Evaluation of antibrowning and antioxidant activities in unripe grapes recovered during bunch thinning

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Abstract

Background and Aims: Research into new systems for controlling enzymatic browning in the agro-food industry has been focused on eco-friendly alternatives to conventional thermal treatments and traditional additives, which could impair the sensory, nutritional and health properties. The use of unripe grapes for reducing alcohol concentration and pH of wines has been previously reported; however, no studies have been made of the evaluation of unripe grapes as potential functional ingredients to control enzymatic browning and to enhance antioxidant properties of plant products.

Methods and Results: Unripe berries were collected in two seasons during bunch thinning of Barbera and Merlot vineyards. Merlot grapes, which had the highest antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl and ferric reducing ability of plasma assays, were also the most effective in preventing enzyme browning, as confirmed by spectrophotometric assays using commercial mushroom tyrosinase, by zymographic techniques on the isoforms isolated from some plant polyphenol oxidases and by in vivo trials on fresh-cut fruits and vegetables.

Conclusions: The juice of unripe grapes showed not only antibrowning but also antioxidant and whitening activities.

Significance: Unripe grapes discarded during bunch thinning of vineyards represent for the agro-food industry a significant source of bioactive compounds that are easy to produce and safe for human health, thus converting these agricultural wastes into value-added products.

Keywords*: antibrowning, antioxidant, bunch thinning, colour, polyphenol oxidase, unripe grapes*

Introduction

Grapes (*Vitis vinifera*) are the world's largest fruit crop with more than 77 million tonnes produced in 2013 as reported by FAOSTAT (Food and Agricultural Organization 2016). After winemaking, three to six million tonnes per year of grape marc was produced in the 2000–2013 period (Food and Agricultural Organization 2016), and all of these wine industry by-products, including skins, seeds, stems and lees, are rich in phenolic antioxidants (Negro et al. 2003, Rockenbach et al. 2011). Additionally, vineyards, which cover a large area worldwide, approximately seven million hectares in 2013 (Food and Agricultural Organization 2016), generate annually a large amount of waste; Spinelli et al. (2012) have suggested an innovative application of the ligno-cellulosic biomass of vineyard pruning residues for achieving industrial biofuel. Also 'green harvesting' may be considered a source of wastes, while bunch thinning is achieved by dropping the unripe grape bunches to increase the quality of production. It is interesting to note that Kontoudakis et al. (2011) reported a possible use of unripe grapes in winemaking for reducing alcohol concentration and pH of wines. The evaluation of such unripe grapes, however, as potential functional ingredients with antibrowning and antioxidant properties has not been undertaken.

Enzyme browning in plant products is associated with many of the qualitative and economic losses in the agro-food industry. The main agent responsible is polyphenol oxidase or tyrosinase (PPO; EC 1.10.3.1), a copper-containing oxidoreductase that catalyses two different reactions involving the oxidation of phenolic substances and subsequent production of quinones that polymerise to brown pigments known as melanins (Seo et al. 2003). The degree of browning is related to the type and concentration of endogenous phenolic substances; presence of oxygen, reducing substances and metallic ions; pH; and temperature that affects PPO activity (Nicolas et al. 1994, Gomes et al. 2014). The enzyme reaction in agro-food products leads not only to colour alteration but also to a reduction in the nutritional and sensory quality as a consequence of quinone condensation with amino acids and proteins (Rapeanu et al. 2006) and of the degradation of phenolic substances, substrates recognised for their health benefits as antioxidants (Quideau et al. 2011, Mihaylova et al. 2014).

Most strategies for controlling enzyme browning have focused on physical and chemical methods to inhibit PPO activity by eliminating the essential components for the reaction, such as oxygen, copper ions, substrate or even the enzyme itself (Queiroz et al. 2008). Recently, research of new PPO inhibitors from natural sources (Loizzo et al. 2012), including dog rose and pomegranate extracts (Zocca et al. 2011), has represented an eco-friendly alternative to thermal treatments and traditional additives, such as ascorbic acid (AA) and its derivatives as well as sulfites (Queiroz et al. 2011), which could impair the sensory, nutritional and health properties (Vally et al. 2009). Currently, there is a growing interest in the conversion of agro-food wastes into value-added products throughout a given product/service lifecycle (Laufenberg et al. 2003). In fact, they are rich in bioactive compounds (Schieber et al. 2001) not only with antibrowning (Zocca et al. 2010, Lante and Tinello 2015) but also with strong antioxidant potential (Moure et al. 2001).

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Hence, the present study is focused on investigating the antibrowning and antioxidant potential of unripe berries collected from bunch thinning of vineyards of two red grape cultivars in order to find a possible recycling use of these agro-food wastes as functional ingredients.

Materials and methods

Chemicals

Catechol, L-3,4 dihydroxyphenylalanine (L-DOPA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu's phenol reagent, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH), gallic acid, polyvinylpolypyrrolidone (PVPP), 4-tert-butyl catechol (t-BC), 2,4,6-tripyridyl-s-triazine (TPTZ) and HPLC standards were purchased from Sigma-Aldrich (St Louis, MO, USA). The Coomassie Plus Protein assay reagent and bovine serum albumin were purchased from Pierce (Rockford, IL, USA). NatureSeal AS1 was obtained from AgriCoat NatureSeal, Berkshire, England.

Preparation of unripe grapes

Unripe berries in the early stages of veraison were collected during bunch thinning of vineyards of two red grape cultivars [*V. vinifera* cvs Barbera (B) and Merlot (M), at the end of July during the 2013 and 2014 seasons (1 and 2, respectively)] at the Cascina Belmonte company (Muscoline, Brescia, Italy) that usually removed three to four bunches per vine producing approximately 2000 kg of waste per hectare annually. Merlot berries harvested at four times starting from veraison up to the ripening stage in the 2013 season (July corresponding to M1, August, September and October) were also used for some analyses in order to compare unripe and ripe grapes. The bunch-thinned grapes and Merlot berries were stored at −20°C in the dark until required.

The skins and seeds were removed and the juice was recovered from unripe and ripe berries in a centrifugal juicer (Moulinex JU655, Groupe SEB, Ecully, France). After measuring the pH value, the grape juice was centrifuged at 3864 *g* for 15 min at 4°C, filtered through a Millipore 0.45 µm filter membrane (Merck Millipore, Billerica, MA, USA) and stored at −20°C in the dark.

Sources of PPO

Commercial mushroom tyrosinase (TYR; 3130 U/mL) was obtained from Sigma-Aldrich. Apples (*Malus domestica* cvs Fuji and Golden Delicious), pears (*Pyrus communis* cv. Abate) and potato tubers (*Solanum tuberosum* cv. Bintje) were purchased at commercial maturity from a local market and stored at 4°C. All fruits and potatoes were washed under running water to eliminate any surface contamination and wiped with blotting paper. Plant PPOs were extracted as reported by Zocca et al. (2010). The protein concentration of buffer dilutions of lyophilised PPO was determined by the Bradford assay using bovine serum albumin as a protein standard (Bradford 1976).

Inhibition of TYR activity

The inhibitory effect of the juice of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) on the activity of commercial mushroom TYR, previously solubilised in 0.1 mol/L sodium citrate buffer at a pH 6.0 to a final concentration of 9390 U/mL, was quantified in accordance with Zocca et al. (2011) using a Varian Cary 50 Bio UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 400 nm and 25°C with 10 mmol/L catechol as the substrate and 0.05% w/v AA as the reference inhibitor. The percent inhibition of TYR activity was calculated as follows (Baurin et al. 2002):

$$\%TYR\ inhibition = [(A_{control} - A_{inhibitor})/A_{control}] \times 100\% \quad (1)$$

where $A_{control}$ = absorbance at 400 nm without inhibitor and $A_{inhibitor}$ = absorbance at 400 nm with inhibitor.

The IC_{50} values (concentration providing 50% inhibition of enzyme activity) of bioactive compounds quantified in the juice of unripe grapes by HPLC analysis were also calculated graphically using a calibration curve in the linear range by plotting their concentration versus the corresponding %TYR inhibition.

The inhibitory kinetics of the juice of all of the unripe grapes were analysed using Lineweaver–Burk plots at different concentration values of catechol substrate in order to calculate the kinetic constants (K_M and V_{max}) and to define the inhibition type as described by Lante and Tinello (2015).

Non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis and PPO zymography

The inhibitory potential of the juice of unripe grapes (B1, B2, M1, M2) and of 0.05% v/v AA as a reference inhibitor was also assessed on the isoforms isolated from commercial mushroom TYR and some plant PPOs. Non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymographic techniques with the L-DOPA/MBTH complex were performed in a Mini Protean II (Bio-Rad-Laboratories, Milano, Italy) at room temperature following the procedure of Zocca et al. (2011). Before electrophoresis, the commercial TYR (1 mg) and the freeze-dried powders of apples, pears and potatoes (400, 200 and 20 mg, respectively) were solubilised with 700 µL of distilled water and 300 µL of Laemmli buffer (1.33 mol/L Tris, pH 7.4, 40% v/v glycerol, 8% w/v SDS) (Laemmli 1970) and centrifuged at 18 659 g for 2 min. Each gel well was loaded as follows: 5 µL of TYR solution (3130 U/mL) and 15 µL of other plant PPOs. The images of zymograms were acquired by a scanner.

Antibrowning effect on fresh-cut fruits and vegetables

The antibrowning potential of the juice of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) was evaluated (Zocca et al. 2011) on fresh-cut apples, pears and potatoes in comparison with that of 0.05% w/v AA as a reference PPO inhibitor. Moreover, the antibrowning effect of the juice of Merlot unripe grapes in the 2013 and 2014 seasons (M1 and M2, respectively) on Golden Delicious apple slices was compared with reference antibrowning formulations including an aqueous solution of 1% (w/v) AA and 0.5% w/v calcium chloride (AAC) as well as 6% (w/v) NatureSeal AS1.

Each fruit and vegetable sample was washed under running water to remove any surface contamination, wiped with blotting paper and manually cut into two symmetrical slices 5 mm thick that were placed in Petri dishes. The surface of a control slice (C) was sprayed with 1 mL of distilled water using a syringe, and another slice was treated with 1 mL of antibrowning formulation. After 15 min at 25°C, the surface of each slice was wiped and treated with 1 mL of 10 mmol/L catechol as a PPO substrate. Browning was observed 10 min after application of catechol by acquiring the images with a digital camera and by measuring the surface colour with a Tristimulus colorimeter (Chroma Meter CR-410, Konica-Minolta, Milan, Italy) in the Commission Internationale de l'Eclairage (L*, a*, b*) colour space (Commission Internationale de l'Eclairage 1976).

The antibrowning effect was expressed as the colour change (ΔE) according to the following equation (Lante et al. 2016):

$$\Delta E = \sqrt{\left(\Delta L^2 + \Delta a^2 + \Delta b^2\right)^2} = \sqrt{(L_t - L_{t0})^2 + (a_t - a_{t0})^2 + (b_t - b_{t0})^2} \quad (2)$$

where L = lightness (100 for white to 0 for black), a = red when positive and green when negative, b = yellow when positive and blue when negative, t = treatment time with catechol and t_0 = initial time before catechol application.

The percent reduction (%RΔE) in colour change was also calculated as follows (Lante et al. 2016):

$$\%R\Delta E = [(\Delta E_{control} - \Delta E_{inhibitor}) / \Delta E_{control}] \times 100 \quad (3)$$

where $\Delta E_{control}$ = colour change of slices not treated with the antibrowning formulation and $\Delta E_{inhibitor}$ = colour change of slices treated with the antibrowning formulation.

Determination of quinone inhibition

The level of 4-tert-butyl-*o*-benzoquinone after the chemical oxidation of t-BC using $NaIO_4$ was detected spectrophotometrically at 400 nm and 25°C (Lante and Tinello 2015). The reaction mixture contained 50 µL of 2 mmol/L $NaIO_4$ and 1.0 mL of 1 mmol/L t-BC in the absence and presence of inhibitors (200 µL). The juice of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) was tested in comparison with AA and citric acid (CA) at the concentration corresponding to 50% (CA50 = 0.0019 mmol/L and CA50 = 0.29 mmol/L, respectively) and 100% of TYR inhibition (CA100 = 0.0039 mmol/L and CA100 = 0.57 mmol/L, respectively).

Whitening effect

The whitening effect was also evaluated by measuring spectrophotometrically the accumulation of brown compounds formed after the chemical oxidation of a catechol solution at 400 nm and 25°C. The reaction mixture contained 50 µL of 4 mmol/L NaOH and 1.0 mL of 10 mmol/L catechol in the absence and presence of inhibitors (200 µL). The juice of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) was tested in comparison with AA and CA at the concentration corresponding to 50% (AA50 = 0.0019 mmol/L and CA50 = 0.29 mmol/L, respectively) and 100% of TYR inhibition (AA100 = 0.0039 mmol/L and CA100 = 0.57 mmol/L, respectively).

Antioxidant activity of the juice of unripe grapes

The antioxidant activity of the juice of unripe grapes (B1, B2, M1 and M2) and of Merlot berries at four harvest times in the 2013 season (July, August, September and October) was estimated using two spectrophotometric assays based on electron transfer (Huang et al. 2005). The DPPH and ferric reducing ability of plasma (FRAP) assays were carried out according to Massini et al. (2016). The antioxidant activity was expressed as Trolox equivalents (mg TE) per millilitre of sample previously diluted in ethanol.

Concentration of phenolic substances of the juice of unripe grapes

The concentration of phenolic substances of the juice of unripe grapes (B1, B2, M1 and M2) and of Merlot berries at four harvest times in the 2013 season was quantified by the Folin–Ciocalteu method and expressed as gallic acid equivalents (mg GAE) per millilitre of sample previously diluted in ethanol (Ercisli 2007).

Analysis of bioactive compounds by HPLC

The bioactive compounds of the juice of unripe grapes (B1, B2, M1 and M2) and of Merlot berries at four harvest times in the 2013 season were characterised by HPLC using a Thermo Finnigan SpectraSystem UV6000LP HPLC system (Thermo Finnigan, San Jose, CA, USA) with diode-array detection. The bioactive compounds were identified by comparing their retention times with those of commercial standards. Before their injection into the column, samples were filtered through Millipore 0.22 µm filter membranes (Merck Millipore)

Organic acids (citric, fumaric, L-malic, oxalic, succinic and tartaric) were quantified with an Aminex HPX-87H column (Bio-Rad Laboratories) according to the method proposed by Nardi et al. (2003). The mobile phase was 0.0025 N sulfuric acid, the temperature of analysis was 60°C, the run time was 60 min and the flow rate was 0.6 mL/min.

The phenolic substances [caffeic acid, (+)–catechin, chlorogenic acid, (-)–epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate (EGCG) and gallic acid] were quantified using a Supelcosil LC-18 column (Sigma-Aldrich) (Zocca et al. 2011). The mobile phase was a mixture of water acidified with sulfuric acid (pH 2.5) and of methanol at different gradient elutions and flow rates in accordance to the procedure of Zocca et al. (2011). The HPLC analysis was carried out at 40°C with a run time of 100 min and a diode-array detection wavelength in the 200 to 600 nm range.

Statistical analysis

All of the data obtained from three replicates were subjected to one-way ANOVA using R software (3.1.2 version) (R Development Core Team 2014) after verifying a normal distribution and homogeneity of variance. Significant differences were determined by Tukey's multiple range test ($P \leq 0.05$).

Results and discussion

Evaluation of the antibrowning potential of the juice of unripe grapes

Whereas the development of new inhibitors for controlling enzymatic browning requires a multidisciplinary approach, the inhibition of PPO by the juice of unripe grapes obtained from Barbera and Merlot in the 2013 and 2014 seasons (B1, B2, M1 and M2, respectively) was widely evaluated through in vitro and in vivo trials.

The inhibitory effect of the juice of unripe grapes on a commercial TYR as a model PPO was quantified spectrophotometrically in comparison with that of 0.05% AA as a reference antibrowning compound, using 10 mmol/L catechol as the phenolic substrate (Figure 1). All of the inhibitors tested significantly decreased ($P \leq 0.001$) the enzyme activity with a TYR inhibition of more than 50% compared with that of the control (C). Although AA exhibited the best anti-TYR performance (85.7%), the juice of all unripe grapes showed greater TYR inhibition than that of pomegranate extract [27.5%; Zocca et al. (2011)] and *Brassicacaea* processing water [23.2%; Zocca et al. (2010)]. Among the unripe grapes, M1 and M2 (68.2 and 67.8% TYR inhibition, respectively) limited the enzyme browning greater than B1 and B2 (56.3 and 58.8% TYR inhibition, respectively). Because the spectrophotometric results achieved from the juice of unripe grapes of the 2013 season were confirmed by those from the 2014 season, the antibrowning effectiveness was related only to the grape cultivar. Moreover, the juice of all of the unripe grapes did not show a lag phase, which was a typical characteristic of reducing agents such as AA (20 s) that inhibited TYR activity by reducing *o*-quinones

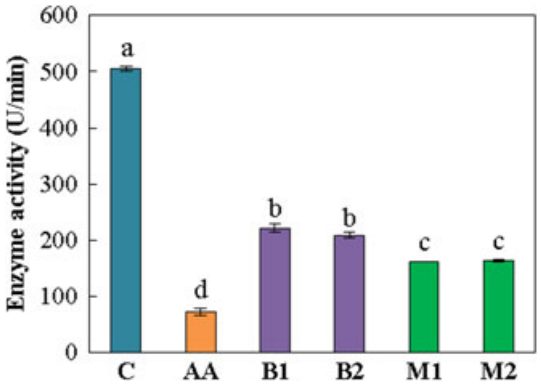

Figure 1. Enzyme activity of commercial mushroom tyrosinase in the absence (C) and presence of 0.05% w/v ascorbic acid (AA) and of the juice of unripe grapes from Barbera (B1, B2) and Merlot vineyards (M1, M2) in the 2013 (B1, M1) and 2014 (B2, M2) seasons using 10 mmol/L catechol as the substrate at 25°C. Histograms are the mean (± standard deviation) of three replicates. Histograms with different letters are statistically different ($P \leq 0.05$), as determined by the Tukey's multiple range test.

to *o*-diphenols and thus slowing the biosynthesis of brown compounds (Ros et al. 1993). In this regard, the level of quinone inhibition was investigated spectrophotometrically in order to confirm the non-reducing capacity of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) in comparison with that of AA and CA at a concentration corresponding to 50% (AA50 and CA50) and 100% (AA100 and CA100) of TYR inhibition. The *o*-quinones can also be formed after the exposure of catechols to oxygen without involving enzyme activity. On the basis of spectrophotometric results (Figure 2), AA at both anti-TYR concentration values (AA50 and AA100) significantly reduced ($P \leq 0.001$) the absorbance value at 400 nm of the control mixture (without inhibitors) and made the corresponding test tube solutions clear, confirming its inhibition of enzyme activity as a reducing agent. Instead, CA at both anti-TYR concentration values (CA50 and CA100) and the juice of all of the unripe grapes did not limit the *o*-quinone production, thus confirming that they acted as real TYR inhibitors. The antibrowning potential, however, could be associated not only with the inhibition of PPO activity but also with the whitening effect on melanins, the polymeric brown pigments derived from enzyme reaction. We also evaluated the whitening capacity of the juice of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) in comparison with that of AA and CA at the concentration corresponding to 50% (AA50 and CA50) and 100% (AA100 and CA100) of TYR inhibition, by spectrophotometrically measuring the brown compounds formed after the chemical oxidation of a catechol solution (Figure 3). The presence of CA and the juice of all unripe grapes significantly reduced ($P \leq 0.001$) the absorbance value at 400 nm of a control mixture (C) by more than 50%. Although both inhibitory concentration values of AC showed the greatest whitening effect (74 and 76% for CA50 and CA100, respectively) by making the corresponding test tube solutions clear, in comparison with that of the control, the juice of unripe grapes from Barbera and Merlot exhibited good whitening performance (58 and 62% for B1 and B2, 57 and 64% from M1 and M2, respectively), thus improving their antibrowning effectiveness.

The mechanism of TYR inhibition was defined by measuring spectrophotometrically the enzymatic kinetic constants using the Lineweaver–Burk plots at several catechol concentration values in the absence and presence of the juice of unripe grapes (Figure 4). The double-reciprocal plots described a family of lines that intersected the vertical axis at different points. In particular, the lines of the juice of unripe grapes were almost parallel to those of the control line. As a consequence, V_{max} and K_M values of all of the unripe grapes decreased in comparison with those of the control (C), confirming an uncompetitive inhibition where the reversible inhibitor reacted only with enzyme–substrate complex.

The antibrowning effects of the juice of unripe grapes from Barbera (B1 and B2) and Merlot (M1 and M2) and 0.05% w/v AA were then tested by carrying out electrophoretic assays on a commercial TYR and some plant PPOs in order to isolate the

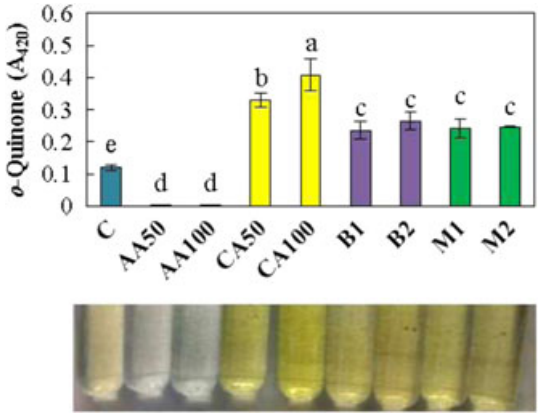

Figure 2. Effect of polyphenol oxidase inhibitors on *o*-quinone production by measuring spectrophotometrically (A_{420}) the amount of 4-tert-butyl-*o*-benzoquinones (t-BC) after the chemical oxidation of 1 mmol/L t-BC at 25°C. Histograms are the mean (± standard deviation) of three replicates. Histograms with different letters are statistically different ($P \leq 0.05$), as determined by the Tukey's multiple range test. The clear solutions of test tubes indicate the inhibitory effect of polyphenol oxidase on *o*-quinone production. AA50 and AA100, ascorbic acid at the concentration corresponding to 50 and 100% of commercial mushroom tyrosinase (TYR) inhibition; B1 and B2, juice from unripe grapes from the Barbera vineyard in the 2013 and 2014 seasons; C, control without inhibitors; CA50 and CA100, citric acid at the concentration corresponding to 50 and 100% of TYR inhibition; M1 and M2, juice from unripe grapes from the Merlot vineyard in the 2013 and 2014 seasons.

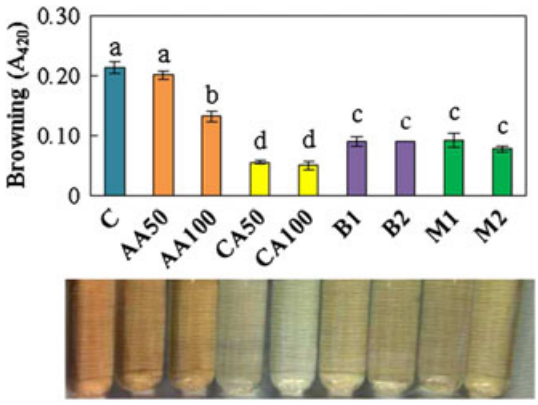

Figure 3. Whitening effect of polyphenol oxidase inhibitors by measuring spectrophotometrically (A_{420}) the amount of brown compounds after the chemical oxidation of 10 mmol/L catechol solution at 25°C. Histograms are the mean (± standard deviation) of three replicates. Histograms with different letters are statistically different ($P \leq 0.05$), as determined by the Tukey's multiple range test. The clear solutions of test tubes indicate the whitening effect of polyphenol oxidase. AA50 and AA100, ascorbic acid at a concentration corresponding to 50 and 100% of commercial mushroom tyrosinase (TYR) inhibition; B1 and B2, juice of unripe grapes from the Barbera vineyard in the 2013 and 2014 seasons; C, control without inhibitors; CA50 and CA100, citric acid at a concentration corresponding to 50 and 100% of TYR inhibition; M1 and M2, juice of unripe grapes from the Merlot vineyard in the 2013 and 2014 seasons.

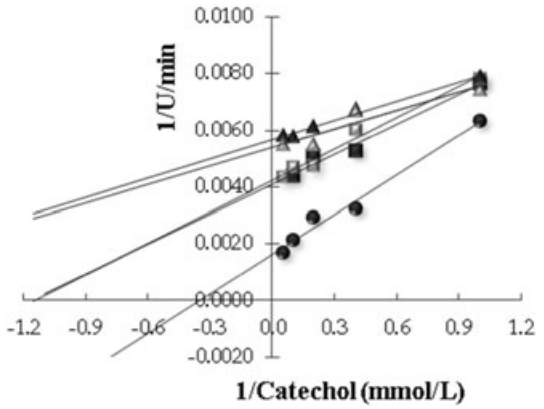

Figure 4. Lineweaver–Burk plots and corresponding kinetic constant values of commercial mushroom tyrosinase in the absence [V_{max} 625 U/min, K_M 2.94 mmol/L, $y = 0.0047x + 0.0016$, $R^2 = 0.9813$ (●)] and presence of the juice of unripe grapes from Barbera [V_{max} 244 U/min, K_M 0.85 mmol/L, $y = 0.0035x + 0.0041$, $R^2 = 0.976$ (■); V_{max} 238 U/min, K_M 0.88 mmol/L, $y = 0.0037x + 0.0042$, $R^2 = 0.9805$ (□)] and Merlot [V_{max} 175 U/min, K_M, 0.40 mmol/L, $y = 0.0023x + 0.0057$, $R^2 = 0.9853$ (▲); V_{max} 185 U/min, K_M 0.41 mmol/L, $y = 0.0022x + 0.0054$, $R^2 = 0.8909$ (Δ)] vineyards in the 2013 (■, ▲) and 2014 (□, Δ) seasons using catechol as the substrate.

corresponding isoforms (Figure 5). The zymographic technique was a valuable tool to visualise the activity of PPO isoforms with/without inhibitors by monitoring the appearance of bands in the gel (Martinez-Alvarez et al. 2008). The juice of all unripe grapes showed a greater inhibitory effect on the activity of the one isoform of TYR and of potato PPO than AA by completely reducing the colour intensity of the corresponding bands (Figure 5a,b). The low correlation between spectrophotometric and zymographic results, especially when 0.05% w/v AA was applied on TYR, could be due to the different specificities towards noncyclisable (catechol) or cyclisable (L-DOPA) diphenolic substrates (Sanchez-Ferrer et al. 1995). The TYR enzyme exhibited a different stereospecificity among phenolic substrates with a greater affinity for dihydroxyphenols, especially for L-isomers (Seo et al. 2003). With potato PPO, the zymographic results were confirmed by in vivo trials on fresh-cut potatoes as the colour change of slices treated with the juice of Merlot and Barbera unripe grapes from the 2013 season (M1 and B1, respectively) was limited 10 min after the application of 10 mmol/L catechol (Figure 5b). A lower inhibitory effectiveness by the juice of unripe grapes was achieved on the other isoform of Golden Delicious and the four isoforms

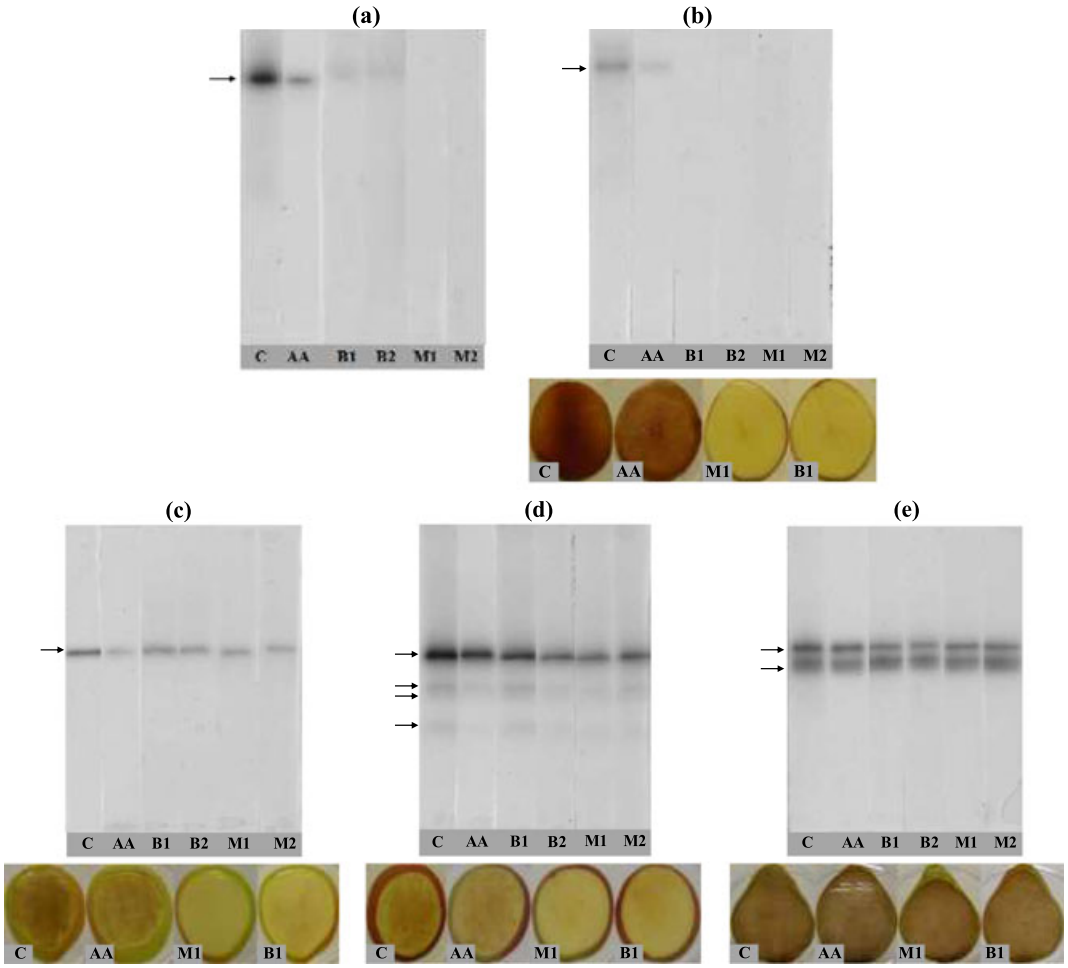

Figure 5. Antibrowning effect of the juice of unripe grapes from Barbera (B1, B2) and Merlot (M1, M2) vineyards in the 2013 (B1, M1) and 2014 (B2, M2) seasons compared with that of the control without inhibitors (C) and 0.05% w/v ascorbic acid (AA) as the reference inhibitor on polyphenol oxidase (PPO) zymograms and corresponding fresh-cut fruits and vegetables. (a) Commercial mushroom tyrosinase (TYR) (16 U per lane); (b) potato PPO (14.62 μg of protein per lane) and slices; (c) Golden Delicious apple PPO (14.62 μg of protein per lane) and slices; (d) Fuji apple PPO (4.84 μg of protein per lane) and slices; and (e) Abate pear PPO (12.76 μg of protein per lane). The arrows indicate the enzymatic isoforms of TYR and plant PPOs on the zymograms.

of Fuji apple PPOs (Figure 5c,d). Moreover, the in vivo assay on fresh-cut apples confirmed the good antibrowning performance of the juice of unripe grapes, especially from Merlot. Additionally, any evident antibrowning effect was observed on the zymogram of Abate pear PPO and on pear slices (Figure 5e) probably because of the low pH of the unripe grapes. In this regard, Gomes et al. (2014) demonstrated that the browning in fresh-cut Rocha pear was not reduced by low pH values in the presence of catechol substrate. The variable antibrowning performance among plant PPOs confirmed that the inhibitory effectiveness was mainly related to the enzyme source, as spectrophotometrically demonstrated by some authors (Zocca et al. 2010, 2011).

The juice from unripe Merlot berries (M1 and M2), which showed the best in vitro performance, was also applied for 15 min at 25°C to Golden Delicious apple slices in order to compare the effectiveness in controlling enzymatic browning in vivo with an aqueous solution of 1% w/v AA and 0.5% w/v AAC as well as 6% w/v NatureSeal (AS1) as reference antibrowning formulations. The colour change (ΔE) on the surface of apple slices was quantified by colorimetric analysis 10 min after catechol addition for visualising the browning in a brief time (Figure 6). The application of all formulations significantly limited ($P \leq 0.001$) the colour change (ΔE) of fresh-cut apples compared with that of untreated samples (C). Although AAC and AS1 were more effective than the juice of unripe grapes, with a reduction in colour change (RΔE) of 96 and 94%, respectively, M1 and M2 showed strong antibrowning potential with RΔE values of 79 and 86%, respectively.

The whitening effect of bunch-thinned grapes (Figure 3) could also explain the low correlation between spectrophotometric and in vivo results by further increasing their antibrowning performance on fresh-cut fruits and potatoes compared with that of the 0.05% w/v AA reference inhibitor.

Evaluation of the antioxidant activity of the juice of unripe grapes

The antioxidant activity of the juice of unripe Barbera (B1 and B2) and Merlot (M1 and M2) grapes was detected using two spectrophotometric assays because different methods can give widely divergent results, as demonstrated by Tabart et al. (2009). Although FRAP showed higher Trolox equivalents (TE) than DPPH, both assays gave essentially the same result (Figure 7). As shown in Figure 7a, the antioxidant activity of all of the unripe berries was statistically relevant ($P \leq 0.001$) depending not on the season but only on the grape cultivar. In particular, Merlot (M1 and M2) had an antioxidant activity twice that of Barbera (B1 and B2) in both DPPH and FRAP assays.

The antioxidant effectiveness of bunch-thinned grapes was associated not only with the composition of the different phenolic substances and organic acids of cultivars (Lima et al. 2014) but also with the stage of ripening of the berries. Thus, the antioxidant activity of Merlot berries collected during bunch thinning at the end of July in the 2013 season (M1) was compared with that of Merlot berries at progressively later harvest times (August, September and October) that corresponded to different ripening stages of grapes before winemaking. The antioxidant activity significantly decreased ($P \leq 0.001$) with increasing harvest times further confirming the best performance of M1 (Figure 7b).

Biochemical characterisation of the juice of unripe grapes

The antibrowning and antioxidant activity of the juice of unripe grapes was mainly related to the composition of their organic acids and phenolic substances that were detected by HPLC analysis (Table 1).

The total amount of organic acids (citric, fumaric, malic, oxalic, succinic and tartaric) resulted in statistically relevant differences ($P \leq 0.01$) among the unripe grapes depending not on the season but only on the grape cultivar (Table 1). In particular, Merlot had a concentration of organic acids higher than that of Barbera (328.4 ± 15.8 and 320.5 ± 0.3 mmol/L for M1 and M2 vs 278.2 ± 0.7 and 290.6 ± 0.8 mmol/L for B1 and B2, respectively). Moreover, the concentration of organic acids of Merlot berries decreased significantly ($P \leq 0.01$) with increasing

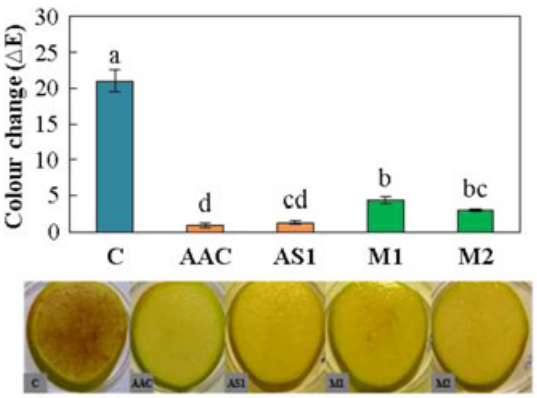

Figure 6. Antibrowning effect of some formulations by measuring with a colorimeter the colour change (ΔE) on the surface of Golden Delicious apple slices 10 min after the application of 10 mmol/L catechol at 25°C. Histograms are the mean (± standard deviation) of three replicates. Histograms with different letters are statistically different ($P \leq 0.05$), as determined by the Tukey's multiple range test. AAC, 1% w/v ascorbic acid and 0.5% w/v calcium chloride; AS1, 6% w/v NatureSeal; C, control without inhibitors; M1 and M2, unripe grapes from the Merlot vineyard in the 2013 and 2014 seasons.

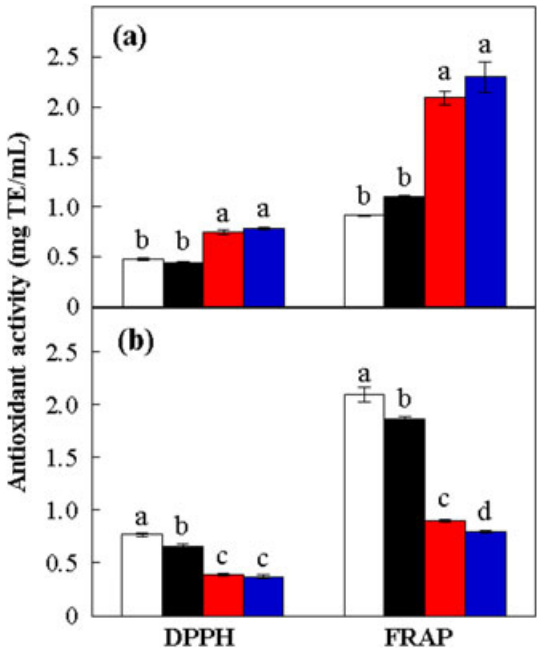

Figure 7. The antioxidant activity detected by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) assays of the juice (a) of Barbera (□, ■) and of Merlot (■, ■) unripe grapes in the 2013 (□, ■) and 2014 (■, ■) seasons and (b) of Merlot berries harvested in July (□), August (■), September (■) and October (■) in the 2013 season. Histograms are the mean (± standard deviation) of three replicates. Histograms with different letters are different ($P \leq 0.05$), as determined by the Tukey's multiple range test.

Table 1. The concentration of organic acids and phenolic substances measured by HPLC in unripe grapes from Barbera and Merlot cultivars in the 2013 and 2014 seasons.

Compounds	IC_{50}†	Concentration				Probability
		B1	B2	M1	M2	
Organic acids (mmol/L)						
Citric acid	289.9	56.5 ± 0.1a	62.9 ± 0.4a	78.1 ± 10.8b	73.6 ± 0.4b	*
Fumaric acid	ND	0.13 ± 0.00	0.13 ± 0.00	0.16 ± 0.02	0.15 ± 0.00	NS
Malic acid	163.8	126.6 ± 0.3a	126.1 ± 0.1a	129.2 ± 1.1b	128.3 ± 0.1b	*
Oxalic acid	1.5	2.5 ± 0.0a	3.2 ± 0.0a	14.7 ± 0.1b	16.8 ± 0.1b	***
Succinic acid	536.7	4.6 ± 0.2a	5.25 ± 0.2a	12.10 ± 2.5b	8.46 ± 0.1b	***
Tartaric acid	293.1	87.9 ± 0.1	93.0 ± 0.	94.1 ± 7.0	93. 2 ± 0.2	NS
Total organic acids		278.2 ± 0.7a	290.6 ± 0.8a	328.4 ± 15.8b	320.5 ± 0.3b	**
Phenolic substances (μmol/L)						
Caffeic acid	955.7	120.0 ± 0.2a	104.5 ± 0.6b	80.7 ± 02.5c	85.3 ± 1.8c	***
Catechin	ND	17.7 ± 0.2a	14.8 ± 0.6a	32.2 ± 0.8b	41.6 ± 0.5c	***
Chlorogenic acid	ND	61.7 ± 0.2a	53.5 ± 0.2a	30.1 ± 0.5b	26.2 ± 0.2b	***
Gallic acid	59.2	2.7 ± 0.2	3.8 ± 0.1	2.7 ± 0.2	2.7 ± 0.1	NS
Epicatechin	ND	9.5 ± 0.1a	6.9 ± 0.1b	18.9 ± 1.6c	18.2 ± 0.7c	***
Epicatechin gallate	383.2	45.6 ± 0.1a	70.7 ± 0.1b	52.7 ± 0.9c	176.6 ± 0.1d	***
Epigallocatechin	615.7	30.1 ± 0.3a	37.1 ± 0.5a	118.8 ± 3.4b	169.6 ± 1.3c	***
Epigallocatechin gallate	421.1	463.9 ± 3.0a	521.2 ± 6.8b	1491 ± 16c	1440 ± 13c	***
Total phenolic substances		751.3 ± 2.8a	812.7 ± 7.0b	1827 ± 17c	1961 ± 16c	***

Significant difference between the concentration of organic acids and phenolic substances at *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. †The inhibitor concentration that reduces the enzyme activity by 50%. Values are the mean (± standard deviation) of three replicates. Values with different letters are statistically different ($P \leq 0.05$), as determined by the Tukey's multiple range test. B1, Barbera 2013 season; B2, Barbera 2014 season; M1, Merlot 2013 season; M2, 2014 season; ND, not detected; NS; not significant difference.

harvest time from July (162.8 ± 21.5 mmol/L) to October (41.3 ± 6.7 mmol/L) in the 2013 season.

The high concentration of organic acids contributed to the lower pH values of Barbera (2.22 for B1 and 2.24 for B2) and of Merlot (2.24 for M1 and 2.18 for M2) bunch-thinned grapes. The low pH could represent an important factor for the control of enzyme browning by reducing the activity below the optimum pH that varied depending on the enzyme source (Rapeanu et al. 2006). In fact, no PPO activity has been spectrophotometrically detected in bunch-thinned grapes (data not shown) as a consequence of the low pH values of unripe berries.

The organic acids detected included some known PPO inhibitors. Son et al. (2000) reported a strong antibrowning activity of oxalic acid defining a competitive inhibition on a catechol–mushroom PPO system with a K_i value of 2.0 mmol/L. The oxalic acid concentration of unripe grapes was greater than the calculated IC_{50} value (1.5 mmol/L). Moreover, the Merlot had an oxalic acid concentration six times higher than that of Barbera (14.7 ± 0.1 and 16.8 ± 0.1 mmol/L for M1 and M2 vs 2.5 ± 0.0 and 3.2 ± 0.0 mmol/L for B1 and B2, respectively). In addition, oxalic acid could also contribute to the antioxidant activity of unripe grapes (Kayashima and Katayama 2002). Son et al. (2001), studying the antibrowning performance of several carboxyl acids on apple slices, confirmed the highest effectiveness not only of oxalic acid but also of tartaric, citric and malic acids that is mostly found in unripe grapes. Meanwhile, fumaric acid, whose concentration in Barbera and Merlot wastes was low, and succinic acid were less effective in controlling enzyme browning. Citric acid, whose concentration in M1 (78.1 ± 10.8 mmol/L) and M2 (73.6 ± 0.4 mmol/L) were higher than that in B1 (56.5 ± 0.1 mmol/L) and B2 (62.9 ± 0.4 mmol/L), is the main acidulant widely used in the agro-food industry. The inhibition of CA was mainly attributed to its capability of reducing the pH in the medium and consequently decreasing the enzyme activity (Liu et al. 2013).

Unripe grapes are also rich in phenolic substances, which include the main antioxidants (Tabart et al. 2009) and PPO inhibitors (Loizzo et al. 2012). The concentration of phenolic substances detected by the Folin–Ciocalteu assay in unripe grapes (Figure 8) was significantly different ($P \leq 0.01$), with greater concentration in Merlot (1.6 ± 0.1 mg GAE/mL for M1 and 1.5 ± 0.1 mg GAE/mL for M2) (Figure 8a) than in Barbera (1.0 ± 0.0 mg GAE/mL for B1 and 1.1 ± 0.1 mg GAE/mL for B2) independent of the season. The concentration of phenolic substances in the Merlot berries increased significantly ($P \leq 0.01$) as harvest time moved from July (1.6 ± 0.1 mg GAE/mL) to October (2.6 ± 0.1 mg GAE/mL) in the 2013 season (Figure 8b).

Among the flavanols (catechin, epicatechin, epicatechin gallate, epigallocatechin and EGCG) and phenolic acids (caffeic, chlorogenic and gallic acids) detected by HPLC, EGCG was the main phenolic substance widely found in unripe grapes (Table 1). In particular, M1 (1491 ± 16 μmol/L) and M2 (1440 ± 13 μmol/L) had an EGCG concentration higher than that of B1 (463.9 ± 3.0 μmol/L) and B2 (521.2 ± 6.8 μmol/L), and they were at least three times the calculated IC_{50} value (421.1 μmol/L). The EGCG concentration in Merlot grapes decreased significantly ($P \leq 0.01$) as harvest time moved from July (1491 ± 16 μmol/L) to October (260 ± 20 μmol/L) in the 2013 season, contrary to the normal phenolic ripeness. Moreover, all unripe grapes showed a greater concentration of EGCG than that of pomegranate extract [700 μmol/L; Zocca et al. (2011)] and green tea infusion [130 μmol/L after 3 min and 200 μmol/L after 20 min of infusion time; Bronner and Beecher

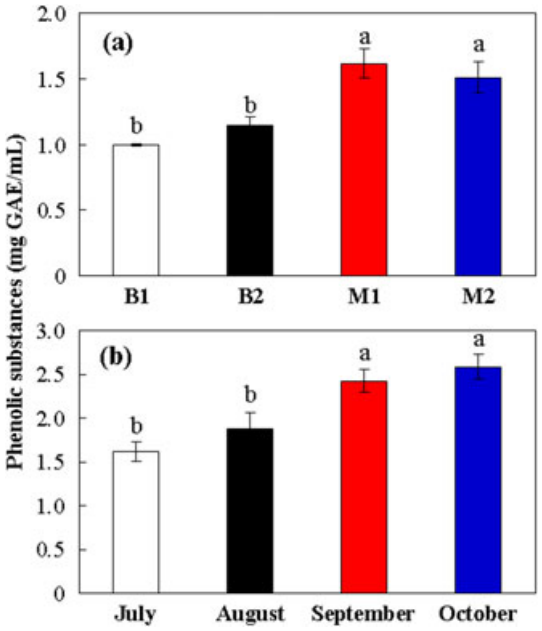

Figure 8. The concentration of phenolic substances detected by the Folin–Ciocalteu assay of the juice (a) of Barbera (□, ■) and of Merlot (■, ■) unripe grapes in the 2013 (□, ■) and 2014 (■, ■) seasons and (b) of Merlot berries harvested in July (□), August (■), September (■) and October (■) in the 2013 season. Histograms are the mean (± standard deviation) of three replicates. Histograms with different letters are statistically different ($P \leq 0.05$), as determined by the Tukey's multiple range test.

(1998)]. Green tea has been widely recognised for its strong antioxidant capacity related mainly to its high catechin concentration (Senanayake 2013). In particular, EGCG (El-Shahawi et al. 2012) showed the greatest antioxidant performance among several phenolic substances (Tabart et al. 2009). Moreover, EGCG behaved as a strong competitive inhibitor towards TYR, thus confirming its antibrowning potential (Loizzo et al. 2012). Catechins, which are also known as depigmenting agents (Parvez et al. 2007), could be involved in the whitening effect of unripe grapes.

Conclusions

The antibrowning and antioxidant activity of bunch-thinned grapes has been related not to the season but to the grape cultivar. This is a consequence of their biochemical composition that differed between the Barbera and Merlot cultivars and remained unchanged between 2013 and 2014, as confirmed by in vitro and in vivo assays. Their whitening potential contributed also to enhance the antibrowning performance of the juice of unripe berries. Hence, bunch-thinned grapes may be valuable for controlling enzyme browning in plant products even if their antibrowning effectiveness mainly depended on the PPO source and the grape cultivar. In detail, the improved performance achieved by Merlot has been associated with its greater concentration of organic acids and of EGCG according to the ripening stage of the grape berries. Further research will be carried out to explore the possible application of unripe grapes from different cultivars to reduce sulfur dioxide addition in the agro-food industry. For this application, the new natural PPO inhibitors are suitable alternatives to traditional additives and thermal technologies that have some drawbacks including a low stability, alteration of sensory and nutritional properties in agro-food products and potential hazards for human health.

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