrate could be hydrogen peroxide, because POD reaction became evident only when exogenous hydrogen peroxide was added, which demonstrated that no hydrogen peroxide was formed upon storage of fresh-cut potatoes for 6 days. This could be explained, at least partially, because the generation of H_2O_2 during PPO-catalyzed reactions is not a general rule (*36*). One of the best phenolics as possible precursor of H_2O_2 generation is catechin, which was not found in these potato cultivars. Chlorogenic acid, one of the most abundant potato PPO substrates, is not as good as catechin at inducing hydrogen peroxide formation (*36*). Therefore, the apparent good correlation between the first slope of POD activity during storage (**Figure 3**; **Table 2**) and the degree of browning did not justify the browning of fresh-cut potatoes.

IEF experiments demonstrated that all cultivars shared approximately the same isoperoxidase pattern before and after minimal processing (**Table 3**) with only minor differences among cultivars. An acidic isoperoxidase (IEP = 3.08) was specifically induced in all of the cultivars because of processing (**Figure 4**). Moreover, the increase in POD activity after 4−5 days (second slope, **Table 2**) was due to the induction of this acidic isoperoxidase as demonstrated in **Figure 5** in the case of the cv. Cara (the same result was found for all of the cultivars; results not shown). The gel from **Figure 5** also shows an apparent induction of the isoenzyme with IEP = 3.67. However, this was an artifact caused by the application point (as demonstrated in **Figure 4**, which does not show such an increment for isoenzyme with IEP = 3.67 in cv. Cara at day 6). The induction of specific isoperoxidases seems to be a common feature in the plant kingdom as a response to stress situations such as wounding (*17*), high salt concentration (*24*), herbicide and pesticide application (*37*), and γ-irradiation (*38*).

Phenylalanine Ammonia-lyase. PAL activity increased after wounding in every potato cultivar (**Figure 6**), which is a typical behavior in the enzyme PAL as a response to this stress (*19*).

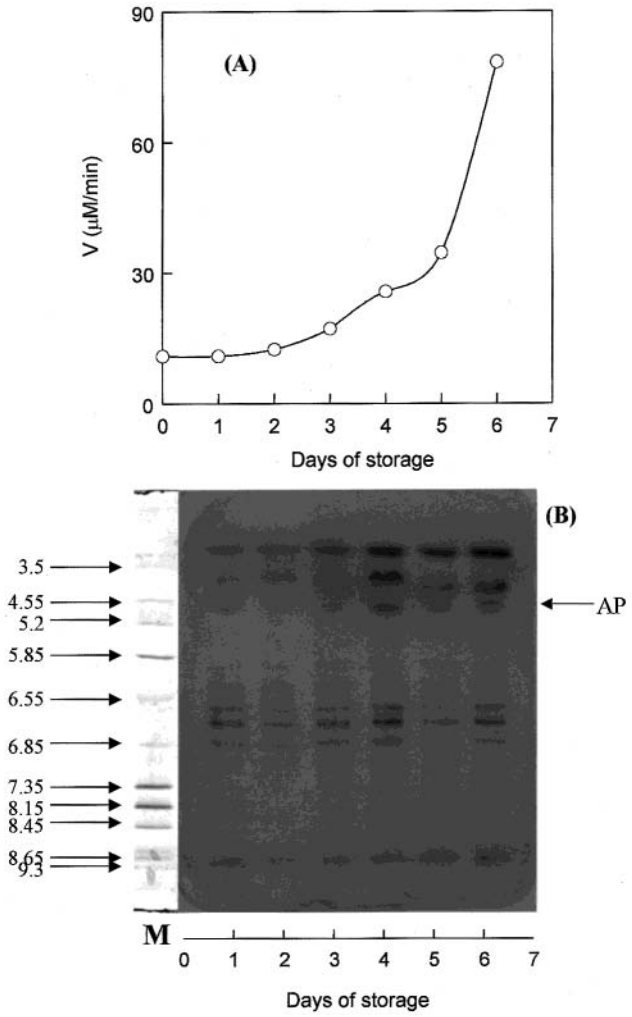

Figure 5. Relationship between the increase in POD activity and POD induction in fresh-cut cv. Cara potatoes upon storage: (A) from **Figure 3**, (○) Cara; (B) isoperoxidase pattern of cv. Cara analyzed every day (AP, application point; M, markers). Conditions for developing POD activity were those described in **Figure 4**: 0.55 μg of protein was applied to the gel on each lane. A representative gel from five replicates is shown.

The induction pattern presented a main peak of maximum activity after 4 days of wounding, which was more evident in the Spunta and Cara cultivars (**Figure 6**). There was a previous peak or "shoulder" of PAL activity after 2 days of wounding except for cv. Agria. This pattern was reproducible in different assays. Probably, the explanation for the presence of these peaks could be the sequential induction of mRNAs for this enzyme as previously reported in potato slices (*39*).

Browning development was correlated to PAL activity for all of the cultivars but only during the first 4 days after wounding (coincident with the maximum PAL activity). The best correlation was found for cv. Cara ($R = 0.96$, $P < 0.01$) and the worst for cv. Spunta ($R = 0.85$, $P < 0.01$). Although PAL activity presented this apparently good correlation, it could not explain the sequence of browning susceptibility (**Figures 1** and **6**).

Ascorbic Acid and Phenolics. In principle, AA, a well-known reducing agent, should be involved in the prevention of melanin formation due to its capability to reduce *o*-quinones to *o*-diphenol precursors. However, correlation coefficients between AA content and browning degree were very poor and not statistically significant at all (results not shown). This result agrees with previous studies, which also report the same lack of correlation in potato tubers upon storage (*28*).

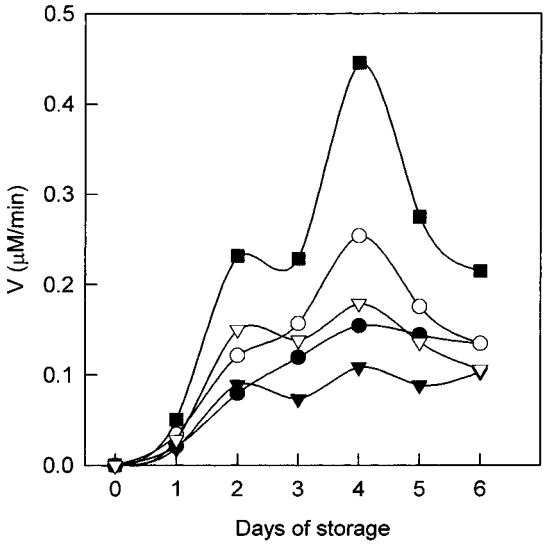

Figure 6. PAL in fresh-cut potatoes upon storage: (●) Agria; (○) Cara; (▼) Liseta; (▽) Monalisa; (■) Spunta. Conditions: 50 mM borate buffer (pH 8.5), 10 mM phenylalanine, and 25 μL of enzymatic extracts (final volume = 3 mL). PAL activity from all cultivars was referred to 1 μg of protein. The mean of three separate experiments is shown. Coefficient of variation was <10%.

Correlation coefficients between browning and total and individual phenolics were also calculated. The main phenolics detected as possible precursors of browning were chlorogenic acid and tyrosine. Minimal processing and further refrigerated storage of potato caused increases in chlorogenic acid content of 2.4-, 2.8-, 3-, 3.5-, and 8.6-fold in cv. Liseta, Spunta, Agria, Monalisa, and Cara, respectively (**Figure 7**). Chlorogenic acid gave the best correlations with browning: Cara ($R = 0.92$, $P < 0.01$), Agria ($R = 0.9$, $P < 0.01$), Liseta ($R = 0.86$, $P < 0.02$), Monalisa ($R = 0.6$, $P < 0.1$), and Spunta ($R = 0.5$, $P < 0.2$). Obviously, coefficients for cv. Monalisa and Spunta were not statistically significant. In the case of Monalisa, chlorogenic acid was better correlated with browning only during the first 2 days after wounding ($R = 0.9$, $P < 0.05$). However, no correlation could be found between chlorogenic acid and browning for the Spunta cultivar. However, the monophenol tyrosine, which was not induced by minimal processing, did not correlate with browning at all for any cultivar, and the content remained almost unchanged throughout the storage (**Figure 7**): Cara (12 ± 3), Agria (13.1 ± 3), Spunta (14.7 ± 2), Liseta (27.5 ± 5), and Monalisa (45.6 ± 3) (mg/100 g of frozen weight ± standard deviation).

Previous studies did correlate blackspot (internal browning) susceptibility in potato tubers with tyrosine content (*9*). However, chlorogenic acid (*o*-diphenolic structure) is a much better substrate than the monophenol tyrosine according to the reaction mechanism and substrate specificity of PPO (*40*). Minimal processing of potatoes involves a cascade of events that result in quick browning reactions. In this case, PPO will catalyze preferentially the oxidation of chlorogenic acid. Therefore, browning reactions in minimally processed potatotes (wounding, short storage) and browning of potato tubers (no wounding, prolonged storage), apparently, do not share rate-limiting steps. Moreover, to fully understand the possible limiting factors in the development of browning in fresh-cut potatoes, more studies involving other important aspects (membrane stability, lipid composition, calcium content, protease activity, agronomic practices, etc.) are required.

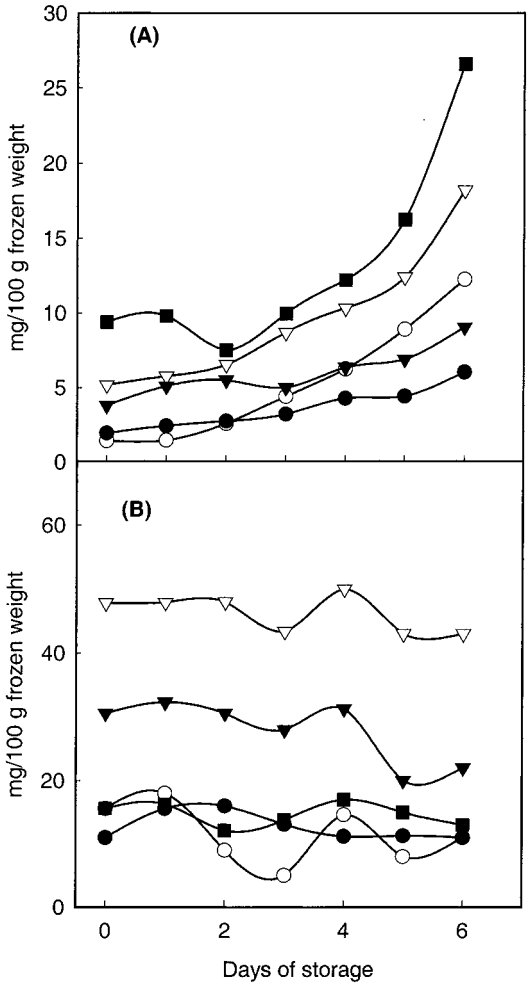

Figure 7. Chlorogenic acid (A) and tyrosine (B) evolution in fresh-cut potato cultivars upon storage. (●) Agria; (○) Cara; (▼) Liseta; (▽) Monalisa; (■) Spunta. HPLC profile was repeated three times. Chlorogenic acid was quantified by comparisons with an external commercial standard of chlorogenic acid at 320 nm. Coefficient of variation in the quantification of chlorogenic cid was <10%. Tyrosine was quantified by comparisons with an external commercial standard of tyrosine at 270 nm. Coefficients of variation in the quantification of tyrosine were 25% for Cara, 22% for Agria, 13.6% for Spunta, 0.18% for Liseta, and 6.6% for Monalisa.

ABBREVIATIONS USED

AA, ascorbic acid; AB, sodium acetate buffer; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DHPPA, 3,4-dihydroxyphenylpropionic acid; DMF, *N*,*N*′-dimethylformamide; IEF, isoelectric focusing; IEP, isoelectric point; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MeOH, methanol; PAL, phenylalanine ammonia-lyase; PB, sodium phosphate buffer; PMSF, phenylmethanesulfonyl fluoride; POD, peroxidase; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; tropolone, 3-hydroxy-2,4,5-cycloheptatrien-1-one; TX-114, Triton X-114.

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