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INNOVATIVE TECHNOLOGIES AND BIOACTIVE COMPOUNDS FOR THE CONTROL OF POLYPHENOL OXIDASE

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Abbreviations

AA	ascorbic acid
AAC	ascorbic acid and calcium chloride
AS1	NatureSeal®
B1	vineyard pruning residues from Barbera cultivar in the 2013 season
B2	vineyard pruning residues from Barbera cultivar in the 2014 season
CA	citric acid
CH	citron hydrosols
CIHs	citrus hydrosols
cv.	cultivar
EGCG	epigallocatechin gallate
FAO	Food and Agricultural Organization in the United Nations
FDA	Food and Drug Administration
GC	gas chromatography
HPLC	high performance liquid chromatography
LED	light emitting diode
LH	lemon hydrosols
M1	vineyard pruning residues from Merlot cultivar in the 2013 season
M2	vineyard pruning residues from Merlot cultivar in the 2014 season
OH	orange hydrosols
PPO	polyphenol oxidase
TYR	tyrosinase; commercial mushroom tyrosinase
UV	ultraviolet
UV-A LED	ultraviolet light at 390 nm with LED source
UV-A	ultraviolet light in the range 315-400 nm
UV-B	ultraviolet light in the range 280-315 nm
UV-C	ultraviolet light in the range 100-280 nm
VPRs	vineyard pruning residues
WHO	World Health Organization

Riassunto

L'imbrunimento enzimatico dei prodotti agro-alimentari in post-raccolta e le problematiche associate alla melanogenesi nel settore cosmetico comportano consistenti perdite qualitative ed economiche. Il principale responsabile di entrambi questi fenomeni è la tirosinasi o polifenol ossidasi (PPO, EC 1.14.18.1), una ossido riduttasi contenente un ione rame all'interno del sito attivo, che catalizza due differenti reazioni enzimatiche di ossidazione di substrati polifenolici e quindi rende possibile la successiva formazione di composti scuri.

Negli ultimi anni, lo studio di nuovi sistemi ecocompatibili per il controllo dell'attività enzimatica si è focalizzato sulle tecnologie non-termiche e sugli inibitori di origine naturale da proporre in alternativa ai convenzionali trattamenti termici ed ai tradizionali additivi chimici. Un impulso alla ricerca in questa direzione è stato dato dalla dimostrazione del loro impatto negativo non solo sulla qualità organolettica e nutrizionale dei prodotti agro-alimentari e sulla stabilità delle formulazioni cosmetiche, ma anche sulla sicurezza in seguito ad ingestione o contatto.

Partendo da questi presupposti il progetto di ricerca alla base di questa tesi di dottorato vuole valutare, attraverso saggi *in vitro* e *in vivo*, l'efficacia anti-imbrunimento di tre possibili sistemi alternativi: una tecnologia UV-A basata su fonte di luce a LED (primo contributo) e due estratti naturali ottenuti da sottoprodotti agro-industriali, gli idrosol degli agrumi (CHIs; secondo contributo) e gli scarti di potatura del vigneto (VPRs; terzo contributo).

Nel primo contributo, il trattamento basato su luce UV-A, alla lunghezza d'onda di 390 nm, è stato applicato, a temperature ambiente, in intervalli fino un'ora complessiva, su fette di mela (Golden Delicious, Granny Smith, Fuji) e pera (Abate Fétel, Decana), utilizzando un prototipo di illuminatore a LED, dove alcuni parametrici fisici, quali numero di diodi, voltaggio e distanza dal campione, sono stati impostati in modo tale da garantire il massimo irraggiamento ($2.43 \cdot 10^{-3}$ Wm^{-2}). La variazione totale di colore (ΔE) e la sua riduzione percentuale ($\%R\Delta E$) sono state misurate utilizzando un colorimetro; le mele trattate mostravano una maggiore percentuale di riduzione del colore rispetto alle pere (rispettivamente 58.3% e 25.5% in media, dopo un irraggiamento di 60 minuti). Le ottime potenzialità inibitorie del trattamento con luce UV-A nei confronti dell'attività PPO sono state confermate anche dalle prove elettroforetiche e zimografiche eseguite su una tirosinasi commerciale di origine fungina (TYR) e sulla PPO estratta dalle fette di mela Golden Delicious dopo l'irraggiamento. Sulla base dei risultati ottenuti, l'efficacia anti-imbrunimento di questa tecnologia non termica, basata su luce UV-A

con fonte a LED dipende non solo da tempo e intensità di irraggiamento, ma anche da tipo e cultivar di frutti utilizzati.

Nel secondo contributo, l'inibizione tirosinasica da parte di tre diversi tipi d'idrosol, co-prodotti durante distillazione in corrente di vapore delle bucce di cedro, arancia e limone (CH, LH, OH, rispettivamente), è stata determinata spettrofotometricamente, utilizzando (+)–epicatechina e L-DOPA come substrati fenolici rappresentanti, rispettivamente, l'imbrunimento enzimatico delle piante e la melanogenesi della pelle. Tutti gli idrosol di agrumi testati mostravano un'inibizione enzimatica di tipo misto (tra 21.8 e 68.9 %), in base al tipo e alla concentrazione di substrato fenolico utilizzato. L'analisi gas cromatografica (GC) degli idrosol di agrumi ha permesso di individuare tra i terpeni alcuni noti inibitori dell'enzima TYR, quali mircene, sabinene, geraniolo e citrale.

Il terzo contributo esamina le potenzialità anti-imbrunimento e antiossidante di alcuni centrifugati di bacche d'uva provenienti dagli scarti di potatura del vigneto di due diverse cultivar, Barbera (B) e Merlot (M), durante le stagioni di vendemmia dell'anno 2013 (1) e 2014 (2). Tra gli scarti di diradamento, quelli di Merlot inibivano maggiormente l'attività dell'enzima commerciale TYR, quantificata allo spettrofotometro in presenza del substrato catecolo, rispetto a quelli di Barbera (68.2% e 67.8% per M1 e M2, rispettivamente; 56.3% and 58.8% per B1 e B2, rispettivamente) mostrando un'inibizione di tipo acompetitiva; i risultati spettrofotometrici sono stati confermati anche dai test su piastra. Le tecniche zimografiche applicate sulle isoforme enzimatiche isolate da TYR e da alcune PPO vegetali (mele Fuji e Golden Delicious; pere Abate Féte; patate Bintje) così come le prove *in vivo,* condotte su diverse fette di frutta (mele Fuji, Golden Delicious e Granny Smith; pere Abate e Decana) verdura (patate Bintje; melanzane) e su fette essiccate di mela Golden Delicious, hanno dimostrato che il grado d'inibizione dipende principalmente dall'origine dell'enzima. Infatti, questo trattamento chimico non si è rivelato efficace nei confronti della PPO di pera. Tuttavia, lo studio effettuato sugli scarti di potatura di vigneto ha messo in luce le loro potenzialità non solo come agenti anti-imbrunimento, ma anche come sbiancanti e antiossidanti; le loro molteplici proprietà possono essere correlate al loro alto contenuto in acidi organici ed epigallocatechin gallato (EGCG).

Nel complesso, questa ricerca dimostra come l'efficacia inibitoria sia legata principalmente non solo all'origine della PPO, ma anche alla dose e al tipo di inibitore applicato. La tecnologia UV-A con fonte a LED, gli idrosol di agrumi e gli scarti di potatura del vigneto rappresentano sistemi sicuri, economici ed a basso impatto ambientale per controllare l'imbrunimento

enzimatico nel settore agro-alimentare e cosmetico. Inoltre, questi estratti naturali, ricchi in composti bioattivi con forti proprietà inibitorie, suggeriscono un possibile impiego alternativo che potrebbe conferire un interessante valore aggiunto a questi sottoprodotti della filiera agro-industriale.

Abstract

The enzymatic browning and melanogenesis are associated respectively with the most of qualitative and economical losses during post-harvest processing in agro-food industry and human skin disorders in cosmetic field. The main responsible is tyrosinase or polyphenol oxidase (PPO, EC 1.14.18.1), a copper-containing oxidoreductase that catalyses two different enzymatic reactions involving polyphenolic substrates and oxygen and producing dark pigments.

Recently, the research of new eco-friendly systems for controlling PPO activity is focused on innovative non-thermal technologies and bioactive compounds to replace the conventional thermal treatments and traditional additives. All of these have critical points related not only to organoleptic and nutritional qualities of agro-food products and stability in cosmetic formulations but also to human health after topical, oral or parenteral exposure.

In this regards, the goal of this study is to evaluate, by *in vitro* and *in vivo* assays, the anti-browning performances of a UV-A LED technology (first contribution) and two natural extracts obtained from agro-food by-products such as citrus hydrosols (CIHs; second contribution) and agricultural wastes like vineyard pruning residues (VPRs; third contribution).

In the first contribution, after fixing the optimal operational conditions of a UV LED illuminator prototype ($2.43 \cdot 10^{-3}$ Wm^{-2} irradiance) in accordance with number of LED diodes, voltage, and distance from sample, the UV-A light (390 nm) treatment at 25 °C over increasing time periods up to 60 min was applied on fresh-cut apples (Golden Delicious, Granny Smith, and Fuji) and pears (Abate Fétel and Decana). The total colour change (ΔE) and its percent reduction (%RΔE) were measured using a colorimeter and the greatest performances were observed in apples with higher %RΔE values than pears (58.3% *vs.* 25.5% on average after 60 min exposure, respectively). Moreover electrophoretic and zymographic techniques on the commercial mushroom tyrosinase (TYR) and PPO extracted from irradiated Golden Delicious apple slices confirmed the inhibitory effects of UV-A light on PPO activity. The anti-browning effectiveness of UV-A LED technology was related to irradiance, exposure time, and fruit type and cultivar.

In the second contribution, three kinds of hydrosols, which have been obtained by subjecting citron, lemon, and orange peels to steam distillation (CH, LH, and, OH respectively), were spectrophotometrically assessed for anti-TYR activity in the presence of (+)–epicatechin and L-DOPA as the model phenolic substrates of plant enzymatic browning and human skin

melanogenesis, respectively. All of the CIHs showed a mixed-type inhibition at varying levels in the 21.8–68.9 % range, depending on substrate type and concentration. The gas chromatography analysis (GC) of their terpene contents indicated that some known TYR inhibitors including myrcene, sabinene, geraniol and citral were present in CIHs.

The third contribution investigate the anti-browning and antioxidant potentials of some grape juices obtained by cold-pressing the berries collected from the VPRs of Barbera (B) and Merlot (M) cultivars during 2013 (1) and 2014 (2) seasons. Among the VPRs, Merlot wastes spectrophotometrically exhibited a greater uncompetitive inhibition towards TYR activity than those of Barbera (68.2% and 67.8% for M1 and M2, respectively; 56.3% and 58.8% for B1 and B2, respectively), in the presence of catechol substrate, as confirmed also by gel diffusion assay. The zymographic techniques on the isoforms isolated from TYR and some plant PPOs (Fuji and Golden Delicious apples; Abate Fétel pears; Bintje potatoes) as well as *in vivo* trials on several fresh-cut fruits (Fuji, Golden Delicious, Granny Smith apples; Abate and Decana pears) vegetables (Bintje potatoes; eggplants), and dried apple slices (Golden Delicious) demonstrated that the inhibitory performances were related mainly to enzyme source. In this regards, this chemical treatment with VPRs was not effective on pear PPO. However, the VPRs showed not only anti-browning but also whitening and antioxidant capacities that were associated mainly with their high organic acids and epigallocatechin gallate (EGCG) contents detected by HPLC analysis.

Overall this research confirms that the inhibitory effectiveness is a function of PPO source and inhibitor type and dose. The UV-A LED technology, CIHs, and VPRs are eco-friendly, safe, and inexpensive systems for effectively inhibiting PPO activity, thus preserving the enzymatic browning in agro-food and cosmetic industries. Moreover, these natural extracts, whose anti-browning performances depends mainly on their bioactive compounds contents, suggest a possible recycling use with high value added of these agro-food by-products.

1 Introduction

1.1 Enzymatic browning in agro-food chain

In recent decades, the market for fresh-cut products is taking hold because of their convenience and healthfulness (Ragaert *et al.*, 2004). The International Fresh-cut Produce Association (IFPA) defines fresh-cut products as fruit or vegetables that have been trimmed and/or peeled and/or cut into 100% usable product which is bagged or pre-packaged to offer consumers high nutrition, convenience, flavour while still maintaining its freshness (Lamikanra, 2002). The main factors affecting the perceived quality of fresh-cut products are texture and appearance (Rico *et al.*, 2007; Toivonen & Brummell, 2008) that depend not only on pre-harvest and genetic factors but also on abiotic stresses during processing, packaging and storage (Hodges & Toivonen, 2008).

Several studies have yet pointed to the most food consumers' preferences for organoleptic features rather than potential health benefits (Verbeke, 2005 & 2006; Urala & Lähteenmäki, 2007; Annunziata & Vecchio, 2011). There is no doubt that the first parameter which food consumers take into account is appearance, an attribute judged on the basis of multiple factors including size, shape, form, colour, condition and absence of defects all of which can be influenced by several pre-harvest factors (Kays, 1999). In particular colour plays a key role in food choice by influencing taste thresholds, sweetness perception, food preference, pleasantness and acceptability (Clydesdale, 1993) and can be used both as a direct and an indirect index of quality (Francis, 1995).

The colour is related to the presence of chromophores pigments divided into four primary classes based on their chemistry: chlorophylls, carotenoids, flavonoids and betalains (Kays, 1999). However, browning and discoloration reactions can affect the colour changes (Toivonen & Brummell, 2008). Non-enzymatic browning such as Maillard reaction, caramelisation, chemical oxidation of phenols and enzymatic browning are the main phenomena occurring in food processing and storage and show several implications in food technology, nutrition and health (Manzocco *et al.*, 2001).

The enzymatic browning in plant products (Figure 1.1) is associated with the most of qualitative and economical losses in agro-food industry (Whitaker & Lee, 1995; Jiang *et al.*, 2004; Degl'Innocenti *et al.*, 2007).

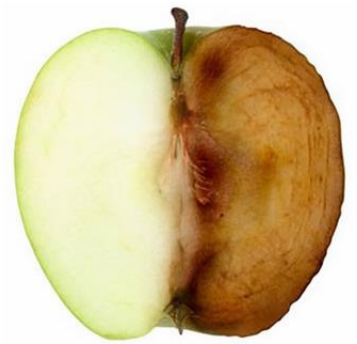


Figure 1.1 Enzymatic browning in the apple.

Browning reactions take place in the presence of oxygen when polyphenolic substrates are exposed to PPO and/or phenol peroxidases as a consequence of mechanical and physical stresses that occur during post-harvest processing (handling, peeling, brushing, cutting, packaging, etc.) and storage leading to the breakdown of cell structure (Hurrel & Finot, 1984). Wounding is the main post-harvest physical damage (Hodges & Toivonen, 2008) that increases the oxidative stress of plants (Hodges *et al.*, 2004) and involves some key enzymes in the metabolism of phenols thus compromising the quality in fruits and vegetables (Tomás-Barberán & Espín, 2001). As regards fresh-cut lettuce, in response to cutting operation, phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the committed enzyme in the phenylpropanoid pathway, produces phenols that are then oxidized by the action of polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) to quinones which spontaneously polymerize to form dark pigments responsible of colour changes (Saltveit, 2000; Degl' Innocenti *et al.*, 2005, 2007). This pattern has been observed also in fresh-cut potato strips (Cantos *et al.*, 2002), broccoli florets (Gong & Mattheis, 2003), jicama cylinders (Aquino-Bolaños *et al.*, 2000), carrots (Goldberg *et al.*, 1985) and lettuce leaf segments (Hisaminato *et al.*, 2001; Murata *et al.*, 2004).

Although several authors have attributed a partial role of POD in enzymatic browning (Underhill & Critchley, 1995; Richard-Forget & Gauillard, 1997; Degl'Innocenti *et al.*, 2005), the main responsible is PPO, a copper-containing oxidoreductase that catalyses two different reactions involving phenolic compounds and oxygen and producing brown- or black-coloured compounds depending on the specific structure of the polyphenolic substrate (Martinez & Whitaker, 1995; Seo *et al.*, 2003; Yoruk & Marshall, 2003; Garcia-Molina *et al.*, 2007; Queiroz *et al.*, 2008). The browning degree depends on type and concentration of endogenous phenolic compounds, the presence of oxygen, reducing substances, and metallic ions and pH and temperature that control PPO activity (Nicolas *et al.*, 1994; Martinez & Whitaker, 1995; Yoruk & Marshall, 2003). The enzymatic reaction in agro-food products leads not only to colour alterations but also to reduced nutritional and organoleptic properties as a consequence of the

quinones condensation with other compounds such as amino acids, proteins, phenols and sugar (Oszmianskii *et al*., 1990; Rapeanu *et al.*, 2006; Queiroz *et al.*, 2008) and the degradation of polyphenolic substrates recognized for their health benefits as antioxidant and anti-inflammatory agents (Stevenson & Hurst, 2007; Quideau *et al*., 2011; Kang *et al*., 2011).

1.2 Enzymatic browning in cosmetic field

The enzymatic browning is also involved in the melanin biosynthesis inside melanocytes in the inner layer of epidermis (Parvez *et al*., 2006). The colour of mammalian skin and hair depends on the melanin synthesis and distribution by keratinocytes that play an essential role in the regulation of melanocyte growth and differentiation (Minwalla *et al*., 2001; Thong *et al*., 2003).

Melanins can be classified in two basic types: eumelanins, which are brown or black, and phaeomelanins, which are yellow or red (Prota, 1995; Slominski *et al*., 2004; Ito & Wakamatsu, 2003). Their metabolic pathways in melanocytes include some oxidative reactions driven by enzyme tyrosinase (TYR, EC 1.14.18.1) on the amino acid tyrosine (Sanchez-Ferrer *et al*., 1995; Olivares *et al*., 2001; Ito, 2003; Wang & Herbert, 2006; Parvez et al., 2007; Ito & Wakamatsu, 2008; Simon *et al*., 2009), as illustrated in Figure 1.2. In the first steps of melanogenesis, TYR catalyses the tyrosine hydroxylation to dihydroxyphenylalanine (DOPA) which was then oxidized to DOPAquinone. Subsequently, DOPAquinone is converted to DOPAchrome through autoxidation, and finally to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) forming eumelanin (brown-black pigment). The latter reaction is performed by DOPAchrome tautomerase and DHICA oxidase. However, in the presence of cysteine or glutathione, DOPAquinone is converted to cysteinylDOPA or glutathioneDOPA producing pheomelanin (yellow-red pigment). The down-regulation expression of tyrosinase-related proteins (e.g. TRP1 and TRP2) may modulate the production of pheomelanin rather than eumelanin (Kobayashi *et al*., 1995; Wang & Herbert, 2006).

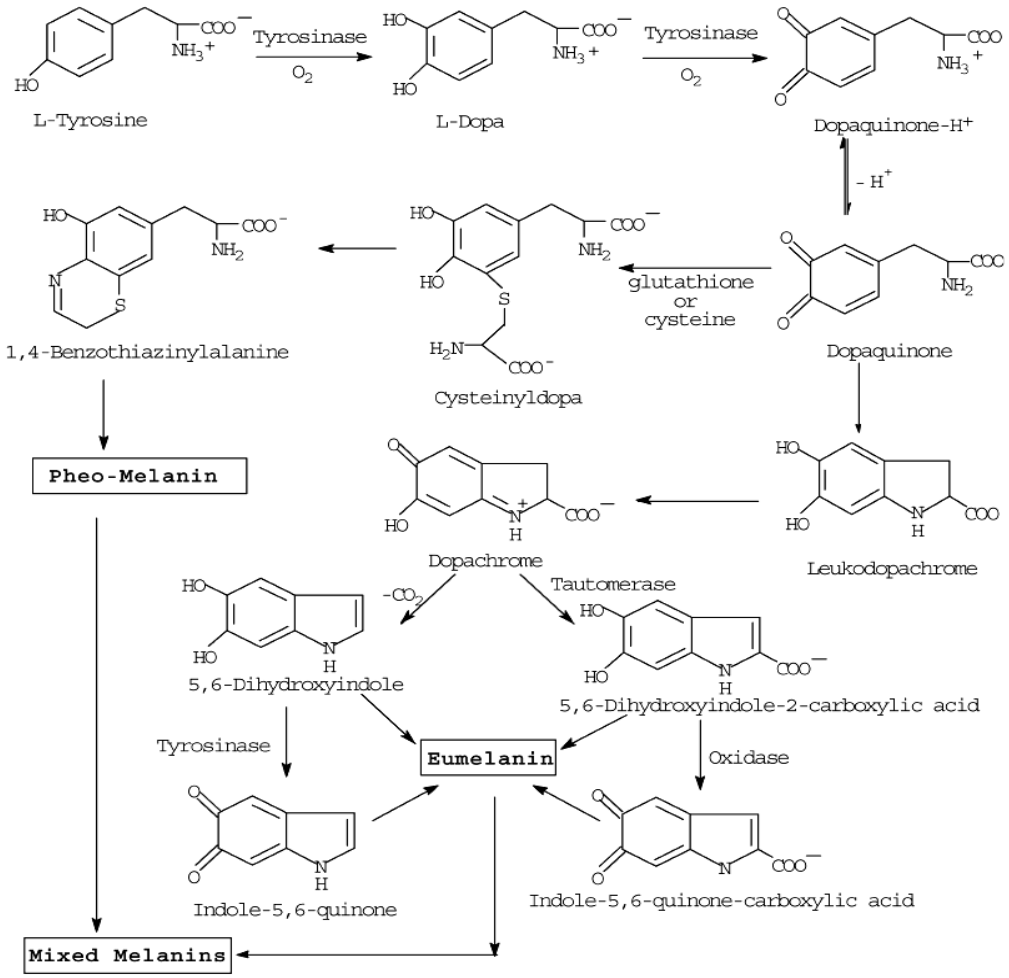


Figure 1.2 The melanogenesis pathway (Seo *et al*., 2003).

Wang & Herbert (2006) review the steps involving TYR maturation from its synthesis in cytosolic ribosomes up to its transportation out of trans-Golgi network to melanosomes which mature in melanocytes (Figure 1.3).

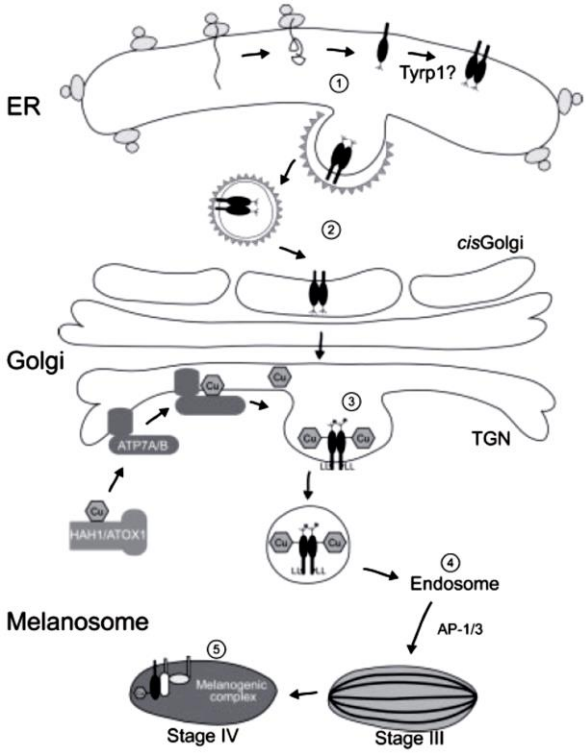


Figure 1.3 Tyrosinase maturation and trafficking through the secretory pathway (Wang & Herbert, 2006). (1) Tyrosinase folds in the ER co-translationally and dimerizes. The quality control system in the ER ensures the folding and assembly are correct. (2) The export-competent tyrosinase is transported to the cis-Golgi network in COPII vesicles. (3) In the trans-Golgi network (TGN), the N-linked glycans are modified further to complex sugars and copper is loaded. (4) Tyrosinase is transported out of the TGN to the melanosomes. (5) The melanogenic complex is formed in melanosomes, which mature through the various stages.

However, the melanogenesis, which is regulated by pH conditions (Halaban *et al.*, 2002), depends also on additional melanogenic factors (Schaffer & Bolognia, 2001; Slominski *et al*., 2004; Schallreuter *et al*., 2007; Gillbro & Olsson, 2011).

The main functions of melanin are related to the absorption of free radicals generated within the cytoplasm and the human skin protection from various types of ionizing radiations, including UV light (Henessy *et al*., 2005; Costin & Hearing, 2007; Lin & Fisher, 2007; Park *et al*., 2009). However, the hyperpigmentation, which is related to an abnormal melanin accumulation because of alterations during melanogenesis and TYR maturation in the mammalian secretory pathway (Wang & Herbert, 2006), is associated to several esthetic problems such as freckles, age spots and melasma (Costin & Hearing, 2007) and dermatological diseases including vitiligo and melanoma (Seo *et al.*, 2003).

1.4 Polyphenol oxidase (PPO, EC 1.14.18.1)

Polyphenol oxidases are copper-containing oxidoreductases that catalyse the oxidation of phenolic compounds in the presence of molecular oxygen as the terminal electron acceptor. They are divided into two main groups on the basis of different diphenolic substrates and molecular properties (Mayer & Harel, 1979; Mayer, 1987):

- *o*-diphenol oxidase: catechol oxidase (CO; EC 1.10.3.1), an intracellular enzyme of plant (Eicken *et al.*, 1998; Rompel *et al.*, 1999a, 1999b; Rompel *et al*., 2012) and fungal origin (Motoda, 1979a, 1979b); tyrosinase (TYR; EC 1.14.18.1, EC 1.10.3.1) widely found in plants and fungi (Vamos-Vigyazo & Haard,1981; Halaouli *et al*., 2006; Mayer, 2006; Marusek *et al*., 2006), bacteria (Claus & Decker, 2006), insects and crustaceans e.g. shrimp (Zamorano *et al.*, 2009) as an intracellular enzyme and also in humans as a trans-membrane enzyme (Kwon *et al*., 1987; Kobayashi *et al.*, 1995; Sanchez-Ferrer *et al.*, 1995; Olivares *et al*., 2001).
- *p*-diphenol oxidase: laccase (EC 1.10.3.2), an extracellular enzyme mainly of fungal origin (Mayer & Stapples, 2002).

The enzymes TYR and CO remove an electron pair from the hydroxyl groups of *o*-diphenolic substrates producing quinones, while laccase remove single electrons from the reducing groups of *p*-diphenolic substrates producing usually free radicals (Figure 1.4).

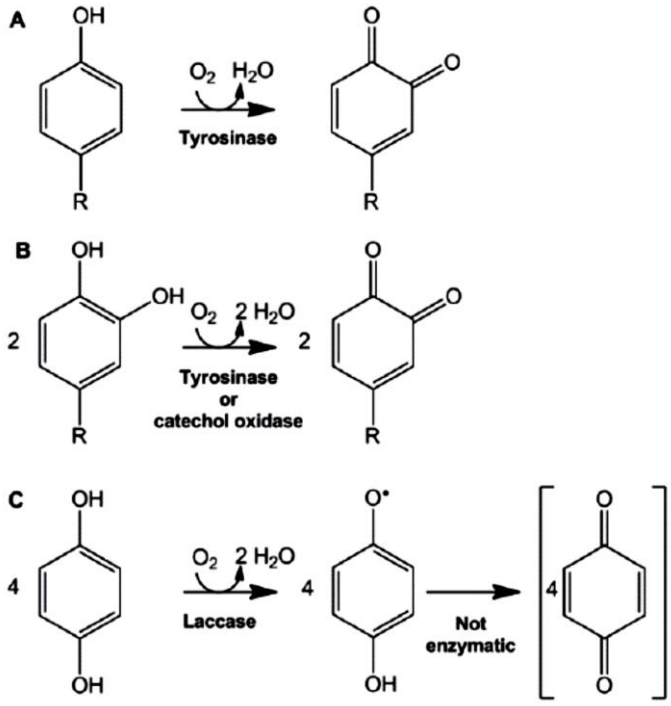


Figure 1.4 Oxidation of diphenolic substrates catalysed by tyrosinase (A and B), catechol oxidase (B), and laccase (C) (Sanchez-Amat & Solano, 1997).

Anyway, the polyphenol oxidase (PPO) label is usually used for all *o*-diphenol oxidases (Marusek *et al.*, 2006).

In a typical plant cell, PPO is localized in cytoplasmic organelles especially at thylakoid membrane of chloroplasts as well as in mitochondria and rarely in peroxysomes, while its phenolic substrates are mostly found in the vacuole and also in the apoplast/cell wall compartment (Figure 1.5). The bond strength between the enzyme and the membrane depends on the tissue type and the ripening stage of plant. For a better PPO extraction, the anionic detergent Triton X-100 is usually used because it is able to modify the enzyme structure by changing the substrate specificity and optimum pH (Weemaes *et al*., 1998).

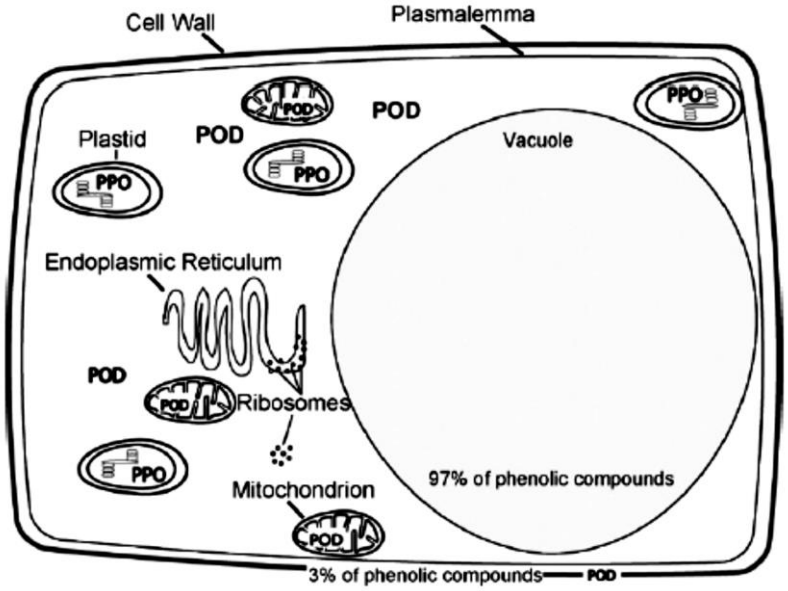


Figure 1.5 Localization of PPO and its phenolic substrates in a typical plant cell (Toivonen & Brummell, 2008). POD: phenol peroxidase; PPO: polyphenol oxidase.

The PPO structure consists of three domains: an N-terminal domain, a central catalytic domain and a C-terminal domain (Seo *et al*., 2003; Marusek *et al*., 2006; Flurkey & Inlow, 2008). Animal and plant PPOs have all three domains, whereas fungal PPOs lack the N-terminal domain. Bacterial PPOs have only the central catalytic domain, but they are produced in association with a caddie protein that acts as a C-terminal domain. In plants, the N-terminal domain is involved in the protein transportation to the chloroplast thylakoid lumen where occurs the PPO maturation. The C-terminal domain covers the PPO active site, which is contained in the central catalytic domain, thus keeping the enzyme inactive in the secretory pathway. PPOs are usually activated by cleavage of the N- and C-terminal domains, or in the case of bacterial PPOs, by removal the caddie protein.

The active site of PPO in the central catalytic domain mainly includes a copper ion (Cu^{2+}) for interacting with both molecular oxygen and its phenolic substrate; it is bound to six or seven histidine residues and cysteine residues which vary according to enzyme origin and play an important role in the formation of disulphide linkages for stabilizing the protein structure (Seo *et al*., 2003; Mayer, 2006). As shown in Figure 1.6, PPOs are binuclear copper oxidases with a T3 copper site containing two copper binding sites, called CuA and CuB, each of which is linked to three histidine residues (Solomon *et al*., 1992; Solomon *et al*., 1996; van Gelder *et al*., 1997; Solomon *et al*. 2001).

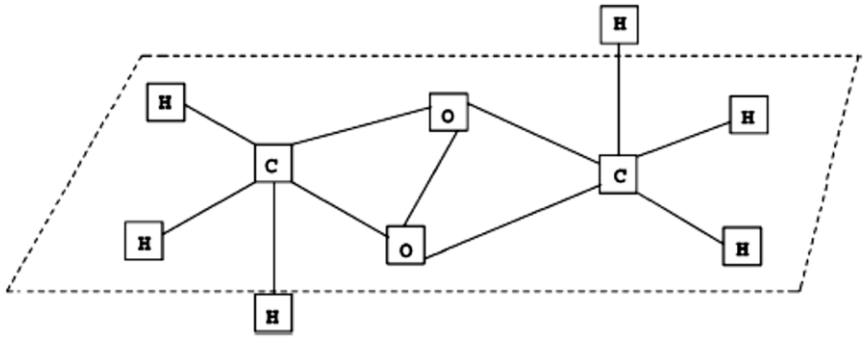


Figure 1.6 Schematic representation of binuclear copper site. C: copper ion. O: oxygen. H: histidine residues (Seo *et al*., 2003).

The PPO enzyme catalyses two different reactions (Sanchez-Ferrer *et al*., 1995; Seo *et al*., 2003; Garcia-Molina *et al*., 2007) including the oxidation of phenolic substrates in the presence of molecular oxygen as cofactor (Figure 1.7):

- cresolase or monophenolase activity (EC 1.14.18.1): *o*-hydroxylation of monophenols (*p*-cresol, tyrosine) to *o*-diphenols;
- catecholase or diphenolase activity (EC 1.10.3.1): oxidation of *o*-diphenols (catechol, L-DOPA, D-DOPA, catechin, chlorogenic acid) to *o*-quinones; these compounds are unstable and therefore polymerize to dark pigments known as melanins, which are responsible of colour alterations.

The first catalytic step has not been observed in CO enzyme (Mayer & Harel, 1979, Walker & Ferrar 1998; Gerdemann *et al*., 2002).

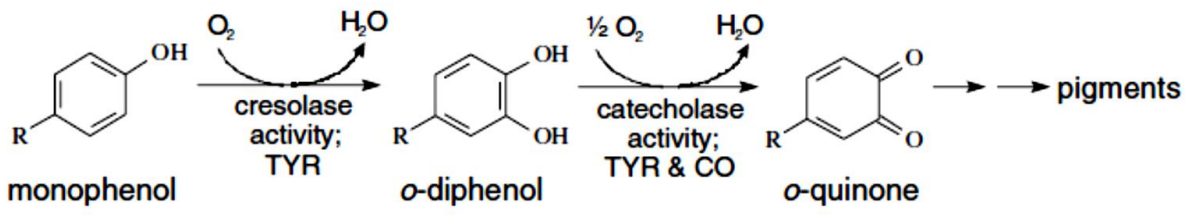


Figure 1.7 The enzymatic browning reactions catalyzed by PPO (TYR and CO) (Marusek *et al*., 2006).

As described in Figure 1.8, the PPO active site can occur in three forms during enzymatic browning reactions: *met*-PPO (Cu^{2+}), *oxy*-PPO (Cu^{2+}) and *deoxy*-PPO (Cu^{1+}) (Martinez & Whitaker, 1995; Sanchez-Ferrer *et al*., 1995; Seo *et al*., 2003; Kim & Uyama, 2005). Initially, PPO reacts with oxygen generating *oxy*-PPO and thus activating the first catalytic step (cresolase/monophenolase activity) because the monophenolic substrate can react only with this oxidixed form of enzyme. The subsequent formation of *o*-diphenol is followed by the release of substrate and the conversion of enzyme active site in *deoxy*-PPO. At this point, the enzyme is ready for another cycle without being converted in latent form. In the second catalytic step (catecholase/diphenolase activity), the diphenolic substrate reacts not only with *oxy*-PPO but also with *met*-PPO. The latter is reduced in *deoxy*-PPO form after the oxidation of *o*-diphenol to *o*-quinone. Then, *deoxy*-PPO reacts with oxygen in order to create *oxy*-PPO by oxidizing another *o*- diphenolic compound. Finally, the enzyme is reduced in its latent form (*met*-PPO) after the transformation of *o*-diphenolic substrate.

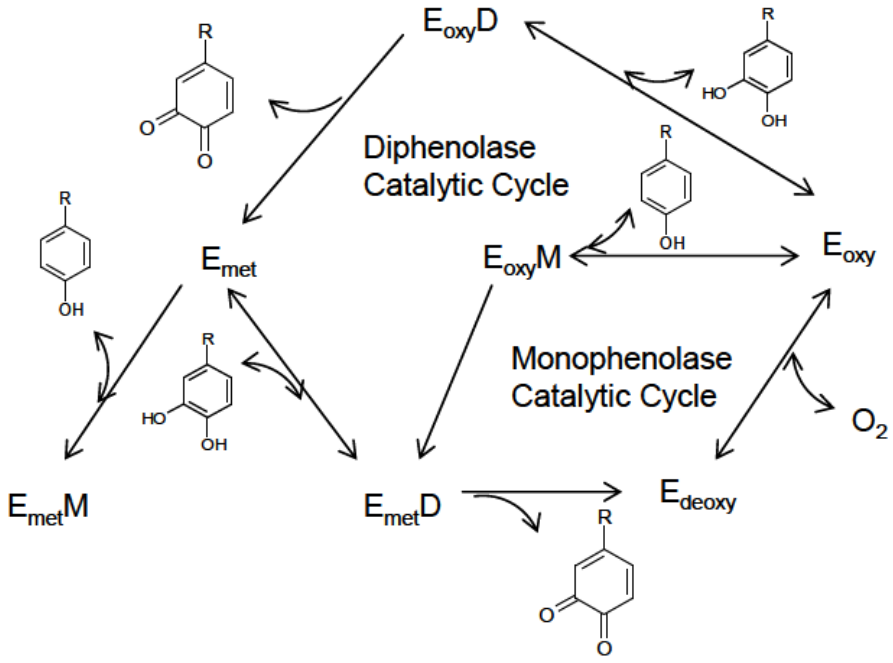


Figure 1.8 Catalytic cycles for cresolase/monophenolase and catecholase/diphenolase activities of PPO (Chang, 2009).

The PPO has also latent forms which make even more difficult the control of enzymatic browning. The latency degree depends on the enzyme origin, the ripening stage of plant , and activator type. Yoruk & Marshall (2003) report several studies showing the activation of PPO latent forms by fatty acids, alcohols, denaturants, detergents, acid and alkaline compounds, proteases, sonication, cold temperatures and mild heat treatments. The anionic detergent, SDS,

has been widely used as the main activator of latent PPO from several plant sources by increasing the enzyme activity as a function of detergent concentration and pH (Chazarra *et al*., 1996; Soyo *et al*., 1998a, b; Nuñez-Delicado *et al*., 2003; Gandía-Herrero *et al*., 2004; Orenes-Piñero *et al*., 2006; Sellés-Marchart *et al*., 2007; Cabanes *et al*., 2007; Saeidian & Rashidzadeh, 2013). As shown in Figure 1.9, the activation of the latent mushroom TYR occurs at low SDS concentrations and pH values over 5.0 (Espín & Wichers, 1999).

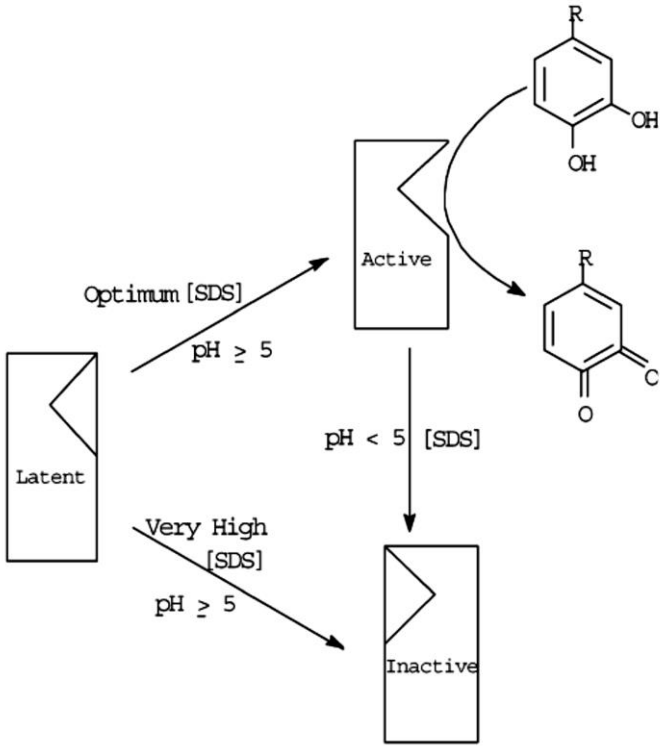


Figure 1.9 pH-dependent activation of latent mushroom TYR by SDS (Seo *et al*., 2003).

The effectiveness in keeping active the PPO latent is related also to its treatment time with activator. In this regard, the PPO activity of various apple cultivars subjected to heating treatment at three different temperatures (68, 73 and 78 °C) significantly decreased at increasing times after an initial activation of a thermal stable latent PPO (Yemenicioglu et al., 1997). The same pattern was confirmed by Soysal (2008) showing an activation of apple PPO at initial stages of a mild heat treatment at 45, 55 and 65 °C. Also trypsin, which was the most effective protease in activating the latent forms of peach (Laveda *et al*., 2001) and grape PPO (Nunez-Delicado *et al*., 2005), exhibited a stronger activation of sago log PPO than ethanol, linoleic acid and SDS only until 10 min treatment time; after that linoleic acid and ethanol showed the best performance until 24 hours (Onsa *et al*., 2000). Moreover, the latent PPO could be activated by pathogen attack as demonstrated by Anderson *et al*. (2010) discovering a seed-decay isolate of *Fusarium avenaceum* as a possible activator of latent PPO in wild oat caryopsis.

The PPO catalytic activity depends on several physical-chemical parameters such as substrate specificity, pH, and temperature that have been associated to the enzyme source (Yoruk & Marshall, 2003).

The enzyme can react with monohydroxyphenols (*p*-cresol and tyrosine), dihydroxyphenols (catechol, L-DOPA, D-DOPA, catechin and chlorogenic acid) and trihydroxyphenols (pyrogallol). However, PPO is the most active towards dihydroxyphenols (Zhang *et al*., 1999; Seo *et al*., 2003; Yoruk & Marshall, 2003; Eidhin *et al*., 2006; Rapeanu *et al*., 2006) with a greater substrate stereo specificity for L-isomers than D-isomers (Espin *et al.*, 1998; Rescigno *et al.*, 2002) on the basis of lower K_m (Michaelis-Menten constant) and higher V_{max} (maximum reaction velocity).

The PPO inactivation is related to protein structure denaturation because of conformational changes in the enzyme catalytic site when pH and temperature values are too much above or below the optimum range of enzyme (Valero & Garcia-Carmona, 1998; Yoruk & Marshall, 2003). The optimum pH, which generally ranges from 4.0 and 8.0, can be affected by various factors such as extraction method, temperature, type of phenolic substrate, and buffer solution used during the detection of enzyme activity (Yoruk & Marshall, 2003).

The optimal pH and temperature values of PPO widely vary according to species and cultivars (Table 1.1, 1.2, and 1.3) and also to enzyme isoforms, whose molecular weights range from 116 to 128 KDa, with a minimum between 26 and 32 KDa (Mayer & Harel, 1979).

Table 1.1 Chemical-physical properties of some PPOs extracted from different fruits.

PPO source	pH	T (°C)	References
Apple (*Malus domestica*) cv. Bramley's Seedling	6.5	30	Eidhin *et al*., 2006
Apple (*Malus domestica*) cv. Golden Delicious	6.0	25	Weemaes *et al*., 1997 Soysal, 2008
Apple (*Malus domestica*) cv. Jonagored	5.0	25	Rocha *et al*., 2000
Apple (*Malus domestica*) cv. Red Delicious	6.2	30	Satjawatcharaphong *et al*., 1983
Apple (*Malus domestica*) cv. Monroe	4.6	30-40	Zhou *et al*., 1993
Banana (*Musa cavendishii*)	7.0	30	Unal *et al*., 2005
Avocado (*Persa Americana*)	7.5-7.6	60-65	Gomez-Lopez, 2001
Barbados cherry (*Malpighia glabra*)	7.2	40	Anil Kumar *et al*., 2008
Chestnut (*Castanea sativa*) cv. Jiangsuluhe	7.0	40-50	Gong *et al*., 2015
Cashew Apple (*Anacardium occidentale*)	6.5	27	Queiroz *et al*., 2011
Coffee (*Coffea arabica*)	6.0-7.0	30	Mazzafera *et al*., 2000
Litchi (*Litchi chinensis*)	7.5	45	Liu *et al*., 2007
Longan (*Dimocarpus longan*)	6.5	35	Jiang, 1997
Apricot (*Prunus aemeniaca*)	8.5	40	Arslan *et al*., 1998
Mango (*Mangifera indica)* cv. Manila	6.0	20-70	Palma-Orosco *et al*.,2014
Marula fruit (*Sclerocarya birrea*)	4.0	60	Mdluli, 2005
Medlar (*Mespilus germanica*)	6.0-7.0	30	Ayaz *et al*., 2008
Grape (*Vitis vinifera*) cv. Napoleon	6.0	30-60	Núñez-Delicado *et al*., 2007
Grape (*Vitis vinifera*) cv. Victoria	5.0	25	Rapeanu *et al*., 2006
Pear (*Pyrus communis*) cv. Ankara Armutu	8.2	35	Ziyan *et al*., 2003
Snake fruit (*Salacca Zalacca*)	6.5	30	Mohd Zaini *et al*., 2013
Wolf apple (*Solanum lycocarpum*)	6.0-6.5	28	Batista *et al*., 2014

Table 1.2 Chemical-physical properties of some PPOs extracted from different vegetables.

PPO source	pH	T (°C)	References
Artichoke (*Cynara scolymus*)	5.0-7.0	40	Doğan *et al.*, 2005
Bean sprouts (*Glycine max*)	9.0	40	Nagai *et al.*, 2003
Butter lettuce (*Lactuca sativa* cv. Capitata	5.5	30-40	Gawlik-Dziki *et al.*, 2008
Celery root (*Apium graveolens*)	7.0	30	Aydemir *et al.*, 2006
Chiense cabbage (*Brassica rapa*)	5.0	40	Nagai *et al.*, 2001
Jerusalem arthichoke (*Helianthus tuberosus*)	7.5-8.0	25-30	Ziyan *et al.*, 2003
Mushrooms (*Agaricus bisporus*)	6.0-8.0	40-45	Simsek *et al.*, 2007
Parsley (*Petroselinum crispum*)	4.0	35-40	Dogru *et al.*, 2012
Potato (*Solanum tuberosum*)	6.6	40	Marri *et al.*, 2003
Radish (*Raphanus sativus*) cv. Sativus	7.0	20-40	Goyeneche *et al.*, 2013
Sweet potato (*Ipomea batas*)	4.0-6.5	60	Lourenco *et al.*, 1992
Yacon root (*Smallanthus sonchifolius*)	5.0-6.6	60-70	Neves *et al.*, 2007

Table 1.3 Chemical-physical properties of some PPOs extracted from different fishes.

PPO source	pH	T (°C)	References
Cuttlefish (*Sepia esculeuta* Hoyle)	7.5	60	Zhou *et al.*, 2004
Deepwater pink shrimp (*Parapenaeus longirostris*)	4.5	30-35	Zamorano *et al.*, 2009

1.5 PPO inhibition by physical treatments

1.5.1 Conventional thermal technologies

The thermal treatments are the most widely used preservative technology in the food industry for processing juices, smoothies, purees, nectar, dehydrated, and canned fruits and vegetables. Blanching, which is generally used as a pretreatment in many processing techniques e.g. freezing and drying, is a common method for controlling enzymatic browning (Severini *et al*., 2003). Blanching is performed by exposing fruits and vegetables to hot boiling water or solutions containing acids and/or salts, steam or microwaves (Devece *et al*., 1999; Severini *et al*., 2003).

An exposure of PPO to high temperatures in the range of 70-90 °C results in enzyme denaturation and subsequent inactivation (Vámos-Vigyázó, 1981; Santos *et al*., 2007) after overcoming its optimum value in the range of 30-40 °C on the basis of PPO source (Table 1.1, 1.2, and 1.3). The time and temperature required for achieving an effective thermal inactivation of PPO depend not only on enzyme origin and latency but also on the presence of sugars and salts that have a protective function toward PPO (Yoruk & Marshall, 2003). Moreover, heating can induce enzyme activation as observed in PPO of western rock lobster hemolymph after processing at the temperatures ranging between 60 and 80 °C (Williams *et al*., 2003). In this case, the maximization of PPO deactivation and the minimization of melanosis in the crustacean tissues required an internal temperature of processed lobsters exceeding 90 °C. The heat-induced activation of enzyme could be related to the presence of thermostable latent forms of enzymes as demonstrated in some studies on apple PPO (Yemenicioglu *et al*., 1997; Soysal, 2008). However, a higher core temperature in pre-cooked Pacific white shrimp has been associated with a lower PPO activity and melanosis score during 7 days of storage at 4 °C (Figure 1.10), but higher cooking loss. Thus, pre-cooking of shrimp for achieving a core temperature of 80 °C, with a holding time of 30 s, could prevent the severe cooking loss (cooking yield of 95.6%) and lower melanosis during storage as a consequence of 96.1% PPO inhibition (Manheem *et al*., 2012).

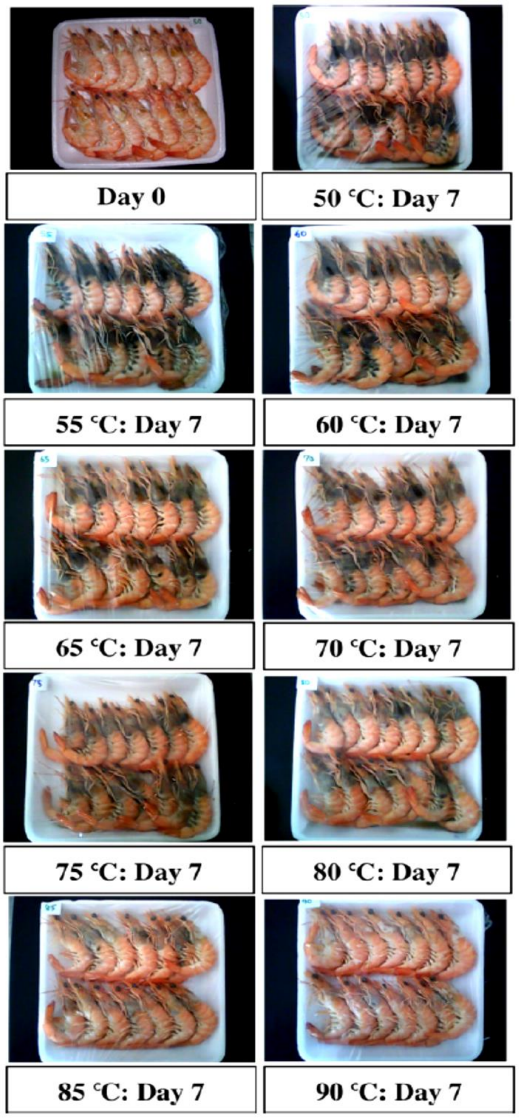


Figure 1.10 Pre-cooked Pacific white shrimp with different core temperatures during 7 days of refrigerated storage at 4°C (Manheem *et al*., 2012).

The time/temperature binomial should be suitable for a complete PPO inactivation (Chutintrasri & Noomhorm, 2006). However, some studies report a negative impact of heat processing on the organoleptic and nutritional qualities of final product (Braddock, 1999) significantly decreasing the content in bioactive compounds such as flavonoids, anthocyanins, carotenoids and ascorbic acid (Blasco *et al*., 2004; Rawson *et al*., 2011). The thermal treatment is also responsible for the most of vitamin losses according to the heating method and type of food (Lešková *et al*., 2006). In particular, the antioxidant vitamin C is the most labile with losses of 41-42% for broccoli and 28-32% for cauliflower after blanching at the temperatures varying between 96 and 98°C for 3 and 4 min respectively (Lisiewska & Kmiecik; 1996) and 44-66% for various vegetables after boiling in the range of 3-5.5 min (Masrizal *et al*., 1997).

Steaming broccoli for 3.5 min leads to better vitamin C retentions in comparison to conventional cooking for 5 min (99.7% *vs* 72.8%; Vallejo *et al*., 2002). The steam blanching is also effective in limiting the colour changes of mango slices by inhibiting PPO activity of 97% after 3 min and 100% after 5 min (Ndiaye *et al*., 2009).

Electromagnetic waves are another valid alternative to conventional heat treatments not only in inhibiting mushroom PPO (Devece *et al*., 1999) but also in preserving the content of antioxidant flavonoids in grapefruit juice (Igual *et al*., 2011). Moreover, a treatment with microwaves at 1000 W for 340 seconds leads to a 90.2% inhibition of kiwi PPO (Benlloch-Tinoco *et al*., 2013). However, the advantage of this treatment is the appearance of non-enzymatic browning during processing because the core temperature reaches 85 °C (Devece *et al*., 1999).

1.5.2 Innovative non-thermal technologies

The non-thermal technologies represent emerging alternatives to conventional treatments in food processing and preservation (Henry *et al*., 1997; Gould, 2000; Raso & Barbosa-Canovas 2003) for improving food safety and shelf-life thanks to the inactivation of enzymes and microorganisms (Mertens & Knorr, 1992; Knorr, 1999; Manas & Pagan; 2005; Morris *et al*., 2007) and simultaneously minimizing the sensory and nutritional losses (Knorr *et al*., 2002; Tiwaria *et al*., 2009; Rawson *et al*., 2011).

Moreover, they are eco-friendly systems because allows producing high quality products with lower environmental impact in terms of energy efficiency, water savings and reduced emission (Pereira & Vicente, 2010).

High hydrostatic pressure processing

The high hydrostatic pressure (HHP) processing or high pressure processing (HPP) is the most widely used preservative treatment that shows a minimal impact on the nutritional and organoleptic qualities of food (Van der Plancken *et al*., 2012; Vervoort *et al*., 2012). Several studies report that HHP technology better retains the sensory and nutritional properties of fresh juices than thermal treatment (Bull et al., 2004; Tiwari *et al*., 2009; Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Keenan *et al*., 2012).

The effectiveness of HPP treatment in controlling enzymatic browning mainly depends on the pressure levels applied on the basis of the target microorganism or enzyme to be inactivated. Bacterial vegetative cells, yeasts and moulds are sensitive to pressures between 200 and 700 MPa, while some enzymes and some bacterial spores may survive pressurization above 1,000 MPa at room temperature (Arroyo *et al*., 1999; Bull *et al*., 2004). In details, pressures levels

lower than 700 mPa partially inactivate PPO (Gomes & Ledward, 1996; Weemaes *et al*., 1998; Keenan *et al*., 2012). Also the treatment time plays an important role in enhancing the PPO inhibition. Garcia-Palazon *et al*. (2004) report that HPP treatments of raspberry puree at lower pressures in the range 400-800 mPa for 5 minutes lead to a PPO activation between 8% and 15%. Instead, increasing the time up to 10 and 15 minutes involves a decreased enzyme activity between 3% and 29%. Moreover, several studies report high-pressure resistances of apple PPO (Bayındırlı *et al*., 2006; Valdramidis *et al*., 2009).

Thus, a suitable combination of pressure and mild initial temperatures (60-90ºC) is requested in order to improve the inhibitory performance (Buckow *et al*., 2009) and optimize the food safety and quality (Van der Plancken *et al*., 2012). Moreover, it allows reducing the treatment times (Ramirez *et al*., 2009; Knoerzer *et al*., 2010; Mújica-Paz *et al*., 2011). The PPO activity in blueberry juice decreases at relatively high pressure-mild temperature (400-600 MPa, 60 °C) and mild pressure-high temperature (0.1-400 MPa, 70-80 °C) combinations (Terefe et al., 2015). However, Terefe *et al*. (2010) have shown a maximum inactivation of strawberry PPO around 23% after treatment at the strongest processing condition (690 MPa, 90 °C).

In this regard, another solution may be the application of pressure levels higher than 700 MPa. The increase in the pressures levels from 800 to 1600 mPa leads to enhanced inactivation of *Agaricus bisporus* PPO in the buffer as well as in the mushroom puree. In details, after treatments at pressures between 1400 and 1600 mPa for a 1 minute the enzyme activity of PPO in the model system decreases by 90.4% and 99.2% respectively, while increases in the mushroom puree (Yi *et al*., 2012).

Pulsed electric field

The non-thermal technology based on pulsed electric field (PEF) is another effective alternative to conventional thermal treatments only for liquid food in order to achieve the microbial and enzymatic inactivation without compromising the organoleptic and nutritional qualities of products (Noci *et al*., 2008). This physical method consists in applying pulsed electric fields of high voltage (typically 20-80 kV/cm) for short time periods (<1 s) to fluid foods placed between two electrodes (Señorans *et al*., 2003).

The PPO inactivation by PEF processing depends not only on treatment parameters, such as electric field strength, pulse number, temperature and frequency (Giner *et al*., 2001, 2002; Marsellés-Fontanet & Martín-Belloso, 2007; Riener *et al*., 2008; Schilling *et al*., 2008; Van Loey, Verachtert, & Hendrickx, 2002; Zhong *et al*., 2007; Moritz *et al*., 2012; Meneses *et al*., 2013) but also on food properties, such as electrical conductivity, ion strength and pH. In details,

Meneses *et al*. (2011) show pH-changes up to 4.04 units already after a treatment time of 34 µs at electric field strength of 10 kV/cm that affect PPO activity.

Cold plasma

Plasma can be described as the "fourth state of matter", generated by applying energy in the form of heat, voltage or electromagnetic fields to a gas, and leading to reactions such as ionisation, excitation and dissociation (Figure 1.11).

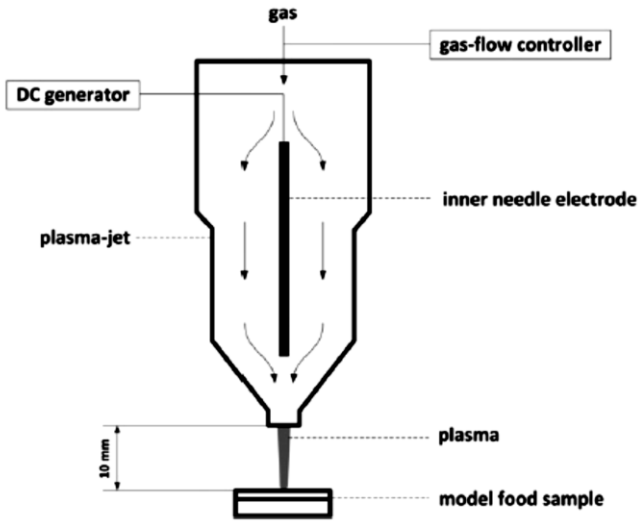


Figure 1.11 Experimental set-up of the cold atmospheric pressure plasma device and model food sample (Surowsky *et al*.; 2013).

During cold plasma treatment, various active components are formed including UV radiation, charged particles and radicals (Laroussi & Leipold, 2004).

In details, the reactive oxygen species (ROS), e.g. molecular oxygen or OH radicals, attack part of the cell membrane thus starting oxidative reactions and leading to the disintegration of unsaturated lipids into lipid peroxides. Other targets can be amino acids like tryptophan, which are sensitive to oxidation, as well as the DNA (Mogul *et al*., 2003). The aromatic amino acids tyrosine, tryptophan and phenylalanine can be found in PPO. In particular, tryptophan emits light in the wavelength range between 300 and 350 nm after excitation at 280 nm. Changes in tryptophan fluorescence can be used as an indicator of oxidation and subsequent conformational changes of protein structures (Gießauf *et al*., 1995; Vivian & Callis, 2001). It is also suggested that plasma immanent species lead to \C\C\ and/or \C\H bond breaking reactions, resulting in the formation of carboxyl and carbonyl groups (Grzegorzewski *et al*., 2010). For the generation of cold plasmas, corona discharges, dielectric barrier discharges and atmospheric pressure plasma jets are common setups (Ehlbeck *et al*., 2010).

The cold plasma technology is effective not only for sterilization (Mishra *et al*., 2014) but also for enzyme inactivation in food. In this regard, Surowsky *et al*. (2013) shows that a treatment with cold plasma composed of Argon and 0.1% oxygen is capable of decreasing PPO activity until 70% after 60 seconds and 90% after 180 seconds in the model food.

UV technology

Ultraviolet light (UV) radiation is classified into three types (Koutchma *et al*., 2007; Bolton 2010): UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). The latter, which shows the greatest microbicide properties (Bintsis *et al*., 2000), is recognized as a simple, eco-friendly way to destroy the most of microorganisms in whole and fresh-cut produce (Allende & Artés, 2003; Allende *et al*., 2006; Birmpa *et al*., 2013). Moreover, the UV-C irradiation with germicidal effect has been widely applied in several agro-food processes and products (Falguera *et al*., 2011a): the air disinfection in meat or vegetables processing (Xu *et al*., 2003; Josset *et al*., 2007); the sterilization of drinking water (Sommer *et al*., 2000; Sutton *et al*., 2000; Hijnen *et al*., 2006) and water waste (Whitby *et al*., 1993; Blatchely *et al*., 1996; Braunstein *et al*., 1996; Oppenheimer *et al*., 1997; Taghipour, 2004); the sterilization of materials for aseptic processing and packaging (Ozen & Floros, 2001; Marquis & Baldeck, 2007); the inhibition of microorganisms on the surface of fresh products e.g. chicken, fish, eggs (Wong *et al*. 1998; Liltved & Landfald, 2000; Hadjock *et al*., 2008) and in liquid food e.g. milk (Matak *et al*., 2004, 2005), liquid egg (Geveke *et al*., 2008) fruit juices (Oteiza *et al*., 2005) or cider (Duffy *et al*., 2000; Basaran *et al*., 2004; Quintero-Ramos *et al*., 2004).

However, in liquid food, the low penetration of UV irradiation, which is associated to the type of fluid and the presence of solutes, can limit the efficacy of this non-thermal treatment (Falguera *et al*., 2011b). In details, a loss of radiation intensity around 30% has been achieved at 40 cm from the surface in distilled water and at only 5 cm in a 10% sucrose solution (Snowball & Hornsey, 1988). In fruit juices, the most of UV absorption occurs in the first 1 mm from the surface (Sizer & Balasubramaniam, 1999). Moreover, the UV treatment has to take into account the different absorption coefficients of dissolved and suspended solids in liquid foods (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Koutchma, 2009). As regard the major components in apple cider, the main sugars including fructose, sucrose, and glucose have high UV absorbance around 200 nm, while malic and ascorbic acids at wavelengths less than 240 nm and 220 and 300 nm respectively (Fan & Geveke, 2007). Ye *et al.* (2007), studying the different absorption coefficients of commercial apple juices in the range 39.1-7.1 cm^{-1}, show that higher vitamin C contents can increase the UV absorption. Moreover, Koutchma *et al*. (2004), studying the effect

of the concentration in suspended solids of model solutions of caramel and dried apple particles on the absorption coefficient, has found an increased UV absorption due to the light scattering by particles. Thus, the presence of solutes in juices and other liquid foods increases the UV absorption and scattering because of their higher optical density than water and simultaneously decrease the light transmittance compromising the effectiveness of this non-thermal treatment. A good solution may be to use a continuous operation in order to ensure that the fluid flows with high turbulence (Koutchma *et al*. (2004), Keyser *et al*., 2008; Falguera *et al*., 2011). Moreover, the choice of the most appropriate light source including incandescent and halogen lamps, arches of carbon and plasma, high intensity discharge lamps (mercurium, sodium, xenon) can optimize the UV treatment (Koutchma, 2009; Falguera *et al*., 2011a).

The UV irradiation is effective not only on microorganisms but also on toxins and enzymes (Falguera *et al*., 2011b). In this regard, several studies have confirmed the inhibitory potential of UV-C on PPO enzyme in model systems and apple derivatives (Manzocco *et al*., 2009; Sampedro & Fan, 2014) and in fresh apple and grape juices (Falguera *et al*., 2011b; Müller *et al*., 2014). Unfortunately, the potential for UV-C treatment is limited because of possible adverse effects in food including the alteration of sensory quality attributes such as colour (Refsgaard *et al*., 1993; Manzocco *et al*., 2008), the reduction of vitamin C (Tran & Farid, 2004) and antioxidant capacity (Li *et al*., 2014) and the formation of furan recognized by the WHO as a potential human carcinogen (Fan & Geveke, 2007; Bule *et al*., 2010; Müller *et al*., 2013; WHO, 2011).

However, UV-C light is not the only effective range in limiting enzymatic browning. As this regard, irradiation for 120 min with a high-pressure mercury lamp of 400 W emitting UV-visible light between 250 and 740 nm (maximum power of emission from 400-450 nm) effectively inactivate PPO in juices from both apples (Falguera *et al*., 2011b) and pears (Falguera *et al*., 2014). Furthermore the treatments show no alterations on pH, the formol index or the content in soluble solids, total phenolics or sugars.

Anyway, the best performance of UV treatment have been achieved mainly at increasing irradiance and exposure time as demonstrated by Müller *et al*. (2014), studying the effect of UV-C (36 W low pressure mercury lamp with maximum peak radiation at 253.7 nm) and UV-B (18.3 W lamp with maximum emission between 290 and 315 nm) on PPO activity in model system and in apple and grape juices, and by Manzocco *et al*.(2009), studying the effect of UV-C (15 W lamps with maximum emission at 253.7 nm) and visible light (fluorescent tubes with maximum emission from 430-560 nm) treatments on PPO activity in model systems and Golden Delicious apples at 28 °C. The same authors report that the UV-C irradiation has been more effective than

visible light in the inactivation of PPO in an aqueous solution showing inhibitions of 40% and 100% after 60 min exposure to 3.9 Wm^{-2} and 13.8 Wm^{-2} irradiance respectively. Contrastingly the visible light treatment, where exposure time was in the order of hours, has been effective only at high doses (12.7 Wm^{-2}). In fact lower irradiances (11.7 and 9.4 Wm^{-2}) lead to an initial PPO activation and only inactivation with increasing exposure time.

Kwak *et al*., (2004) hypothesize the mechanism of action for the anti-browning effect of UV-visible treatment might stem from the degradation of melanoidins, the polymeric brown compounds that result from PPO activity. Ibarz *et al*., (2005) has corroborated the hypothesis studying the effect of UV-visible irradiation in apple, peach and lemon juices whose increased brightness has been attributed to the photochemical destruction of brown pigments.

Moreover, the PPO inactivation via visible and UV light exposure has been associated mainly with direct photo-oxidation arising from the absorption of light by amino acid residues (Trp, Tyr, His, Phe, Met, Cys), the resulting protein denaturation and the formation of high molecular weight aggregates (Davies & Truscott, 2001; Davies, 2003; Lante *et al*., 2013) analyzed by HPLC gel permeation by Manzocco *et al*. (2009).

1.6 PPO inhibition by chemical treatment

1.6.1 Classification of chemical inhibitors

Most of the strategies for controlling the enzymatic browning focus on chemical methods to inhibit PPO activity by eliminating the essential components for reaction such as oxygen, copper ion, substrate or even the enzyme itself (Queiroz *et al*., 2008).

The PPO inhibitors are classified into six main groups (Chang, 2009):

- reducing agents: compounds (e.g. ascorbic acid) that act indirectly on enzyme by reducing *o*-quinones, thus avoiding the formation of melanins;
- *o*-quinone scavengers: compounds (e.g. sulphur containing compounds) that react with *o*-quinones to form colourless products;
- alternative enzyme substrates: compounds (e.g. some phenolic compounds) that show a good affinity for the enzyme, thus preventing the formation of dark pigments;
- nonspecific enzyme inactivators: compounds (e.g. acids or bases) that non-specifically denature the enzyme, thus inhibiting its activity;
- irreversible inhibitors ("suicide substrates"): compounds that inhibit the enzyme irreversibly by the formation of a covalent bond during the catalytic reaction (Figure 1.12);
- reversible inhibitors ("true inhibitors"): compounds that reversibly bind to the enzyme thus reducing its catalytic capacity.

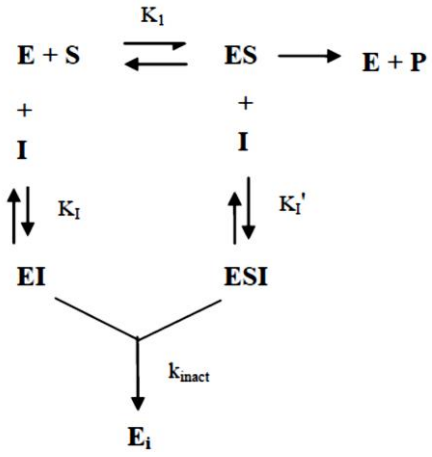


Figure 1.12 Action mechanism of irreversible inhibitors. E and Ei are the enzyme and the inactivated enzyme, respectively; S, I, and P are the substrate, inhibitor, and product, respectively; ES, EI and ESI are the intermediates.

The latter group, which includes the major PPO inhibitors, is classified once again into four types (Figure 1.13):

- competitive inhibitors: compounds that bind to the active site of the free enzyme by preventing the binding with the substrate;
- uncompetitive inhibitors: compounds that react only with enzyme-substrate complex;
- mixed inhibitors: compounds that bind free enzyme or the enzyme-substrate complex;
- non-competitive inhibitors: compounds that react equally with free enzyme or with enzyme-substrate complex.

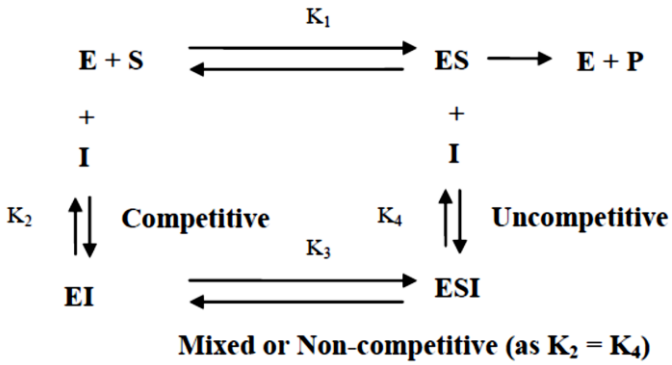


Figure 1.13 Action mechanism of reversible inhibitors. E, S, I, and P are the enzyme, substrate, inhibitor, and product, respectively; ES is the enzyme-substrate complex; EI and ESI are the enzyme-inhibitor and enzyme-substrate-inhibitor complexes, respectively (Chang, 2009).

The anti-browning effectiveness depends mainly on inhibitor type and concentration, identified with IC_{50} index (inhibitor concentration providing 50% inhibition of enzyme activity), and PPO source.

1.6.2 Traditional food additives

Reducing agents

The reducing agents, that include the main food additives, indirectly inhibit PPO by reducing *o*-quinones to colourless diphenols e.g. ascorbic acid or reacting irreversibly with *o*-quinones to form colourless compounds e.g. sulphur containing compounds (Figure 1.14).

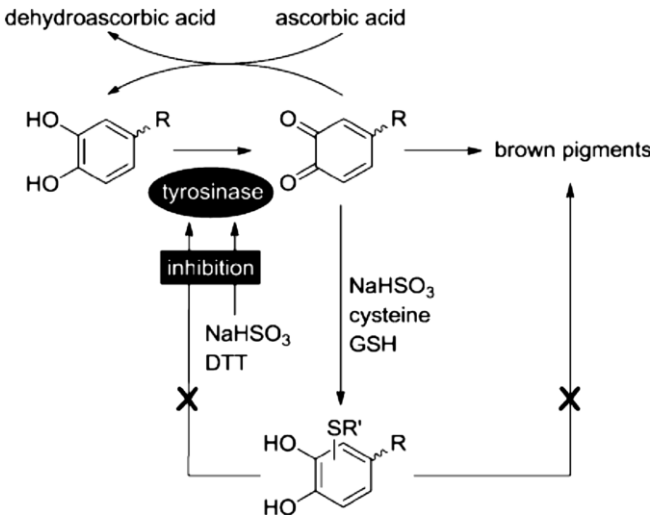


Figure 1.14 Action mechanism of reducing agents (Kuijpers *et al*., 2012).

The **L-ascorbic acid** (Figure 1.15) acts as strong antioxidant and anti-browning agent (Altunkaya & Gökmen, 2008; Queiroz *et al*., 2011; Sun *et al*., 2012).

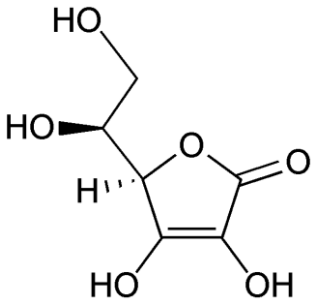


Figure 1.15 Chemical structure of L-ascorbic acid.

It inhibits PPO by kidnapping copper ion in the enzyme active site (Sapers & Miller, 1998) and mainly by reducing coloured *o*-quinones to colourless diphenolic substrates (Kuijpers *et al*., 2012) as described in Figure 1.16. Ali *et al.* (2015) report that the reducing capacity of ascorbic acid can be related to its concentration. In details, it reduces the formed quinone at high concentration (>1.5%), while at lower concentrations acts as competitive inhibitor (Ki = 0.26 ± 0.07 mM).

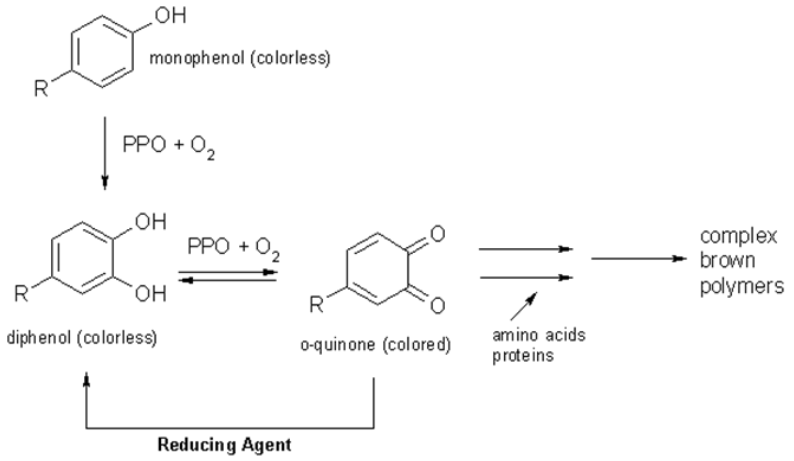


Figure 1.16 Action mechanism of ascorbic acid as reducing agent.

Anyway, the action mechanism of ascorbic acid as reducing agent is responsible of the accumulation in *o*-diphenols, which will indirectly lead to the activation of oxidative reactions because of the low stability of ascorbic acid in aqueous solutions (Ros *et al*., 1993). To solve this problem, derivatives compounds (Hsu *et al*., 1999). Among ascorbic acid derivatives, the ascorbic-2.phosphate shows the highest anti-browning potential on apple slices (Son *et al*., 2001) The magnesium-L-ascorbil-2-phosphate (MAF) is stable in aqueous solutions reducing PPO activity. However its use is limited mainly to the dermatological field by controlling the activity of human TYR and subsequent melanogenesis (Kameyama *et al*., 1996; Curto *et al*., 1999).

In agro-food industry, ascorbic acid is often combined with other chemical inhibitors. It show a strong synergistic effect with citric acid (Pizzocaro *et al*., 1993), oxalic acid (Son *et al*., 2001), cysteine and cinnamic acid (Özoğlu & Bayındırlı, 2002), 4-hexylresorcinol (Luo & Barbosa-Cánovas, 1997; Guerrero-Beltrán *et al*., 2005).

The **sulphur containing compounds** including sulphites, cysteine, glutathione and Mailard reaction products strongly inhibit enzymatic browning (Eissa *et al*., 2006; Kuijpers *et al*., 2012) by irreversibly reacting with *o*-quinones to form colourless compounds (Figure 1.14).

The **sulphiting agents** such as sulphite salts and sulphur dioxide (SO_2) can be formed in aqueous solutions or in foods by reacting with carbohydrate, protein and lipid molecules (Vally *et al*., 2009). In aqueous solutions they are subjected to a pH-dependent equilibrium (Figure 1.17). At low pH, the equilibrium leads to sulphurous acid (H_2SO_3), at intermediate pH towards bisulphite ions (HSO_3^-), while at high pH the formation of sulphite ions (SO_3^{2-}) is favoured. The proportion of free and bound sulphites varies in different foods and depends on the temperature, pH, macromolecular composition of the food and the concentration in sulphites Vally *et al*., 2009).

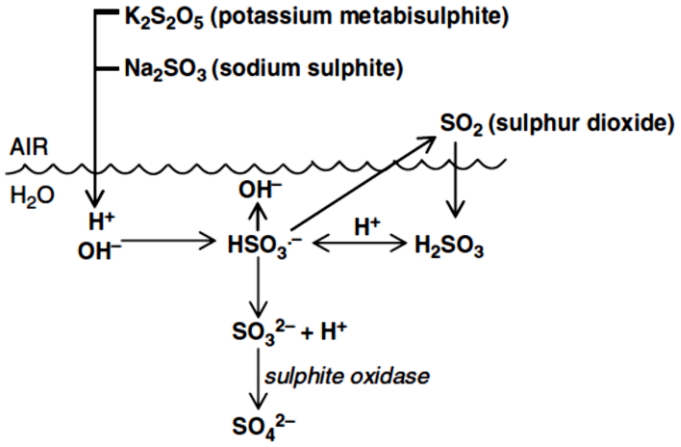


Figure 1.17 Sulphites (Vally *et al*., 2009).

SO_2, which is added to machine-harvested grapes and to wine after malolactic fermentation is one of the most versatile and efficient additives used in winemaking because of its antiseptic and antioxidant properties (Bartowsky, 2009; Oliveira *et al*., 2011). In addition, it prevents the wine browning by inactivating enzymes such as polyphenol oxidase, peroxidase and proteases and also by inhibiting Maillard reaction (Ribéreau-Gayon *et al*., 2006a, 2006b). Eissa *et al*. (2006) report an anti-browning effect of sulphites similar to 4-hexyl resorcinol and higher than ascorbic acid. Madero & Finne (1982) report a competitive effect of bisulphite on PPO by binding the sulfhydryl groups of the enzyme active site. Instead, Ferrer *et al*., (1989) show that bisulphite acts as reducing agent by reacting with intermediate quinones forming sulfoquinones and by irreversibly inhibiting PPO. The concentration in sulphites for controlling enzymatic browning varies according to the food and the time required for inhibiting PPO (Taylor *et al*., 1986). In details, potato, rich in monophenolic substrates such as tyrosine, require a low amount of sulphites, while avocado, rich in diphenolic substrates, need higher quantities.

Moreover, the anti-browning effectiveness depends also on the sulphite type, concentration and PPO source. In this regard, Queiroz *et al*. (2011), studying the effect of some sulphites on cashew apple PPO, show that sodium sulphite completely inhibits enzyme activity at the concentration of 1 mM, while sodium metabisulphite at 2.5 mM. The latter has been effective on Anamura banana PPO at lower concentration equal to 0.01 mM (Ünal, 2007). Among sulphites, sodium hydrogen sulphite ($NaHSO_3$) simultaneously acts as reducing agent by irreversibly reacting with *o*-quinones to form colourless addition products (sulfochlorogenic acid) and inhibitor of mushroom tyrosinase in a time-dependent way (Kuijpers *et al*., 2012).

However, the sulphites content in foods and drinks has been restricted because of potential health hazards ranging from dermatitis, urticaria, flushing, hypotension, abdominal pain and

diarrhoea to life-threatening anaphylactic and asthmatic reactions in sensitive individuals after topical, oral or parenteral exposure (McEvily *et al*., 1992; Timbo *et al*., 2004; Rangan & Barceloux, 2009; Vally *et al*., 2001, 2009; Oliphant *et al*. 2012; Stohs & Miller 2014).

The International Organization of Vine and Wine (OIV) has reduced the maximum concentration authorized in wines, which is nowadays 150 mg/L for red wines and 200 mg/L for white wines (Regulation (EC) No 607/2009). The World Health Organization (WHO) and Food and Agricultural Organization of the United Nations (FAO) have set the "Codex General Standard for Food Additives" (GSFA, Codex STAN 192-1995; last revision in 2015 year) that has been adopted by the Codex Alimentarius Commission. The maximum sulphites levels (sulphur dioxide, sodium sulphite, sodium hydrogen sulphite, sodium metabisulphite, potassium metabisulphite, potassium sulphite, calcium hydrogen sulphite, potassium bisulphite, sodium thiosulfate) in some foods and drinks are reported in Table 1.4.

Table 1.4 Maximum levels of sulphites in some foods and drinks (Codex Alimentarius, 2015).

Food category	Max level	Year adopted
Peeled, cut or shredded fresh and vegetables*, seaweeds, and nuts and seeds	50 mg/kg	2006
Frozen vegetables*, seaweeds, and nuts and seeds	50 mg/kg	2006
Dried vegetables*, seaweeds, and nuts and seeds;	500 mg/kg	2006
Herbs and spices	150 mg/kg	2006
Vegetables* and seaweeds in vinegar, oil, brine, or soybean sauce and seaweeds in vinegar, oil, brine, or soybean sauce	100 mg/kg	2006
Canned or bottled (pasteurized) or retort pouch vegetables* and seaweeds	50 mg/kg	2006
Vegetables*, seaweed, and nut and seed purees and spreads (e.g. peanut butter)	500 mg/ kg	2006
Vegetable*, seaweed, and nut and seed pulps and preparations (e.g., vegetable desserts and sauces, candied vegetables)	30 mg/kg	2011
Vegetable nectar	50 mg/kg	2006
Surface-treated fresh fruit	30 mg/kg	2011
Frozen fruit	500 mg/kg	2007
Dried fruit	100 mg/kg	2011
Fruit preparations, including pulp, purees, fruit toppings and coconut milk	100 mg/kg	2012
Jams, jellies, marmalades	100 mg/kg	2008
Candied fruit	100 mg/kg	2006
Fruit juice	50 mg/kg	2005
Cider and perry	200 mg/kg	2006
Grape wines	350 mg/kg	2006
Snacks - potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	50 mg/kg	2006
Fresh and frozen fish including molluscs, crustaceans, and echinoderms	100 mg/kg	2006

* including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera.

Moreover, the excessive use of SO_2 may affect not only the human health but also the wine quality because of unpleasing flavours and aromas and turbidity during storage (Li *et al*., 2008).

Even if the complete replacement of SO_2 remains unsuccessful, some studies suggest a reduction of SO_2 by combining with another hurdle treatment based on the application of chemical compounds and non-thermal technologies (Li *et al*., 2008; Bartowsky, 2009; Oliveira *et al*., 2011; Santos *et al*., 2012; Falguera *et al*., 2013).

The c**ysteine** (Figure 1.18)**,** which is a nonessential amino acid found in a wide range of agro-food products including cereals, show a strong PPO inhibition (Altunkaya & Gökmen 2008) with a low IC_{50} value equal to 2 mM in the catechol-mushroom PPO system (Son *et al*., 2000).

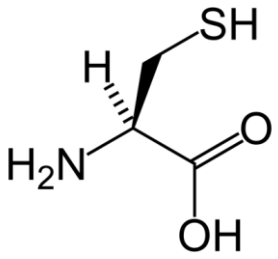


Figure 1.18 Chemical structure of cysteine.

Several studies confirm that this sulphur amino acid not act directly on PPO structure but on *o*-quinones to give colourless adducts (Friedman & Molnar-Perl, 1990; Richard-Forget *et al*., 1992; Friedman & Bautista, 1995; Ding *et al.*, 2002; Peñalver *et al*., 2002; Garcia-Molina *et al*., 2005). The sulfhydryl group of cysteine forms a sulphide adduct, as a result of a nucleophilic addition reaction with quinones, which inhibits the formation of dark compounds (Figure 1.14). In fact, this addition compound known as cysteine-quinone (CQAC) acts as a competitive inhibitor showing more affinity for the PPO active site. Richard-Forget *et al*. (1992) report that the anti-browning effect of CQAC is related mainly to the initial concentration of cysteine and the ratio cysteine/polyphenols. If the ratio cysteine/polyphenols is higher there is no browning because all polyphenols are degraded in CQAC. Instead if the ratio cysteine/polyphenols is lower there is a partial conversion of *o*-quinones in the sulphide adduct; as a consequence, the free *o*-quinones will react with CQAC regenerating polyphenols by coupled oxidation and producing brown compounds (Figure 1.19).

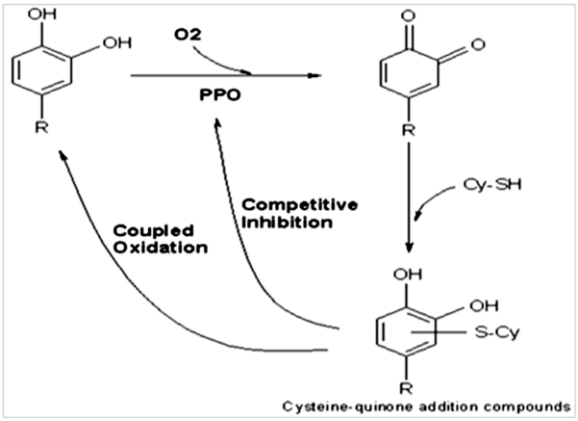


Figure 1.19 Effect of cysteine and cysteine-quinone addition compound (CQAC) on the enzymatic oxidation of *o*-diphenols (Marshall *et al.*, 2000).

Ali *et al.* (2015) have investigated the browning inhibition mechanisms by cysteine in the catechol-lettuce PPO system showing that, at higher concentrations (≥1.0 %), the sulphur amino acid reacts with the formed quinone to give colourless products, while, at lower concentrations, it acts as competitive inhibitor (K_i =1.11±0.18 mM).

On the other hand, high cysteine contents may negatively affect the organoleptic properties of food products producing unpleased off-flavour (Mathew & Parpia, 1971).

The **glutathione** (GSH, Figure 1.20), whose application as food additive is permitted in China, is effective on enzymatic browning (Jiang & Fu, 1998) by reacting irreversibly with o-quinones to form colourless compounds (Figure 1.14). Its anti-browning effectiveness has been demonstrated on the meat of *Clanis bilineata* (Wu, 2013), mushroom slices (Xia, 2013), apple slices (Son *et al*., 2001) litchi fruit (Jiang & Fu, 1998) and white wines (El Hosry *et al*., 2009). Wu (2014) report a 99.4% inhibition of PPO activity in grape juice treated with 0.04% glutathione during processing and accelerated browning.

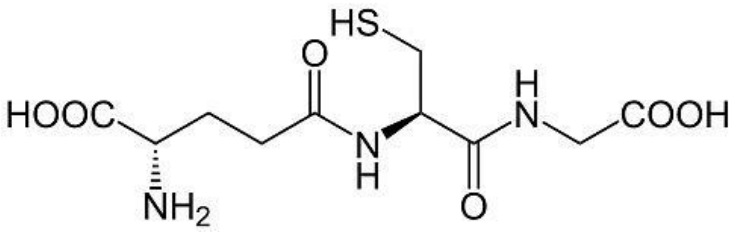


Figure 1.20 Chemical structure of glutathione.

Also the **Maillard reaction products** (MRPs), which derived from non-enzymatic browning reactions in heated products containing a sugar-amino acid combination (Manzocco *et al*., 2011), can effectively control the enzymatic browning thanks to their reducing (Figure 1.14), chelating and oxygen-scavenging properties (Roux *et al*., 2003) .

Billaud *et al*., 2003, studying the effect of L-cysteine, D-glucose, D-fructose aqueous solutions and equimolar mixtures (1 M) of hexose/cysteine on apple PPO as a function of temperature (80-110 °C), heating time (0-48 h) and various amounts of reagents, show an enhanced inhibitory efficacy at increasing times and temperatures and decreasing concentrations in hexoses. Moreover, Roux *et al*. (2003), investigating the action mechanism of the previously MRPs, define a mix-type inhibition and show a greater effectiveness of the glucose/cysteine model solution. The MRPs derived from glucose/cysteine model system were also more effective than those derived from ribose/cysteine model system in controlling the enzymatic browning of Red delicious apple slices and pulp (Eissa *et al*., 2006).

Generally, the inhibitory potential of MRPs varies according to the type and concentration of amino acids and sugars and the type of phenolic substrate as widely demonstrated by Lee & Park (2005) on potato PPO.

Acidulants

The acidulants are nonspecific enzyme inactivators by lowering the pH below the optimum value of PPO that varied depending on the enzyme source (Yoruk & Marshall, 2003). Polycaboxylic acids e.g. citric, tartaric, and malic and succinic acids act as PPO activity either by lowering the pH or chelating the copper at the enzyme active site (Sedaghat & Zahedi, 2012). Among organic acids, oxalic, tartaric, citric and malic acids show strong anti-browning performance, while fumaric and succinic acids the lowest one (Son *et al*., 2001).

Citric acid (Figure 1.21) is the most widely used organic acid in agro-food industry especially in combination with ascorbic acid (Pizzocaro *et al*., 1993). The inhibition of citric acid has been attributed mainly to its capability of unfolding the conformation of enzyme structure (Liu *et al*., 2013) and consequently decreasing enzyme activity (Queiroz *et al*., 2011; Sun *et al*., 2012). Ali *et al*. (2015) report a PPO non-competitive inhibition with K_i equal to 2.07±0.36 mM.

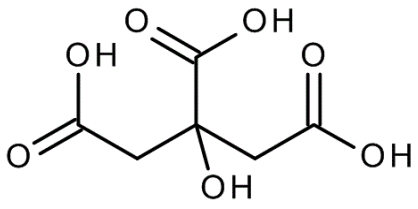


Figure 1.21 Chemical structure of citric acid.

Among organic acids, **oxalic acid** (Figure 1.22) has the strongest anti-browning potential (Son *et al*., 2001) with IC_{50} value (1.1 mM) lower than that of citric acid (150 mM) and tartaric acid (200 mM) and also cysteine (2.0 mM) and glutathione (3.7 mM) in the catechol-mushroom PPO system (Son *et al*., 2000). Son *et al*. (2000) define a competitive inhibition on catechol-mushroom PPO system with a K_i value of 2.0 mM.

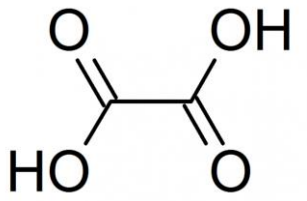


Figure 1.22 Chemical structure of oxalic acid.

Complexing agents

EDTA (Figure 1.23) inhibits PPO activity by chelating the copper ion in the active site. The best inhibitory effect is obtained when the carboxylic groups are in a dissociated state at high pH values (Dziezak, 1986).

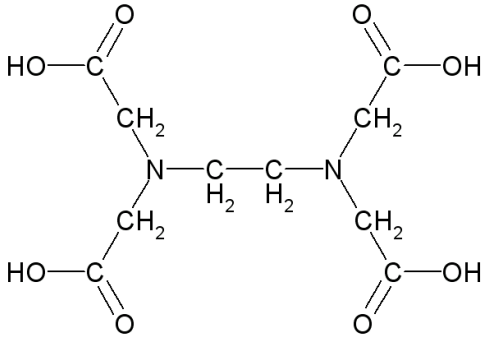


Figure 1.23 Chemical structure of EDTA.

Cyclodextrins (Figure 1.24) are cyclic oligosaccharides formed during the bacterial digestion of starch (Astray *et al*., 2009). It consists of six, seven or eight glucopyranose units that are linked by 1-4 glycosidic bonds forming various cylindrical structures known as α,β,γ-cyclodextrins (CDs) (Nunez-Delicado *et al*., 2005).

The core is hydrophobic while the outside structure is hydrophilic. The hydrophobic core can form complexes with several molecules, including phenolic substrates, thus preventing their oxidation to quinones and the consequent formation of brown pigments (Cai *et al*., 1990; Irwin *et al*., 1994). The CDs are effective in the control of enzymatic browning in apple products (Irwin *et al*., 1994; Hicks *et al*., 1996; Pilizota & Subaric, 1998; Gacche *et al*. 2003; Ozoglu & Bayindirli, 2004;). López-Nicolás *et al*. (2007a, 2007) show The anti-browning effectiveness of

α,β,γ-CDs and maltosyl-β-CD has been demonstrated also on fresh apple (López-Nicolás *et al*., 2007a), banana (López-Nicolás *et al*., 2007b), peach (López-Nicolás *et al*., 2007c) and pear (López-Nicolás *et al*., 2007d) juices. Moreover, the maltosyl-β-CD can enhance the ability of ascorbic acid to prevent the enzymatic browning due to its protective effect against ascorbic acid oxidation as secondary antioxidant. The β-cyclodextrin in combination with ascorbic and citric acids improves the quality of precooked vacuum packed potatoes by limiting the browning surface (Lante & Zocca, 2010).

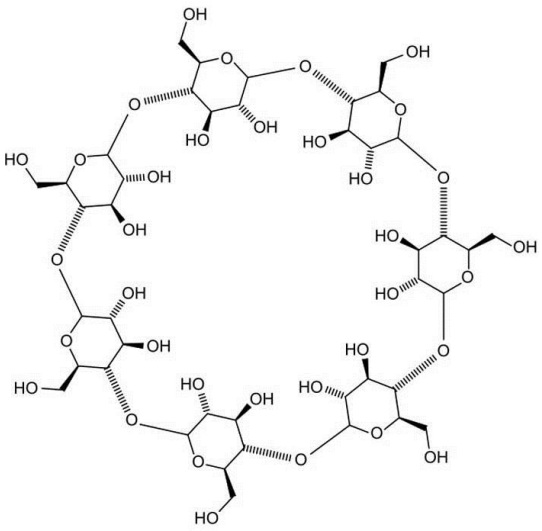


Figure 1.24 Chemical structure of cyclodextrines.

1.6.3 Anti-browning formulations for fresh-cut fruits and vegetables

Edible coatings and dipping treatments are the main ways to apply PPO inhibitors to fresh-cut products (Rojas-Graü *et al*., 2009; Oms-Oliu *et al*., 2010).

Edible coatings may contribute to extend the shelf-life of fresh-cut products by reducing moisture and solute migration, gas exchange, respiration and oxidative reaction rates, as well as by reducing or even suppressing physiological disorders (Rojas-Graü *et al*., 2009). Their main advantage is that several active ingredients such as antimicrobial and antioxidant compounds can be incorporated into the polymer matrix and consumed with the food, thus enhancing safety or even nutritional and sensory attributes. Several studies report various edible coatings based on the incorporation of anti-browning agents for fresh-cut fruits (Table 1.5).

Table 1.5 Edible coatings containing anti-browning agents for some fresh-cut fruits (Oms-Oliu *et al*., 2010).

Fresh-cut fruits	Coating materials	Anti-browning agents	References
Apple	Apple puree/pectin alginate	0.5% AA + 0.5% CA	McHugh & Senesi (2000)
		2.5% MA + 1% NAC + 1% GSH + 0.7% lemongrass or 0.3% cinnamon oil + 2% CaL	Raybaudi-Massilia *et al*. (2008a)
	WPC	1% AA+1% $CaCl_2$	Lee *et al.* (2003)
	Alginate/gellan alginate/apple puree	1% NAC + 2% $CaCl_2$	Rojas-Graü *et al*. (2008b)
		1% NAC + 2% $CaCl_2$ + 0.3–0.6% vainillin or 1–1.5% lemongrass or 0.1–0.5% oregano oil	Rojas-Graü *et al*. (2008a)
	WPC-BW	1% AA or 0.5% cys	Perez-Gago *et al*. (2006)
	Alginate/pectin/methylcellulose	1% AA + 0.5% CA + 0.25% $CaCl_2$	Wong *et al*. (1994)
Melon	Alginate	2.5% MA + 0.3% palmarosa oil + 2% CaL	Raybaudi-Massilia *et al*. (2008b)
	Alginate/pectin	2% $CaCl_2$	Oms-Oliu *et al*. (2008a)
Papaya	Alginate/gellan	1% AA+2% $CaCl_2$	Tapia *et al.* (2008)
Pear	Methylcellulose Alginate/pectin	1% AA + 0.1% PS + 0.25% $CaCl_2$	Olivas *et al.* (2003)
		0.75% NAC + 0.75 GSH + 2% $CaCl_2$	Oms-Oliu *et al*. (2008b)
Pineapple	Alginate	1% CA+1% AA+2% $CaCl_2$	Montero-Calderón *et al.* (2008)

BW: beeswax. AA: ascorbic acid. MA: malic acid. CaCl2: calcium chloride. CaL: calcium lactate. CA: citric acid. GSH: glutathione. NAC: N-acetylcysteine. cys: cysteine. PS: potassium sorbate. WPC: whey protein concentrates.

Dipping treatments after peeling and/or cutting are effective not only for microbial inactivation (Martín-Belloso *et al*., 2006) but also for PPO inhibition. In this regard, the most common commercial anti-browning formulation for fresh-cut products is a mixture of calcium salts with ascorbic acid that act respectively to keep cell structure integrity and to control PPO activity (Pizzocaro *et al*., 1993; Gorny *et al*., 1999; Soliva-Fortuny *et al*., 2001, 2002a, 2002b; Rupasinghe *et al*. 2005). As reported by Lante & Zocca (2010), dipping potato slices into β-cyclodextrin can improve the brightness of precooked, vacuum-packed potatoes and may be useful for other minimally-processed products.

Some thiol-containing substances, for example N-acetylcysteine and reduced glutathione have been proposed as browning inhibitors for apple, potato (Molnar-Perl & Friedman, 1990a; Friedman *et al*., 1992; Rojas-Grau *et al*.,2006).

Carboxylic acids have been usually used thanks to their anti-browning activity. Citric acid displays a double inhibitory effect by reducing pH and chelating copper in the active site of PPO (Son *et al*., 2001). Furthermore, oxalic acid and oxalacetic acid have a higher anti-browning potential. In details, the immersion of banana and apple slices in oxalic acid solutions results effective on enzymatic browning (Son *et al*., 2001; Yoruk *et al*., 2004).

Among resorcinol derivatives the 4-hexylresorcinol has been proved to be effective in controlling browning on fresh-cut apples and pears (Monsalve-Gonzalez *et al*., 1993; Dong *et al*., 2000; Son *et al*., 2001; Oms-Oliu *et al*., 2006; Rojas-Grau *et al*., 2006) Its anti-browning effectiveness can increase especially in combination with reducing agents (Monsalve-Gonzalez *et al*., 1993; Luo & Barbosa-Canovas, 1997; Dong *et al*., 2000; Arias *et al*., 2008). Another possible combination with sodium erythorbate has been suggested to prevent fresh-cut pears from enzymatic browning (Sapers & Miller, 1998).

Table 1.6 Dipping treatments with anti-browning agents for some fresh-cut fruits (Oms-Oliu *et al*., 2010).

Fresh-cut fruits	Anti-browning agents	References
Apple	0.5% CaL	Alandes *et al*. (2006)
	0.001M HR+0.5M IAA+0.05M CaP 0.025M cys	Buta *et al*. (1999)
	7% CaA	Fan *et al*. (2005)
	0.01% HR + 0.5% AA	Luo and Barbosa-Cánovas (1997)
	1% AA + 0.2% CA or 0.5% NaCl	Pizzocaro *et al*. (1993)
	4% CaP	Quiles *et al*. (2007)
	1% NAC + 1% GSH + 1% LCa	Raybaudi-Massilia *et al*. (2007)
	0.75% AA + 0.75% $CaCl_2$	Rocha *et al*. (1998)
	1% AA + 0.5% $CaCl_2$	Soliva-Fortuny *et al*. (2001)
	0.05% kojic acid	Son *et al*. (2001)
	0.5% AA + 1% $CaCl_2$ + 0.1%PA	Varela *et al*. (2007)
Banana	0.5M CA+0.05M NAC	Moline *et al*. (1999)
Kiwifruit	1% $CaCl_2$ or 2% CaL	Agar *et al*. (1999)
Peach	2% AA + 1% CaL	Gorny *et al*. (1999)
Pear	2% AA + 0.01% HR + 1% $CaCl_2$	Arias *et al*. (2008)
	0.01%HR+0.5%AA+1%CaL	Dong *et al*. (2000)
	2% AA + 1% CaL + 0.5% cys	Gorny *et al*. (2002)
	0.75% NAC + 0.75% GSH	Oms-Oliu *et al*. (2006)
	4%NaE+0.2%$CaCl_2$+100ppmHR	Sapers & Miller (1998)
	1% AA + 0.5% $CaCl_2$	Soliva-Fortuny *et al*. (2002)
Mango	0.001M HR + 0.5M IAA	González-Aguilar *et al*. (2000)
	3% $CaCl_2$	Souza de *et al*. (2006)
Melon	2.5% CaL	Luna-Guzmán & Barrett (2000)
	1% AA + 0.5% $CaCl_2$	Oms-Oliu *et al*. (2007)
Watermelon	2% $CaCl_2$	Mao *et al*. (2005)

AA: ascorbic acid. CaA: calcium ascorbate. $CaCl_2$: calcium chloride. CaL: calcium lactate. CaP: calcium propionate. cys: cysteine. CA: citric acid. HR: 4-hexylresorcinol. IAA: isoascorbic acid. NAC: N-acetylcysteine. PA: propionic acid. NaCl: sodium chloride. NaE: sodium eritorbate.

1.6.4 Skin whitening agents

The modulation of melanogenesis is an important strategy for the control of skin hyperpigmentation (Briganti *et al*., 2003) by inhibiting TYR activity (Hearing & Tsukamoto, 1991) with several whitening compounds of natural (Maeda & Fukuda, 1991; Seo *et al*., 2003; Solano *et al*., 2006; Smit *et al*., 2009) or synthetic origin (Ha *et al*., 2011; Han *et al*., 2012; Chung *et al*., 2013).

Among the natural hypo pigmenting agents, kojic acid and arbutin are the most used in cosmetic field (Parvez *et al*., 2006, 2007) although they can exhibit side effects (Maeda and Fukuda, 1991).

Kojic acid (5-hydoxy-4-pyran-4-one-2-methyl; Figure 1.25) is one of the metabolites produced by various fungal or bacterial strains such *Aspergillus* and *Penicillium* and has been used in many countries as a skin-whitening agent because of its tyrosinase inhibitory activity on melanin synthesis (Ohyama & Mishima, 1990; Curto *et al*., 1999; Son *et al*., 2001).

The PPO inhibition by kojic acid depends on the enzyme source. In details, kojic acid partially inhibits the apple and potato PPOs in comparison to fungal TYR (Chen *et al*., 1991a). Moreover, it show a competitive inhibition for apple and potato PPOs and monophenolic substrates (L-tyrosine) while has a mixed-type inhibition for PPO extracted from crustaceans and for diphenolic substrates (L-DOPA). The kojic acid reversibly inhibits enzyme activity by sequestering the oxygen required for the enzymatic browning reactions and by reducing *o*-quinones to *o*-diphenols (Chen *et al*., 1991b). Burdock *et al*. (2001) confirm the inhibitory efficacy of kojic acid by preventing the conversion of *o*-quinone to D,L-DOPA and dopamine to its corresponding melanin. Ha *et al*. (2001) report a mix-type inhibition of kojic acid towards mushroom TYR with IC_{50} value equal to 0.014 mM.

However, its use in cosmetics has been limited because of the skin irritation due to its cytotoxicity and instability on storage. Chen *et al*., 1991b report that it is more effective at low and at room temperatures. Several kojic acid derivatives have been synthesized in order to improve the stability of kojic acid and strongly increase the TYR inhibition (Lee *et al*., 2006; Noh *et al*., 2007).

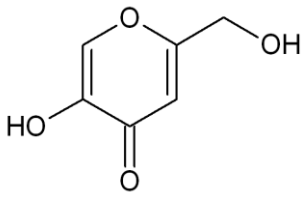


Figure 1.25 Chemical structure of kojic acid.

Arbutin (hydroquinone-O-β-D-glucopyranoside; Figure 1.26) is hydroquinone glycoside isolated from the fresh fruit of the California buckeye, *Aesculus californica* (Kubo & Ying, 1992). Arbutin has two isomers, α and β (Sugimoto *et al*., 2007). Although only β-arbutin is able to inhibit TYR activity, the α-anomer results more effective against melanoma mouse (Funayama *et al.*, 1995). However, Yang *et al*. (1999) report that the control of melanogenesis is mainly due to the inhibition of melanosomal tyrosinase activity rather than the suppression of enzyme's synthesis and expression. The β-arbutin acts as competitive inhibitor of fungal TYR (Tomita *et al*., 1990) with IC_{50} equal to 0.04 mM (Yagi *et al*., 1987).

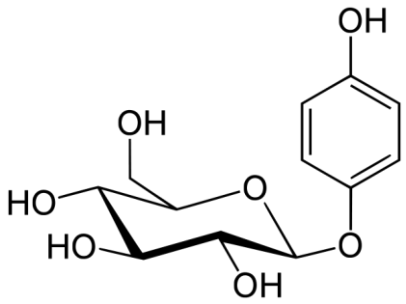


Figure 1.26 Chemical structure of arbutin.

Hori *et al*. (2004) report that the inhibitory action of arbutin could be related to its ability to act as alternative monophenolic substrate by bind the *met*a-TYR form (E_{met}) producing an inactive complex (Emet-arbutin) which represent the dead-end pathway of TYR oxidation avoiding the formation of melanins (Figure 1.27). This approach is supported also by several authors indicating that the monophenolic substrates can react only with *oxy*-PPO (Wilcox *et al*., 1985; Martinez & Whitaker, 1995; Sanchez-Ferrer *et al*., 1995; Seo *et al*., 2003; Kim & Uyama, 2005).

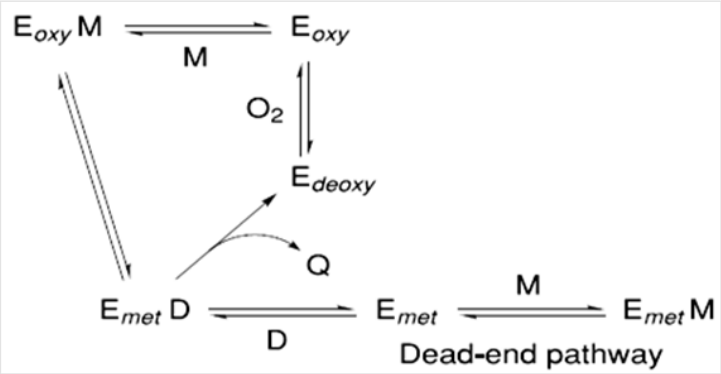


Figure 1.27 Action mechanism of Tyr on monophenolic substrates as arbutin. M: monophenol. D: diphenol. Q: quinone. E_{met}: *met*-TYR. E_{oxy}: *oxy*-TYR. E_{deoxy}: *deoxy*-TYR (Hori *et al*., 2004).

The inhibitory capacity of arbutin depends also on its structural conformation. In fact, the arbutin is not able to completely bind to the hydrophobic proteins in the enzyme active site because of the polar block due to its glucose molecule (Decker *et al*., 2000). Because of the monophenolic substrate has to rearrange in enzyme the active site during hydroxylation step of browning reactions, the glucose molecule may be a hurdle (Wilcox *et al*., 1985).

However, arbutin can be oxidized as a monophenolic substrate extremely slow rate, and the oxidation can be accelerated as soon as catalytic amounts (0.01 mM) of L-DOPA became available as a cofactor. (Hori *et al*., 2004). A possible solution may be the combination of arbutin with L-ascorbic acid, thus synergistically improving the anti-browning effect and avoiding the arbutin oxidation (Hori *et al*., 2004).

1.6.5 Bioactive compounds

Recently, the research of new PPO inhibitors from natural sources (Kim & Uyama, 2005; Chang, 2009; Loizzo *et al*., 2012) including dog rose and pomegranate extracts (Zocca *et al*., 2011) and other plant extracts (Baurin *et al*., 2002; Masuda *et al*., 2005; Wessels *et al*., 2014) is becoming an eco-friendly alternative to thermal treatments and traditional additives, such as ascorbic acid and its derivatives as well as sulphites (Queiroz *et al*., 2011), which have critical points related not only to the organoleptic quality but also to nutritional and health claims (Vally *et al*., 2009).

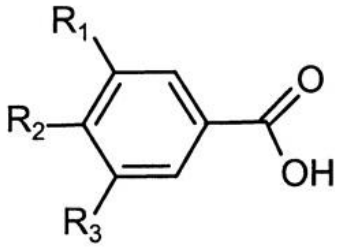
Currently, there is a growing interest in the conversion of agro-food wastes into value-added products throughout a given product/service lifecycle (Laufenberg *et al*., 2003). In this regards, agro industrial wastes and by-products are rich in bioactive compounds (Schieber *et al.*, 2001) not only with strong antioxidant (Moure *et al.*, 2001; Balasundram *et al*., 2006; Wijngaard *et al*., 2009). but also with anti-browning potentials as observed in *Brassicacea* processing water

(Zocca *et al*., 2010), citrus peels (Sasaki & Yoshizaki, 2002; Matsuura *et al*., 2006), and pomegranate peels (Basiri *et al*., 2015).

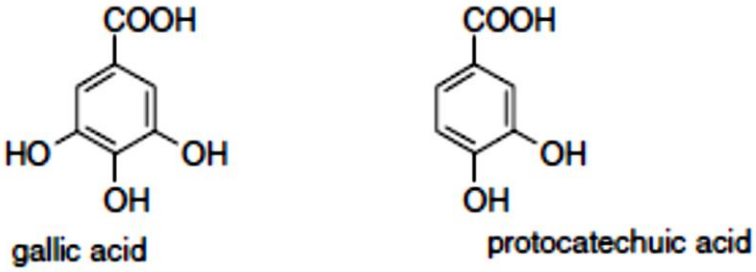
Polyphenols

Polyphenols, which are the main bioactive compounds in fruits and vegetables (Manach *et al*., 2014), are mostly found in the peels usually discarded (Gorinstein *et al*., 2001; Gil *et al*., 2002; Wolfe, *et al*., 2003).

Phenolic compounds, the secondary metabolites from the pentose phosphate, shikimate, and phenylpropanoid pathways in plants, structurally consists of an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Balasundram *et al*., 2006; Quideau *et al*., 2011). On the basis of chemical structure, polyphenols are divided into several classes, where phenolic acids, including hydroxybenzoic (C6-C1; Figure 1.28) and hydroxycinnamic acids (C6-C3; Figure 1.29), and flavonoids (C6-C3-C6; Figure 1.30) are mostly found in various fruits and vegetables by-products (Table 1.7, Table **1.8**).

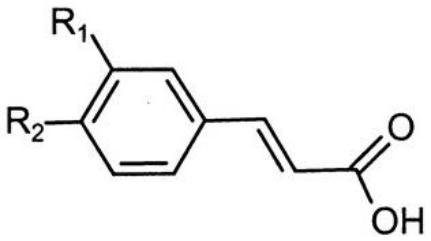


(a)

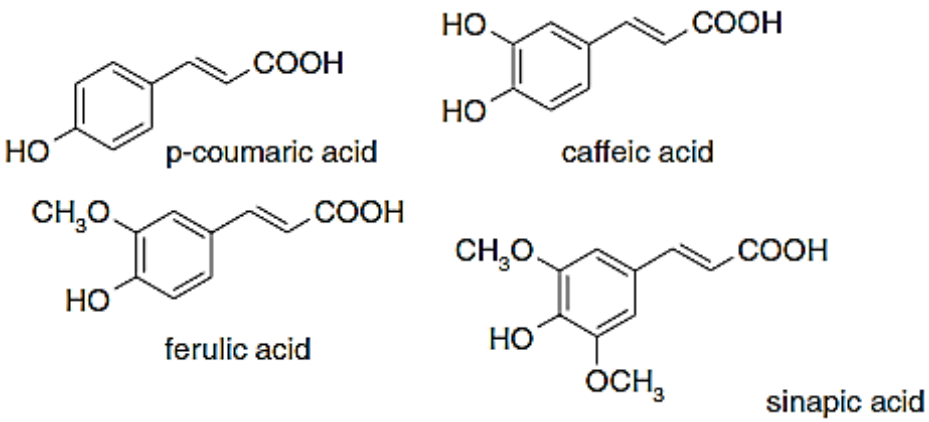


(b)

Figure 1.28 Chemical structure (a) and some examples (b) of hydroxybenzoic acids.

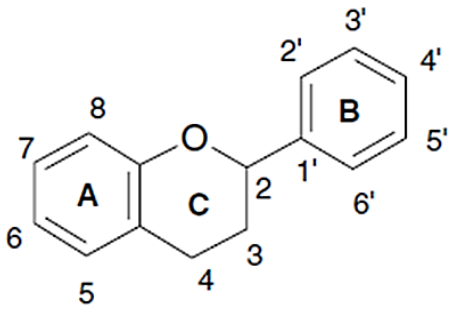


(a)



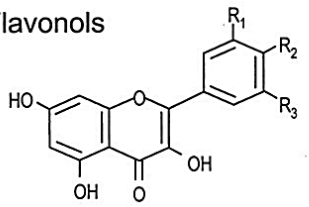
(b)

Figure 1.29 Chemical structure (a) and some examples of hydroxycinnamic acids (b).



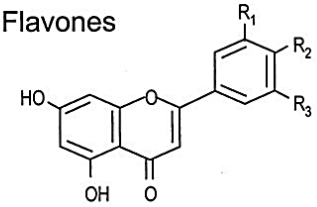
(a)

Flavonols



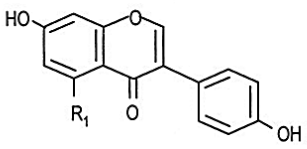
R_2 = OH; R_1 = R_3 = H : Kaempferol
R_1 = R_2 = OH; R_3 = H : Quercetin
R_1 = R_2 = R_3 = OH : Myricetin

Flavones



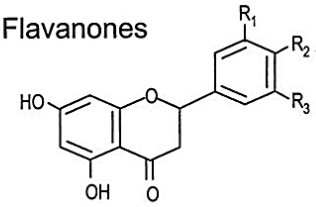
R_1 = H; R_2 = OH : Apigenin
R_1 = R_2 = OH : Luteolin

Isoflavones



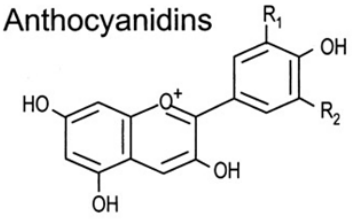
R_1 = H : Daidzein
R_1 = OH : Genistein

Flavanones



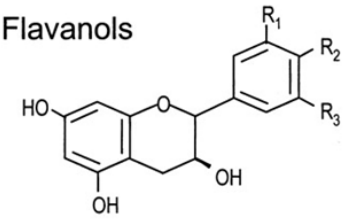
R_1 = H; R_2 = OH : Naringenin
R_1 = R_2 = OH : Eriodictyol
R_1 = OH; R_2 = OCH_3: Hesperetin

Anthocyanidins

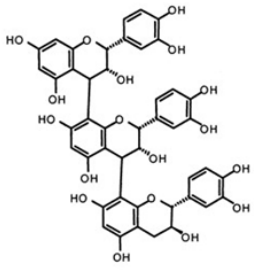


R_1 = R_2 = H : Pelargonidin
R_1 = OH; R_2 = H : Cyanidin
R_1 = R_2 = OH : Delphinidin
R_1 = OCH_3 ; R_2 = OH : Petunidin
R_1 = R_2 = OCH_3 : Malvidin

Flavanols



R_1 = R_2 = OH; R_3 = H : Catechins
R_1 = R_2 = R_3 = OH : Gallocatechin



Trimeric procyanidin

(b)

Figure 1.30 Chemical structure of a flavonoid molecule (a) and six classes of flavonoids (b).

Table 1.7 Main polyphenols in some fruit by-products (Schieber *et al.*, 2001).

Fruit by-products	Polyphenols	References
Apple pomace	Hydroxy cinnamic acids Flavonoids (catechins, phloretin glycosides, procyanidins, quercetin glycosides)	Lu & Foo (1997, 1998); Foo & Lu (1999); Lommen *et al.* (2000); Schieber *et al.* (2001);
Citrus seeds and peels	Flavonoids (eriocitrin, hesperidin, narirutin,naringin)	Mouly *et al.* (1994); Bocco *et al.* (1998); Coll *et al.* (1998); Gorinstein *et al.* (2001)
Grape pomace	Flavonoids (anthocyanins, catechins, flavonol glycosides) Phenolic acids and alcohols Stilbenes (resveratrol)	Mazza (1995); Lu & Foo (1999, 2000)
Grape skins	Flavonoids (catechin, epicatechin, epicatechin gallate, epigallocatechin)	Souquet *et al.* (1996)
Grape seeds	Flavonoids (procyanidins)	Kallithraka *et al.* (1995) Fuleki & Ricardo da Silva (1997) Saito *et al.* (1998); Jayaprakasha *et al.* (2001)

Table 1.8 Main polyphenols contents in some vegetables by-products (Schieber *et al.*, 2001).

Vegetable by-products	Polyphenols	References
Olive mill waste waters	Hydroxycinnamic acids Phenolic alcohols (hydroxytyrosol, oleuropein tyrosol)	Visioli *et al.* (1999); Rodis *et al.* (2002); Ranalli *et al.* (2003); Obied *et al.* (2005)
Onion wastes	Flavonoids (quercetin glycosides)	Hertog *et al.* (1992); Price & Rhodes (1997); Waldron (2001)
Potato peels	Phenolic acids (chlorogenic, gallic, protocatechuic, and caffeic acids)	Onyeneho & Hettiarachchy (1993); Rodriguez *et al.* (1994)

Polyphenols are recognized mainly for several health benefits thanks to their strong antioxidant activity (Stevenson & Hurst, 2007; Quideau *et al*., 2011).

Moreover, they have been demonstrated effective in controlling the enzymatic browning (Chang, 2009). Among the phenolic compounds, **flavonoids** include the main PPO inhibitors (Table 1.9). Kubo *et al*. (2000) report that flavonoids strongly inhibit PPO because of their ability to chelate copper ions in the active site, only if the hydroxyl group in 3 position is free.

Table 1.9 Inhibitory activity of some flavonoids on the activity of mushroom TYR (Kim & Uyama, 2005).

Flavonoids	Inhibition type	IC_{50} (mM)	References
Flavanols			
(-)-Epigallocatechin	competitive	0.035	No *et al*. (1999)
(-)-Epicatechin gallate	competitive	0.017	No *et al*. (1999)
(-)-Epigallocatechin gallate	competitive	0.034	No *et al*. (1999)
Flavonols			
Quercetin	competitive	0.070	Kubo *et al*., (2000)
Kaempferol	competitive	0.230	Kubo *et al*., (2000)
Morin	competitive	2.320	Kubo *et al*., (2000)
Flavones			
Luteolin	noncompetitive	0.190	Kubo *et al*., (2000)
Luteolin 7-*O*- glucoside	noncompetitive	0.500	Kubo *et al*., (2000)
Isoflavans			
Glabridin	noncompetitive	0.004	Nerya *et al*., (2003)
Glabrene	mixed-type	7.600	Nerya *et al*., (2003)
Isoliquiritigenin	mixed-type	0.047	Nerya *et al*., (2003)

2 Objectives

The research of new systems for controlling enzymatic browning in agro-food and cosmetic industries is focused on eco-friendly alternatives to conventional thermal treatments and traditional additives, which have critical points related not only to the organoleptic quality but also to nutritional and health claims.

This PhD project investigates by *in vitro* and *in vivo* assays the inhibition of polyphenol oxidase or tyrosinase (PPO), which is mainly involved in plant browning and skin melanogenesis, using innovative non-thermal technologies and bioactive compounds from agro-food by-products and wastes

The general working plan is structured in three contributions each of which is outlined into main steps.

The first contribution (A) shows the anti-browning effectiveness of a non-thermal technology based on UV-A light irradiation (390 nm) with LED source on some fresh-cut fruits.

A1. Setting of an illuminator prototype in order to fix the optimal operational conditions in terms of number of LED diodes, voltage, distance from sample, and treatment time. These issues has been carried out by monitoring through a colorimeter the colour changes on the surface of Golden Delicious apple slices with/without UV irradiation.

A2. Study of inhibitory effects of this non-thermal technology on commercial mushroom tyrosinase (TYR) and PPO extracted from Golden Delicious apple slices before and after irradiation, using electrophoretic and zymographic techniques.

A3. Anti-browning evaluation of UV-A LED treatment on several fresh-cut apples (Fuji, Golden Delicious, and Granny Smith) and pears (Abate Fétel and Decana) by measuring the colour changes on the surface of UV-treated/untreated slices.

The second contribution (B) is focused on assessing the anti-TYR activities of some citrus hydrosols (CIHs), which are co-produced during the distillation of citrus peels, in order to find a possible recycling use of these by-products in agro-food and cosmetic industries.

B1. Steam distillation of citron, orange, and lemon peels to produce three different CIHs (CH, OH, and LH, respectively).

B2. Spectrophotometric assays to define type and degree of TYR inhibition by CIHs. (+)-Epicatechin and L-DOPA are selected as the model phenolic substrates of plant enzymatic browning and skin melanogenesis.

B3. GC analysis of terpene contents in CIHs to evaluate the bioactive compounds responsible of anti-browning performances.

The third contribution (C) is addressed at investigating the anti-browning and antioxidant potentials in vineyard pruning residues (VPRs) with the aim to convert these agro-food wastes into value-added products.

C1. Cold pressing of berries, collected from the pruning residues of two red grape cultivars (Barbera, and Merlot) during the 2013 and 2014 seasons. The samples are tested as follows.

C2. Spectrophotometric assays, catechol gel diffusion test, zymographic techniques on the isoforms isolated from some plant PPOs (Fuji and Golden Delicious apples; Abate Fétel pears; Bintje potatoes), *in vivo* trials on fresh-cut fruits (Fuji, Golden Delicious, and Granny Smith apples; Abate Fétel and Decana pears) vegetables (Bintje potatoes; eggplants) and dried slices of Golden Delicious apples are carried out to evaluate the anti-browning potential of the juice recovered from the VPRs.

C3. Assessment of the antioxidant activity by spectrophotometric assays based on electron transfer (DPPH and FRAP) and total phenolic contents by Folin-Ciocalteu method.

C3. HPLC analysis of organic acid and polyphenols contents in VPRs to identify bioactive compounds with anti-browning effectiveness.

3 First contribution:

UV-A LED technology

SCIENTIFIC PUBBLICATION IN ISI-INDEXED JOURNAL

Title: UV-A light treatment for controlling enzymatic browning of fresh-cut fruits

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3.1 Introduction

The main factors affecting the perceived quality of fresh-cut produce are texture and appearance (Rico *et al.*, 2007; Toivonen & Brummell, 2008). There is no doubt that the first parameter which consumers take into account is appearance, an attribute which is judged on the basis of multiple factors including size, shape, form, colour, condition and absence of defects all of which can be influenced by several pre-harvest factors (Kays, 1999). In particular colour plays a key role in food choice by influencing taste thresholds, sweetness perception, food preference, pleasantness and acceptability (Clydesdale, 1993) and colour can be used both as a direct and an indirect index of quality (Francis, 1995).

While the market for fresh-cut products is increasing due to their convenience and healthfulness (Ragaert *et al.,* 2004), browning represents a problem for fresh-cut fruit and vegetables, especially white-fleshed fruit such as apples and pears. Browning reactions are mainly driven by PPO (EC 1.14.18.1), a copper-containing oxidoreductase that catalyses two different reactions involving phenolic compounds and oxygen. These reactions produce quinones that react further and lead to the accumulation of melanin and the development of brown- or black-coloured compounds depending on the specific structure of the polyphenolic substrate (Martinez & Whitaker, 1995; Seo *et al.*, 2003; Yoruk & Marshall, 2003; Garcia-Molina *et al.*, 2007; Queiroz *et al.*, 2008). PPO is an ubiquitous enzyme found in plants and fungi (Vamos-vigyazo & Haard, 1981; Halaouli *et al.*, 2006; Mayer, 2006; Marusek *et al.*, 2006), bacteria (Claus & Decker, 2006), in the exoskeleton of insects and crustaceans such as shrimp (Zamorano *et al.,* 2009) and also in the human epidermis (Kobayashi *et al.*, 1995; Sanchez-Ferrer *et al.*, 1995; Olivares *et al.*, 2001). In a typical plant cell it is localized in cytoplasmic organelles like chloroplasts while its phenolic substrates are mostly in the vacuole but also in the apoplast/cell wall compartment (Toivonen & Brummell, 2008). Enzymatic browning of fruit and vegetables takes place in the presence of oxygen when polyphenolic substrates are exposed to PPO and/or phenol peroxidases as a consequence of mechanical stress caused by post-harvest handling such as brushing, peeling, cutting and crushing which lead to the breakdown of cell structure (Saltveit, 2000; Degl' Innocenti *et al.*, 2005).

Most strategies that have been employed to control cut-edge browning have focused on physical and chemical methods to inhibit PPO activity by eliminating essential components such as oxygen, copper ion, substrate or even the enzyme itself.

With regards to chemical inhibition of browning, the data show carboxylic acids such as oxalic and oxalacetic acids, ascorbic acid derivatives such as ascorbic acid 2-phosphate, thiol-containing compounds such as cysteine, glutathione and N-acetylcysteine, phenolic acids such as

kojic acid, sodium metabisulphite and 4-hexyl resorcinol have the best effects on apple slices (Son, *et al.,* 2001; Eissa *et al.,* 2006). The most common commercial anti-browning formulation for fresh-cut products is a mixture of calcium salts with ascorbic acid that act respectively to keep cell structure integrity and to control PPO activity (Rupasinghe *et al.,* 2005). Dipping treatments and edible coatings are the main ways to apply PPO inhibitors to fresh-cut fruit (Rojas-Graü *et al.,* 2009; Oms-Oliu *et al.,* 2010). As reported by Lante & Zocca (2010), dipping potato slices into β-cyclodextrin improved the brightness of precooked, vacuum-packed potatoes and may be useful for other minimally-processed products.

Some other traditional anti-browning additives, such as ascorbic acid and its derivatives and sulfites, have become considered less useful because of drawbacks including low stability and potential health hazards (McEvily *et al.,* 1992; Rangan & Barceloux, 2009).

Therefore there is active research into the discovery of PPO inhibitors from natural sources (Kim & Uyama, 2005; Chang, 2009; Loizzo *et al.,* 2012) such as dog rose and pomegranate (Zocca, *et al.,* 2011) and other plant extracts (Wessels *et al.,* 2014) and from by-products of the agro-food industry such as Brassicacea processing water (Zocca, *et al.,* 2010) and citrus hydrosols (Lante & Tinello, 2015).

Aside from conventional thermal- and chemical-based strategies to preserve cut produce there are alternative non-thermal technologies that are gaining interest. These emerging strategies can be used not only to control microbiological activity to extend the shelf-life of fresh-cut products (Morris *et al.,* 2007; Falguera *et al.,* 2011) but also to control enzymatic browning and thus preserve the organoleptic and nutritional qualities of produce better than conventional processes. For example, PPO activity has been shown to be effectively controlled by combined treatment with ultrasound and ascorbic acid (Jang & Moon, 2011), pulsed electric fields (Meneses *et al.,* 2013), cold plasma (Surowsky *et al.,* 2013) and pulsed light (Manzocco *et al.,* 2013).

Ultraviolet light (UV) radiation is classified into three types (Bintsis, *et al.,* 2000): UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). The latter is recognized as a simple way to destroy most microorganisms in whole and fresh-cut produce (Allende & Artés, 2003; Allende, *et al.,* 2006; Birmpa, *et al.,* 2013). Indeed several studies have confirmed its potential as PPO inhibitor in model systems and apple derivatives (Manzocco *et al.,* 2009), in fresh apple juices (Falguera, *et al.,* 2011) and in mushroom extracts (Sampedro & Fan, 2014). Unfortunately, the potential for UV-C treatment is limited because of possible adverse effects in food including the alteration of sensory quality attributes such as colour (Refsgaard *et al.,* 1993; Manzocco *et al.,* 2008), the reduction of antioxidant capacity (Li *et al.,* 2014) and the formation

of furan recognized by the WHO as a potential human carcinogen (Fan & Geveke, 2007; Bule *et al.*, 2010; Müller *et al.*, 2013; WHO, 2011). However, UV-C light is not the only effective range in limiting enzymatic browning. As this regard, irradiation for 120 min with a high-pressure mercury lamp of 400 W emitting UV-visible light between 250 and 740 nm (maximum power of emission from 400-450 nm) effectively inactivated PPO in juices from both apples (Falguera, *et al.*, 2011) and pears (Falguera, *et al.*, 2014). Furthermore the treatments did not induce variations in pH, formol index and the contents of soluble solids, total phenolics and sugars. Kwak *et al.* (2004) hypothesized the mechanism of action for the anti-browning effect of UV-visible treatment might stem from the degradation of melanoidins, the polymeric brown compounds that result from PPO activity. Ibarz *et al.* (2005) corroborated the hypothesis studying the effect of UV-visible irradiation in apple, peach and lemon juices. In their study the researchers found increased brightness that was attributed to the photochemical destruction of brown pigments.

The current study was designed to investigate the effectiveness of UV-A irradiation for the control of PPO activity in fresh-cut apples and pears. There are many sources of UV light (Koutchma, 2009; Falguera, *et al.*, 2011) but we chose to use LED technology because it is an inexpensive and eco-friendly source.

3.2 Materials and methods

3.2.1 Reagents

L-3,4-ihydroxyphenylalanine (L-DOPA), 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH), polyvinylpolypyrrolidone (PVPP), sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NatureSeal® AS1 was obtained from AgriCoat NatureSeal, Berkshire, England.

3.2.2 Sample preparation

Commercial mushroom tyrosinase (TYR, EC 1.14.18.1; 3,130 U/mg) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Aliquots equal to 10 ml of TYR aqueous solution (1 mg/ml; 3,130 U/ml) were introduced into Petri plates with 60 mm diameter without cover (layer of 3 mm thickness) and submitted to UV-A LED treatment.

Three varieties of apples (*Malus domestica* L cv. Golden delicious, Granny Smith, Fuji) and two varieties of pears (*Pyrus communis* L cv. Abate Fétel Fétel, Decana) were purchased at commercial maturity from a local market between January and May of 2014 and stored at 4 °C. Fruits were washed under running water to remove any surface contamination, wiped with blotting paper and manually cut into two symmetrical slices of 5 mm thickness of which one was

subjected to UV-A LED treatment while the other was left untreated as a control. Each fresh-cut slice was placed into a 60 mm diameter Petri plate.

3.2.3 UV-A LED illuminator prototype

A prototypic UV-A LED illuminator was designed in order to study the effect of UV-A light (390 nm) on enzymatic browning of fresh-cut fruits. The illuminator was constructed from a 50 mm diameter polyethylene tube at one end of which "pin-in-hole" LED (Light Emitting Diode; Bivar, Inc. Thomas, Irvine, California, USA) diodes with emission peaks at 390 nm and emission angles of 30° were installed (Figure 3.1).

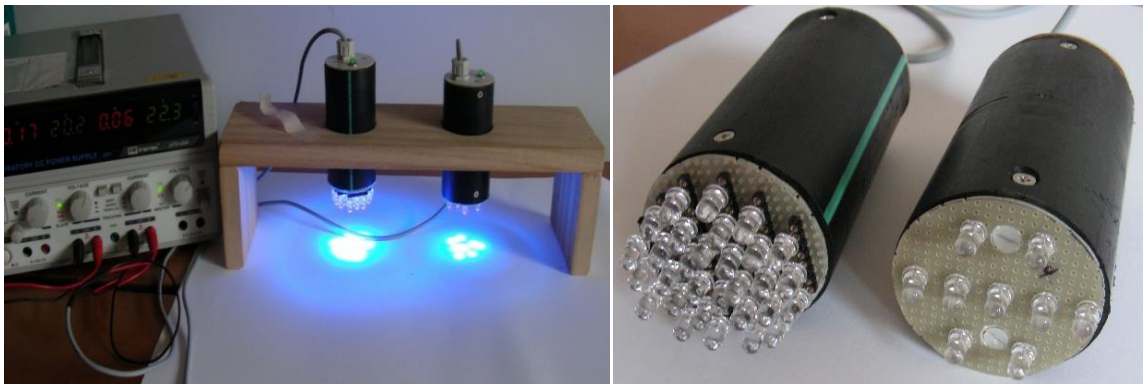

Figure 3.1 UV-A illuminator prototypes with 9 LEDs and 30 LEDs.

The tube was placed on a support in order to adjust its distance from the fruit slices. The illuminator was shielded from visible light during treatment to avoid any interference with the external environment.

3.2.4 Setting of UV-A LED illuminator

Three physical parameters of prototype illuminator, the number of LED diodes, voltage and distance from sample, were assessed by evaluating the anti-browning effect of UV-A light on the surface of Golden Delicious apple slices.

In one set of experiments two illuminator levels (with 9 (L9) or 30 (L30) LED diodes) were tested at three different voltages (10 (V10), 15 (V15), and 20 (V20) volts) at a constant distance of 0.5 cm from the sample. In a second set of experiments the L30 illuminator was placed at four different distances from the surface of the apple slices (5, 3, 1, and 0.5 cm) at a constant voltage of 20 V.

The UV-A light treatments were carried out in triplicate at 25 °C over increasing time periods up to 60 min. The irradiance of both illuminators was measured using the radiometer ILT IL-1700 (International Light Technology, MA, USA) equipped with a UV-A light probe at the research center Plast-optica (Amaro, Udine, Italy) and was expressed as Wm^{-2}.

3.2.5 Irradiation of fresh-cut fruits

After determining the best operational conditions for UV-A LED treatment (the L30 illuminator at 20 V set 0.5 cm from the slices with an irradiance of $2.43 \cdot 10^{-3}$ Wm^{-2}) the fresh-cut apple and pear slices were exposed to UV-A light irradiation in triplicate at 25 °C for increasing time periods up to 60 min. Moreover, the anti-browning effect of UV-A LED treatment for 30 min at 25°C on Golden Delicious apples slices was compared in the same experimental conditions with other chemical treatments. In details, an aqueous solution of 1% (w/v) ascorbic acid and 0.5% (w/v) calcium chloride (AAC) as suggested by Soliva-Fortuny *et al*. (2002) and 6% (w/v) NatureSeal® (AS1, AgriCoat NatureSeal, Berkshire, England) were applied in accordance to Zocca *et al*. (2011).

3.2.6 Temperature

The surface temperature of the fruit slices was measured before and after irradiation by a thermocouple probe BABUC/M (LSI LASTEM, Settala, Premenugo, Milan, Italy).

3.2.7 Colour measurement

Colour analyses on the sliced surfaces of fresh-cut fruits subjected to UV-A LED treatment and their corresponding controls were carried out using a Tristimulus colorimeter (Chroma Meter CR-410, Konica-Minolta, Milan, Italy) in the CIE 1976 (L*,a*,b*) colour space. The instrument was standardized against a white tile before measurements. Colour change (ΔE), calculated as the Euclidean distance between two points in the three-dimensional space defined by L*, a* and b*, were used to estimate the anti-browning effect of UV-A LED treatment and were expressed according to the following equation:

$$\Delta E = \sqrt{(\Delta L^2 + \Delta \alpha^2 + \Delta b^2)^2} = \sqrt{(L_t - L_{t0})^2 + (\alpha_t - \alpha_{t0})^2 + (b_t - b_{t0})^2}$$

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 = exposure time of UV-A light treatment, and t_0 = initial time of UV-A light treatment.

The percent reduction (%RΔE) in colour change was also used to evaluate the anti-browning potential and was calculated as follows:

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

where $\Delta E_{control}$ = colour change of samples not subjected to UV-A LED treatment,

$\Delta E_{treatment}$ = colour change of samples subjected to UV-A LED treatment.

3.2.8 PPO zymography

The inhibitory effect of UV-A LED treatment at the best operational conditions (the L30 illuminator at 20 V set 0.5 cm from the slices with an irradiance of $2.43 \cdot 10^{-3}$ Wm^{-2}) on PPO activity was evaluated through electrophoretic and zymographic techniques (Zocca *et al.*, 2011) after irradiating, at 25 °C for 30 min, TYR solutions and Golden Delicious apple slices. Apple PPO was extracted by blending fruit slices with an aqueous solution (1:1) containing 1% (w/v) PVPP and 0.5% (w/v) Triton X-100 (Weemaes *et al.*, 1998); then the mixture was centrifuged at 48,400 rpm for 15 min at 4°C and filtered through Whatman paper N°1 in a Büchner funnel under vacuum. The protein content of apple extracts was determined by Bradford (1976) assay.

The electrophoretic analysis was carried out in triplicate in a Mini Protean II (Bio-Rad, Milano, Italy) at room temperature. Non-reducing SDS-PAGE was performed according to Zocca *et al.* (2011) using 12% polyacrylamide gel at 100 V. The running buffer was composed of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS.

Before electrophoresis, TYR and apple PPO solutions were solubilized with 700 µl of distilled water and 300 µl of Laemmli buffer (1.33 M Tris pH 7.4, 60% v/v glycerol, 8% w/v SDS; Laemmli, 1970) and centrifuged at 14,000 rpm for 2 min. Each gel lane was loaded with irradiated and untreated enzyme solutions as follows: 5 µl of tyrosinase and 15 µl of apple PPO.

After electrophoresis, the gels were exhaustively washed for 15 min in 50 ml of 0.2 M sodium acetate buffer (0.2 M sodium acetate/0.2M acetic acid) at pH 5.0 and was incubated at 25°C for 30 min with 50 ml sodium acetate buffer at pH 5.0 containing 5 mM L-DOPA and 3 mM MBTH (Núñez-Delicado *et al.*, 2005). The presence of deep pink bands in treated gel lanes was associated to PPO activity. The gel images were acquired using scanner.

3.2.9 Statistical analysis

Statistical analysis was performed by subjecting all of the data obtained from three replicates to one-way analysis of variance (ANOVA) using the SAS software package (Statistical Analysis System Institute Inc., 2014) after verifying normal distribution and homogeneity of variance. The average data were analyzed by the procedure PROC GLM. Significant difference was determined by Bonferroni's multiple range test ($P \leq 0.05$).

3.3 Results and discussion

3.3.1 Optimizing the operational conditions of UV-A LED treatment

The effects of three physical parameters of the UV-A LED illuminator prototype, number of LED diodes, voltage and distance from sample, on ΔE of the surface of Golden Delicious apple slices were assessed at room temperature at different exposure times (10, 20, 30, 40, 50 and 60

min). As reported in Table 3.1, the effect of experimental treatment on ΔE data was significant ($P \leq 0.05$) after 30 min of irradiation. Specifically the L30V15 and L30V20 treatments yielded significant differences and the L30V20 irradiation for 60 min achieved the best performance with a %RΔE of treated apple slices (2.86 ± 0.16) of approximately 60% compared to untreated ones (6.83 ± 1.80) (Table 3.1).

Table 3.1 Effect of UV-A LED treatment obtained by combining illuminator LEDs number and voltage after setting 0.5 cm distance from sample on the colour change (ΔE) of Golden Delicious apple slices for increasing exposure times at 25°C.

	Treatment[1]							
Time (min)	**Control**	**L9V10**	**L9V15**	**L9V20**	**L30V10**	**L30V15**	**L30V20**	***P*-value**[2]
10	2.11[a] ±1.27	2.47[a] ±1.13	1.66[a] ±0.29	2.24[a] ±1.23	1.56[a] ±0.61	1.35[a] ±0.50	1.12[a] ±0.69	NS
20	3.84[a] ±1.69	4.15[a] ±1.38	3.30[a] ±0.40	3.43[a] ±1.33	2.80[a] ±0.95	2.13[a] ±0.52	1.72[a] ±0.62	NS
30	4.98[a] ±1.77	5.07[a] ±1.53	4.09[a] ±0.52	4.02[a] ±1.57	3.54[a] ±1.12	2.47[a] ±0.55	2.13[a] ±0.46	NS
40	5.79[a] ±1.82	5.66[a] ±1.64	4.68[ab] ±0.67	4.31[ab] ±1.03	3.97[ab] ±1.10	2.65[b] ±0.49	2.42[b] ±0.33	*
50	6.41[a] ±1.80	6.07[a] ±1.70	4.93[ab] ±0.78	4.52[ab] ±0.95	4.27[ab] ±1.12	2.87[b] ±0.55	2.67[b] ±0.26	*
60	6.83[a] ±1.80	6.36[a] ±1.70	5.17[ab] ±0.91	4.66[ab] ±0.82	4.48[b] ±1.11	2.90[b] ±0.47	2.86[b] ±0.16	**

[1] Control: no UV-A light treatment. L9V10: combination of 9 LEDs and 10 V. L9V15: combination of 9 LEDs and 15 V. L9V20: combination of 9 LEDs and 20 V. L3V10: combination of 30 LEDs and 10 V. L30V15: combination of 30 LEDs and 15 V. L30V20: combination of 30 LEDs and 20 V.

[2] 'NS' $P > 0.05$; '*' $P \leq 0.05$; '**' $P \leq 0.01$.

[a, b] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

The L30V20 illuminator was selected for further testing and was placed at different distances between 0.5-5 cm from Golden Delicious apple slices. Table 3.2 shows distance had an insignificant effect on ΔE data regardless of exposure time.

Table 3.2 Effect of the distance between L30V20 illuminator and sample surface on the colour change (ΔE) of Golden Delicious apple slices for increasing exposure times at 25°C.

	Distance (cm)				
Time (min)	**0.5**	**1**	**3**	**5**	***P*-value[1]**
10	1.12 ±0.69	1.25±0.28	1.23±0.44	1.11±0.50	NS
20	1.72±0.62	1.83±0.35	1.90±0.39	1.69±0.50	NS
30	2.13±0.46	2.10±0.37	2.25±0.32	1.99±0.46	NS
40	2.42±0.33	2.30±0.40	2.48±0.35	2.19±0.42	NS
50	2.67±0.26	2.41±0.36	2.63±0.33	2.36±0.38	NS
60	2.86±0.16	2.59±0.46	2.79±0.35	2.50±0.37	NS

[1] 'NS' $P > 0.05$.

As reported in Table 3.3, the increase in the number of LED diodes and the voltage that led to a greater anti-browning effect of UV-A light treatment was associated with an increase in the irradiance of illuminator. At a distance of 0.5 cm the irradiance of the L30V20 irradiator was $2.40 \cdot 10^{-3}$ Wm^{-2}, 26 times higher than that of the L9V10 irradiator, $9.23 \cdot 10^{-5}$ Wm^{-2}. Changing the distance from 0.5 to 5 cm did not significantly affect the irradiance, hence the lack of anti-browning effect.

Table 3.3 Irradiance (Wm^{-2}) of L9 and L30 illuminators set at different voltages and distances from the sample surface.

Distance (cm)	**L9[1]**			**L30**		
	V10[2]	**V15**	**V20**	**V10**	**V15**	**V20**
0.5	$9.23 \cdot 10^{-5}$	$7.80 \cdot 10^{-4}$	$1.50 \cdot 10^{-3}$	$1.85 \cdot 10^{-4}$	$1.28 \cdot 10^{-3}$	$2.40 \cdot 10^{-3}$
1	$8.09 \cdot 10^{-5}$	$6.80 \cdot 10^{-4}$	$1.30 \cdot 10^{-3}$	$1.60 \cdot 10^{-4}$	$1.11 \cdot 10^{-3}$	$2.07 \cdot 10^{-3}$
3	$4.93 \cdot 10^{-5}$	$4.08 \cdot 10^{-4}$	$7.80 \cdot 10^{-4}$	$1.69 \cdot 10^{-4}$	$1.17 \cdot 10^{-3}$	$2.20 \cdot 10^{-3}$
5	$5.17 \cdot 10^{-5}$	$4.15 \cdot 10^{-4}$	$7.78 \cdot 10^{-4}$	$1.92 \cdot 10^{-4}$	$1.34 \cdot 10^{-3}$	$2.50 \cdot 10^{-3}$

[1] L9, L30 refer to UV-A light illuminators with 9 and 30 LED lamps, respectively.
[2] V10, V15, V20 refer to treatment voltages of 10, 15, and 20 V, respectively.

Thus the best experimental conditions of UV-A LED treatment were: illuminator L30, V20 voltage, 0.5 cm distance from sample and $2.43 \cdot 10^{-3}$ Wm^{-2} irradiance. Figure 3.2 shows the ΔE of treated (L30V20) and untreated (CL30V20) Golden Delicious apple slices as function of exposure time. It is apparent that the standard deviation was higher in CL30V20 and in the first time points of L30V20 as a consequence of the variability in plant samples despite the maximum standardization of the trial. The increase over time of ΔE values of L30V20 treated samples (61% at 60 min) was less than that of CL30V20 (75% at 60 min). Overall, the %RΔE of treated samples compared to corresponding controls was 28% at 10 min, 49% at 30 min and 53% at 60

min. It is clear that the anti-browning performance of treatment was related to exposure time nearly all the reduction in colour change was seen already after 30 min.

In our study the effectiveness of UV-A LED treatment was a function mainly of increasing irradiance and exposure time, and these results corroborate those by Manzocco *et al.* (2009) studying the effect of UV-C (15 W lamps with maximum emission at 253.7 nm) and visible light (fluorescent tubes with maximum emission from 430-560 nm) treatments on PPO in model systems and Golden Delicious apples at 28 °C. They found the UV-C was more effective than visible light in the inactivation of PPO in an aqueous solution showing inhibitions of 40% and 100% after 60 min exposure to 3.9 Wm^{-2} and 13.8 Wm^{-2} irradiance respectively. Contrastingly the visible light treatment, where exposure time was in the order of hours, was effective only at high doses (12.7 Wm^{-2}) and in fact lower irradiances (11.7 and 9.4 Wm^{-2}) caused an initial PPO activation and only inactivation with increasing exposure time. The PPO inactivation via visible and UV light exposure was associated with direct photo-oxidation arising from the absorption of light by amino acid residues (Trp, Tyr, His, Phe, Met, Cys), the resulting protein denaturation and the formation of high molecular weight aggregates (Davies & Truscott, 2001; Davies, 2003; Lante *et al.*, 2013) analyzed by HPLC gel permeation by Manzocco *et al.* (2009).

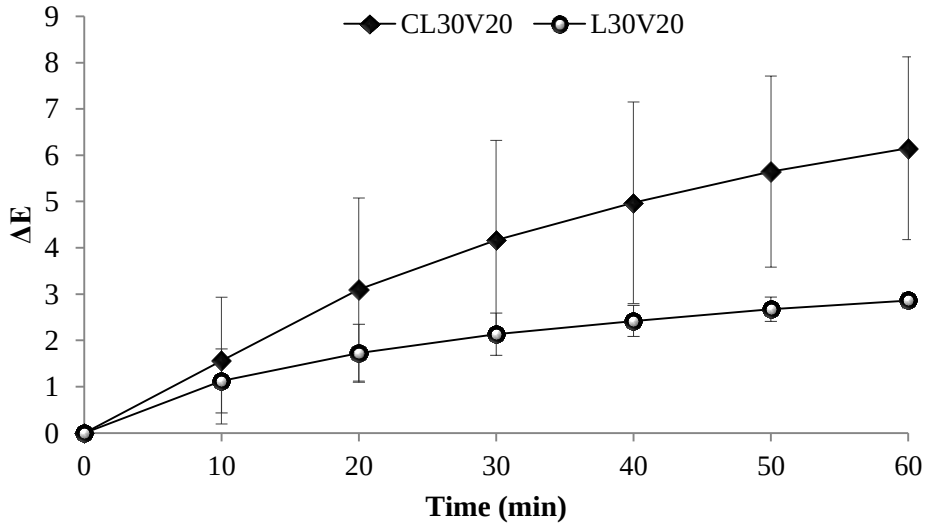

Figure 3.2 Colour change (ΔE) of Golden Delicious apple slices treated over time with a UV-A illuminator with 30 LEDs set to 20 V (L30V20) and corresponding controls (CL30V20).

3.3.2 Evaluation of PPO inhibition by UV-A LED treatment

The inhibitory effect of UV-A LED treatment on PPO activity was confirmed using non reducing SDS-PAGE electrophoresis and zymographic analysis in order respectively to isolate enzymatic isoforms and visualize the appearance of coloured bands in the gel lanes as indicator of enzyme activity (Figure 3.3). The UV-A LED irradiation at the best experimental conditions

fixed previously was carried out at room temperature for 30 min on a model solution of TYR and on Golden Delicious apple slices subjected to subsequent PPO extraction. The zymograms of TYR (Figure 3.3A) and apple PPO (Figure 3.3B) showed only one enzymatic isoform whose activity decreased after irradiation reducing significantly the colour intensity of corresponding band (UV) in comparison to the untreated control (C).

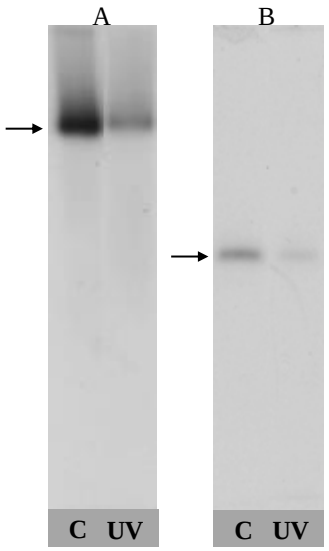

Figure 3.3 SDS-PAGE 12% zymograms of TYR (33 U per lane, A) and PPO extracted from Golden Delicious apple slices (7.38 µg of protein loaded per lane, B) before (C) and after UV-A LED irradiation at 25°C for 30 min with L30V20 illuminator (UV).

3.3.3 UV-A LED treatment of fresh-cut apples and pears

The UV-A LED treatment was applied for 60 min to fresh-cut slices obtained from 3 apple cultivars (Golden Delicious, Fuji, and Granny Smith) and 2 pear cultivars (Abate Fétel, and Decana). As reported in Figure 3.4 the effect of fruit type on %RΔE data was statistically significant regardless of the exposure time ($P \leq 0.001$). In particular, the apple results (63.6 ± 5.2 % for Fuji, 58.4 ± 2.9 % for Golden Delicious, and 52.8 ± 0.8 % for Granny Smith after 60 min irradiation) differed from those of fresh-cut pears (26.3 ± 3.4 % for Abate Fétel and 22.8 ± 4.7 % for Decana after 60 min irradiation) and the higher %RΔE values reflect a stronger anti-browning effect. Of the fresh-cut apples, the Fuji and Golden Delicious were most responsive to treatment and reached half %RΔE after only 20 min exposure (52.3 ± 7.0 and 49.3 ± 4.0, respectively). The effectiveness of UV-A LED treatment has already been demonstrated among several fruit cultivars by Falguera *et al.* (2011) and Falguera *et al.* (2014). These studies showed PPO was inactivated at different rates in various fresh apple and pear juices that were subjected to UV-visible treatment for 120 min with a high-pressure mercury lamp of 400 W which was

placed at 22.5 cm distance from juice surface and emitted in a range between 250 and 740 nm with a resulting incident energy of $3.88 \cdot 10^{-7}$ E·min^{-1}.

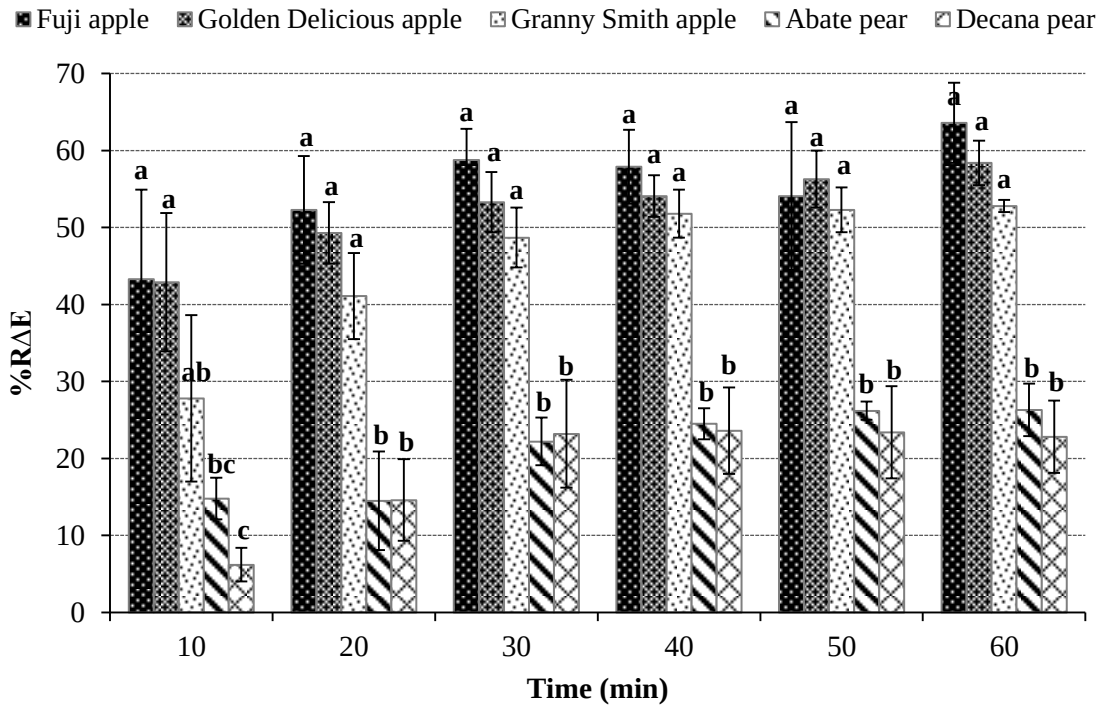

Figure 3.4 Effect of UV-A LED treatment with L30V20 illuminator on the reduction in colour change (%RΔE) of apple and pear fresh-cut slices for increasing exposure times at 25°C.

The anti-browning effect of UV-A on Golden Delicious apple slices was also compared with other chemical treatments. As reported in Figure 3.5, L30V20 illuminator showed similar performance to AAC by decreasing significantly ($P \leq 0.001$) the colour change of apple slice surface up to 60% after 30 min irradiation (Figure 5). Moreover the inhibitory potential of UV-A LED treatment was stronger than 6% (w/v) AS1 commercial formulation whose efficacy in preventing the browning of fresh-cut fruits was demonstrated by several authors (Abbott *et al.*, 2004; Toivonen, 2008; Rößle, *et al.*, 2009).

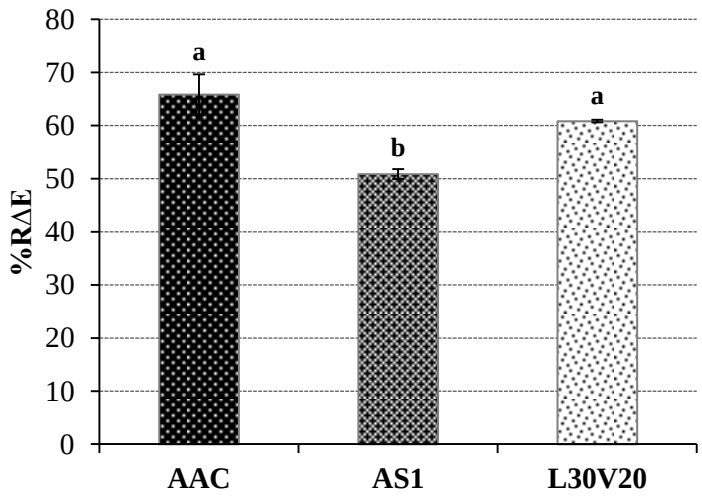

Figure 3.5 Reduction in colour change (%RΔE) of Golden Delicious apple slices treated for 30 min at 25°C with AAC and AS1 as anti-browning references and L30V20 illuminator.

The surface temperature of the fresh-cut fruit slices was measured in order to exclude any anti-browning effects caused by the thermal denaturation of PPO. The surface temperature of treated fresh-cut fruit slices averaged 22.0 ± 0.6 °C before irradiation and 26.0 ± 0.6 °C after 60 min exposure time as a consequence of slight heating by the LED diodes whose temperature rose from 20.1 °C to 29.5 °C during treatment. Optimum temperature for PPO activity is a function of the plant source and variety (Yoruk & Marshall, 2003). The optimum temperature for PPO activity in the current study, which we extracted from Golden Delicious apples, was 50 °C in the presence of a catechol substrate at pH 5.0 (data not shown), a value higher than that measured in Monroe apple peels (30°C) under the same assay conditions (Zhou, *et al.,* 1993). The same authors also showed that apple PPO was stable up to 40 °C, rapidly inactivated above 50 °C and completely inactivated in the range of 70-80 °C as a function of time. This finding was further confirmed by Yemenicioglu *et al.* (1997) and Soysal (2009). Ankara pear PPO, which has an optimum temperature in the range of 20-45 °C, showed a similar trend of thermal inactivation (Ziyan & Pekyardimci, 2004). On the basis of these results the non-significant temperature rise in the sliced surface of fresh-cut apples and pears subjected to UV-A LED treatment confirmed that the anti-browning effect was completely due to UV irradiation.

3.4 Conclusions

The main results of the fist contribution can be summarised as follows.

- The optimal operational conditions of the UV-A LED illuminator prototype were:

  - LEDs number: 30;
  - voltage: 20 V;
  - distance of illuminator from sample: 0.50 cm;
  - irradiance: $2.43 \cdot 10^{-3}$ $Wm^{-2.}$

- The anti-browning effectiveness of UV-A light (390 nm) was related to exposure time at 25°C. In details, the %RΔE of treated Golden Delicious apple slices, previously subjected to UV-A LED treatment with illuminator prototype at the best operational conditions, quickly increased until 30 min (28% at 10 min, 49% at 30 min), after that slowly increased until 60 min (53%). Thus, a time irradiation of 30 min was sufficient to achieve the best anti-browning performances.

- The zymographic results confirmed the inhibitory effects of this non-thermal technology on the one isoform of TYR and PPO extracted from Golden Delicious apple slices after irradiation.

- The UV-A LED treatment was more effective in limiting the colour changes of fresh-cut apples, especially from Fuji and Golden Delicious, than fresh-cut pears as confirmed by the highest %RΔE values (58.3% *vs.* 25.5% on average after 60 min exposure, respectively). Thus, the anti-browning effectiveness was also a function of fruit type and cultivar.

While UV-A light irradiation is less powerful than UV-C, browning can be effectively controlled by UV-A light without compromising the organoleptic and nutritional properties of fresh-cut fruits. Moreover the use of LED diodes meets the needs for energy saving and reduced environmental impact. Therefore, UV LED technology may be an eco-friendly alternative approach to conventional thermal treatments and traditional additives that cause possible adverse effects and involve high energetic costs.

4 Second contribution:

Citrus hydrosols

SCIENTIFIC PUBBLICATION IN ISI-INDEXED JOURNAL

Title: Citrus hydrosols as useful by-products for tyrosinase inhibition

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4.1 Introduction

Hydrosols are by-products coproduced during water or steam distillation of plant material. Since they contain trace amounts of essential oils as well as other hydrophilic dissolved compounds, hydrosols are commonly used for aroma therapeutic and cosmetic purposes (Inouye *et al.*, 2008). Moreover, several authors (Sagdic, 2003; Tajkarimi *et al.*, 2010; Lin *et al.*, 2011; Tornuk *et al.*, 2011, 2014) showed the possibility of using herbs and spice hydrosols in drinks, food preservation and as a convenient sanitizing agent during the washing of fresh-cut fruits and vegetables. When considering the use of hydrosols for food applications, a very important characteristic is that they are considered free from side effects for humans, as is the case for essential oils, which are listed in the Code of Federal Regulation as generally recognized as safe (GRAS) (FDA, 2013; Kabara, 1991).

In particular, citrus oils, which are the focus of this study, are mainly used for the flavouring of fruit beverages, confectioneries, and soft drinks, as well as for the perfuming of eau de cologne, soaps, cosmetics and household products (Raeissi *et al.*, 2008). They are also employed in medical treatments and are known to have antimicrobial properties, including antifungal, antibacterial, antiviral and antiparasitic activities (Rehman *et al.*, 2007). Citrus peel essential oils are contained in oil sacs, or vesicles, located in the outer rind or flavedo of the fruit. The peel oil is a by-product of citrus juice extraction usually recovered by mechanical separation, known as cold-pressing, hydro distillation or steam distillation (Lota *et al.*, 2002). Distillation is an economical way to recover the oils, with a better yield (0.21%) than cold pressing (0.05%) (Ferhat *et al.*, 2007). Moreover Sahraoui *et al.* (2011) extracted essential oil from orange peels with microwave steam distillation in comparison to the conventional steam distillation. Results confirm the effectiveness of this technique which allows the reduction of time and energy of extraction without causing changes in the volatile oil composition.

During distillation, citrus peels are exposed to boiling water or steam to release their essential oils through evaporation. As steam and essential oil vapours condense, both are collected and separated in a vessel, and hydrosols are recovered and usually discarded.

Citrus is the most abundant fruit crop in the world (about 131 million tons in 2012 (FAOSTAT, 2013) and the amount of waste obtained from citrus fruits accounts for 50% of the whole fruit (Braddock, 1995; Chon & Chon, 1997). As reported by Sahraoui *et al.* (2011) transformation of citrus wastes allows balancing their processing cost with value added output and environmental protection. To our knowledge, no study demonstrated yet the ability of citrus hydrosols (CIHs) to control the enzymatic browning that may be considered a bridge between the food and cosmetic fields. On the basis of these considerations, CIHs could have great

potential for further commercial use because TYR (EC 1.14.18.1) is the main enzyme responsible for the browning reaction of fruits and vegetables, and it is involved in the initial reaction of melanin pigment synthesis (Kim & Uyama, 2005). This enzymatic reaction can lead to alteration of colour and a partial loss of the antioxidant capacity of some foods due to the generation of more *o*-quinone and melanisation in animals. The most frequently used inhibitors of enzymatic browning in industry include ascorbic acid and various forms of sulphite-containing compounds. The latter have applications for a broad range of products and are strong anti-browning and antimicrobial agents (Fan *et al.,* 2009). However, adverse side effects such as high toxicity towards cells and low stability when exposed to oxygen and water limit their application (Schurink *et al.,* 2007). Recently, TYR inhibitors from natural sources have become popular as food and cosmetic additives to prevent enzymatic browning (Loizzo *et al.,* 2012; Parvez *et al.,* 2007).

As reported by Zocca *et al.* (2011), dog rose and pomegranate extracts obtained with minimal processing can be used as anti-browning agents to preserve the quality of fresh-cut vegetables and fruit. In addition, products enriched with bioactive compounds such as those present in dog rose hips and pomegranate may prove to be an effective tool to both develop functional foods and to increase the overall intake of plant products. Exploiting the promise of the wastewater agro-food industry, Zocca *et al.* (2010) suggested that also Brassicacea processing water, that is a source of bioactive compounds, may be useful for the control of enzymatic browning throughout a given product/service lifecycle.

The effectiveness of natural products is mainly attributed to their high content of bioactive components as organic acids, glucosinolates and polyphenols. In particular, polyphenols represent a diverse group of compounds containing multiple phenolic functionalities and identified as specific inhibitors of TYR (Chang, 2009). Furthermore polyphenols have been recognized as having many health benefits mainly due to their antioxidant activity (Kang *et al.*, 2011; Lante *et al.,* 2011; Lante & Friso, 2013; Mihaylova *et al.,* 2014).

The purpose of the present work is to investigate possible new uses as anti-browning agents for three different CIHs that are usually discarded even if may contribute to extra business profit as natural additives, providing a connection between the needs of improve quality and reduce chemical substances in the food and cosmetic fields.

4.2 Materials and methods

4.2.1 Reagents

Commercial mushroom tyrosinase (TYR, EC 1.14.18.1), L-3,4-dihydroxyphenylalanine (L-DOPA), (−)-epicatechin, *tert*-butylcatechol (*t*-BC), hexane, acetone, n-dodecane and GC standards such as myrcene, *α*-terpinene, (R)-limonene, terpinolene, sabinene, *α*-terpineol, geraniol, citral (mixture of *cis* and *trans* isomers, ≥96%), and *β*-citronellol were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2 Preparation of the citrus hydrosols (CIHs)

Citrons (*Citrus medica* L.), lemons (*Citrus limon* L. Burm. cv. Femminello), and oranges (*Citrus sinensis* L. Osbeck cv. Tarocco) were purchased at commercial maturity from a local store. All citrus fruits were produced organically.

Fruit was washed under running water to remove any surface contamination and wiped with blotting paper. After that fruit was peeled so as to separate the inner layer (albedo) from the outer one (flavedo). This fraction, rich in oil glands, was cut into small pieces (about 0.5 cm), crushed in liquid nitrogen, weighed and distilled. For laboratory-scale experiments, citrons, oranges and lemon peels (100 g) were distilled by adding 300 ml of distilled water with Cazenave equipment consisting of a steam generator, a flask with steam pipe, a distillation column and a condenser.

Two distillations were done for each fruit. Distillates (250 ml) were collected and analysed to determine terpene content and TYR inhibition capacity in the CIHs. After steam distillation, the oleous phase was completely separated from water by centrifugation at 4 °C at 12,000 rpm for 5 min. Citron hydrosol (CH), lemon hydrosol (LH), orange hydrosol (OH) were kept in air-tight sealed glass vials, covered with aluminium foil at 4 °C until further analysis.

4.2.3 TYR activity inhibition

Commercial TYR was dissolved in 0.1 M sodium citrate buffer at pH 6.0 to a final concentration of 1,336 U/ml. TYR activity was assayed spectrophotometrically at 475 and 440 nm with 10 mM L-DOPA or (−)- epicatechin respectively in a sodium citrate buffer. The solution used for a blank included 1.0 ml of L-DOPA or (−)-epicatechin at different concentrations, 300 µl of distilled water and 10 µl of commercial TYR (added last). Sample reaction mixtures were obtained by substituting 300 µl of inhibitors (CH, LH, and OH) with distilled water. Absorbances (OD) at 440 nm and 475 nm were monitored at 25 °C using a UV/Vis spectrophotometer (JASCO 7800, Tokyo, Japan). The enzymatic activity was calculated as absorbance variation per minute (ΔOD/min) considering the linear part of kinetic curve.

The percent inhibition of TYR activity was calculated as follows (Baurin *et al.*, 2002):

$$\%\text{TYR inhibition} = [(\Delta OD_{control} - \Delta OD_{inhibitor})/\Delta OD_{control}] \times 100\%$$

where $\Delta OD_{control}$ = absorbance variation per minute at 440 and 475 nm without inhibitor and ΔOD_{sample} = absorbance variation per minute at 440 and 475 nm inhibitor.

L-Ascorbic acid (AA) was used as a positive control. In this case, the enzyme activity was calculated in the linear part of the curve after the lag phase (Alam *et al.*, 2011). The assay was carried out in air-saturated aqueous solutions. Inhibitory kinetics of samples were analysed using Lineweaver–Burk plots. The kinetic data were plotted as the reciprocal of initial velocity of enzymatic reaction corresponding to enzyme activity ($1/V_0$ equal to $1/\Delta OD/min$) on the Y-axis against the reciprocal of substrate concentration (1//[S]) on the X-axis, according to the method of Lineweaver–Burk, and the Michaelis–Menten constant (K_M) and maximum velocity (V_{max}) were determined with variable substrate concentrations in the standard reaction mixture.

4.2.4 GC analysis

Terpene quantification was performed by liquid–liquid extraction and GC analysis. To determine terpene content of CIHs, a GC 8000 Top CE Instruments (Thermo Finnigan) gas chromatograph equipped with a flame ionization detector (FID) and AS 2000 auto injection sampler was used. Hydrogen was used as a carrier gas with a flow rate of 22.4 cm/s and flux of 0.37 ml/min at 297 Pa. The column was a DB-1 (40 m × 0.1 mm I.D. and 0.2 μm film thicknesses; Agilent J&W, USA). Injector and detector temperatures were 250 °C. The oven temperature program was as follows.

CIHs samples (9 ml) were transferred to test tubes containing 3 ml of hexane (volume ratio of 3:1). They were sealed with rubber caps and agitated using a vortex mixer for 1 min. After phase separation, 0.950 ml of hexane was transferred to tubes containing the internal standard (50 μl), and 1 μl of this solution was injected into a gas chromatograph. As an internal standard, n-dodecane dissolved in acetone (100 mg/100 ml) was used. Identification of terpenes was done using a reference mixture of volatile compounds in hexane. Quantification of terpenes was expressed as mg/ml of CIHs.

4.2.5 Determination of quinone inhibition

The level of *o*-quinone was determined as previously described (Waite, 1976). *Tert*-butylcatechol was oxidized using $NaIO_4$, and the formation of 4-*tert*-butyl-*o*-benzoquinone was then spectrophotometrically detected by measuring its accumulation at 400 nm. The reaction mixture contained 2 mM (50 μl) of $NaIO_4$ and 1 mM (1.0 ml) of *t*-BC in the absence and presence of inhibitors (100 μl).

4.2.6 Statistical analysis

Statistical analysis was performed by subjecting all of the data obtained from three replicates to one-way analysis of variance (ANOVA). Significant difference was determined by Tukey's multiple range test ($P \leq 0.05$) using the CoHort software package (CoHort Software, Monterey, CA, USA).

4.3 Results and discussion

4.3.1 Determination of TYR activity inhibition by CIHs

To assess, *in vitro*, the potential application of CIHs as food and cosmetic additives, commercial TYR was employed. The CIHs (CH, LH and OH) obtained by steam distillation of citrus peels were capable of inhibiting L-DOPA (Table 4.1) and (−)-epicatechin (Table 4.2) oxidation to varying degrees. The choice of these two types of enzyme substrates was motivated by the consideration that the first is considered a model of initial melanisation, while the second is a reasonable approximation of plant enzymatic browning (Liu *et al.*, 2010; Oszmianskii & Lee, 1990). Results showed that there was a significant difference ($P \leq 0.05$) between the inhibition capacity of OH and LH/CH using the two different phenol substrates. OH showed potent inhibitory action on TYR activity with an IC_{50} value of 5.9 μg (300 μl of OH) of total identified terpenes, using all (−)-epicatechin substrate concentrations. For 1 mM L-DOPA, CH and LH showed an IC_{50} value of 18.04 and 38.57 μg of terpenes, respectively. CIHs in the range of 100–300 μl exhibited a concentration-dependent inhibitory effect on substrate oxidation induced by TYR (data not shown). In comparison to these terpenes, a higher concentration of AA was necessary to obtain similar inhibition results; 18.04 μg of CH terpenes resulted in a 62.16% inhibition using 1 mM L-DOPA and the same value was reached with 82.5 μg of AA (300 μl at the concentration 0.027% w/v). Using (−)-epicatechin, 105 μg of AA (300 μl at the concentration 0.035% w/v) was required in comparison to 5.9 μg of OH terpenes. In addition, TYR in the presence of AA developed a lag period as reported by Jeon *et al.* (2005), while CIHs showed no lag phase. Therefore, the inhibitory effect of CIHs was better than AA against TYR. The selection of AA is not only due to the fact that it is a well-known TYR inhibitor (Ros *et al.*, 1993) but also due to the fact that its effect on TYR has been exhaustively studied because of its extensive use in food processing (Golan-Goldhirsh & Whitaker, 1984).

Table 4.1 The inhibitory effect of CIHs on commercial TYR, at different concentrations of L-DOPA.

	Inhibitors[1]			
	C	**CH**	**LH**	**OH**
L-DOPA 1 mM				
ΔOD_{475}/min	$0.101^{a} \pm 0.001$	$0.038^{c} \pm 0.002$	$0.041^{c} \pm 0.005$	$0.056^{b} \pm 0.002$
Inhibition (%)	–	62.2	59.1	44.5
L-DOPA 2.5 mM				
ΔOD_{475}/min	$0.114^{a} \pm 0.002$	$0.069^{b} \pm 0.002$	$0.072^{b} \pm 0.002$	$0.081^{b} \pm 0.006$
Inhibition (%)	–	39.1	36.7	28.9
L-DOPA 3 mM				
ΔOD_{475}/min	$0.118^{a} \pm 0.005$	$0.075^{c} \pm 0.002$	$0.072^{c} \pm 0.001$	$0.085^{b} \pm 0.005$
Inhibition (%)	–	36.3	38.7	28.1
L-DOPA 10 mM				
ΔOD_{475}/min	$0.122^{a} \pm 0.003$	$0.091^{b} \pm 0.006$	$0.087^{b} \pm 0.002$	$0.095^{b} \pm 0.003$
Inhibition (%)	–	24.7	28.4	21.8
L-DOPA 20 mM				
ΔOD_{475}/min	$0.149^{a} \pm 0.008$	$0.093^{c} \pm 0.008$	$0.095^{c} \pm 0.006$	$0.110^{b} \pm 0.001$
Inhibition (%)	–	37.3	36.2	25.9

[1] C: control without inhibitors. CH: 300 µl of citron hydrosol. LH: 300 µl of lemon hydrosol. OH: 300 µl of orange hydrosol.

[a, b, c] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

Table 4.2 The inhibitory effect of CIHs on commercial TYR, at different concentrations of (−)-epicatechin.

	Inhibitors[1]			
	C	**CH**	**LH**	**OH**
(-)-epicatechin 0.5 mM				
ΔOD_{440}/min	$0.259^{a} \pm 0.010$	$0.144^{c} \pm 0.006$	$0.156^{c} \pm 0.005$	$0.107^{b} \pm 0.003$
Inhibition (%)	–	44.4	39.6	58.6
(-)-epicatechin 1 mM				
ΔOD_{440}/min	$0.397^{a} \pm 0.009$	$0.243^{c} \pm 0.006$	$0.241^{c} \pm 0.008$	$0.124^{b} \pm 0.002$
Inhibition (%)	–	39.0	39.5	68.9
(-)-epicatechin 2.5 mM				
ΔOD_{440}/min	$0.443^{a} \pm 0.012$	$0.279^{c} \pm 0.023$	$0.290^{c} \pm 0.014$	$0.164^{b} \pm 0.005$
Inhibition (%)	–	32.8	34.9	63.0
(-)-epicatechin 3 mM				
ΔOD_{440}/min	$0.422^{a} \pm 0.017$	$0.273^{c} \pm 0.009$	$0.274^{c} \pm 0.006$	$0.157^{b} \pm 0.001$
Inhibition (%)	–	35.2	35.1	62.7
(-)-epicatechin 5 mM				
ΔOD_{440}/min	$0.441^{a} \pm 0.010$	$0.304^{c} \pm 0.009$	$0.323^{c} \pm 0.016$	$0.203^{b} \pm 0.007$
Inhibition (%)	–	31.1	26.8	54.1

[1] C: control without inhibitors. CH: 300 μl of citron hydrosol. LH: 300 μl of lemon hydrosol. OH: 300 μl of orange hydrosol.

[a, b, c] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

The nature of TYR inhibition can be determined by measuring the enzyme inhibition kinetics using the Lineweaver–Burk plots with varying concentrations of phenols as the substrates. Figure 4.1 displays the kinetic behaviour of TYR-catalysed oxidation of different concentrations of L-DOPA and (−)-epicatechin, in the absence and presence of inhibitors, using the same volume (300 μl) of CIHs. When looking at data obtained using L-DOPA as inhibitor, the double-reciprocal plot yields a family of lines with different slopes and different intercepts, and they intersect one another in the first quadrant. Such kinetic behaviour indicates that the various CIHs are mixed-type inhibitors of TYR activity. In other words, the kinetic analysis suggests that the various compounds in CIHs reduce the affinity of the substrate for the enzyme but do not bind to the active site. A similar inhibition pattern was observed with (−)-epicatechin, with the exception of OH, where the affinity of the substrate for the enzyme slightly increased.

A Lineweaver–Burk plot of the results showed that CIHs changed both K_M and V_{max} of L-DOPA and (−)-epicatechin oxidation (Table 4.3).

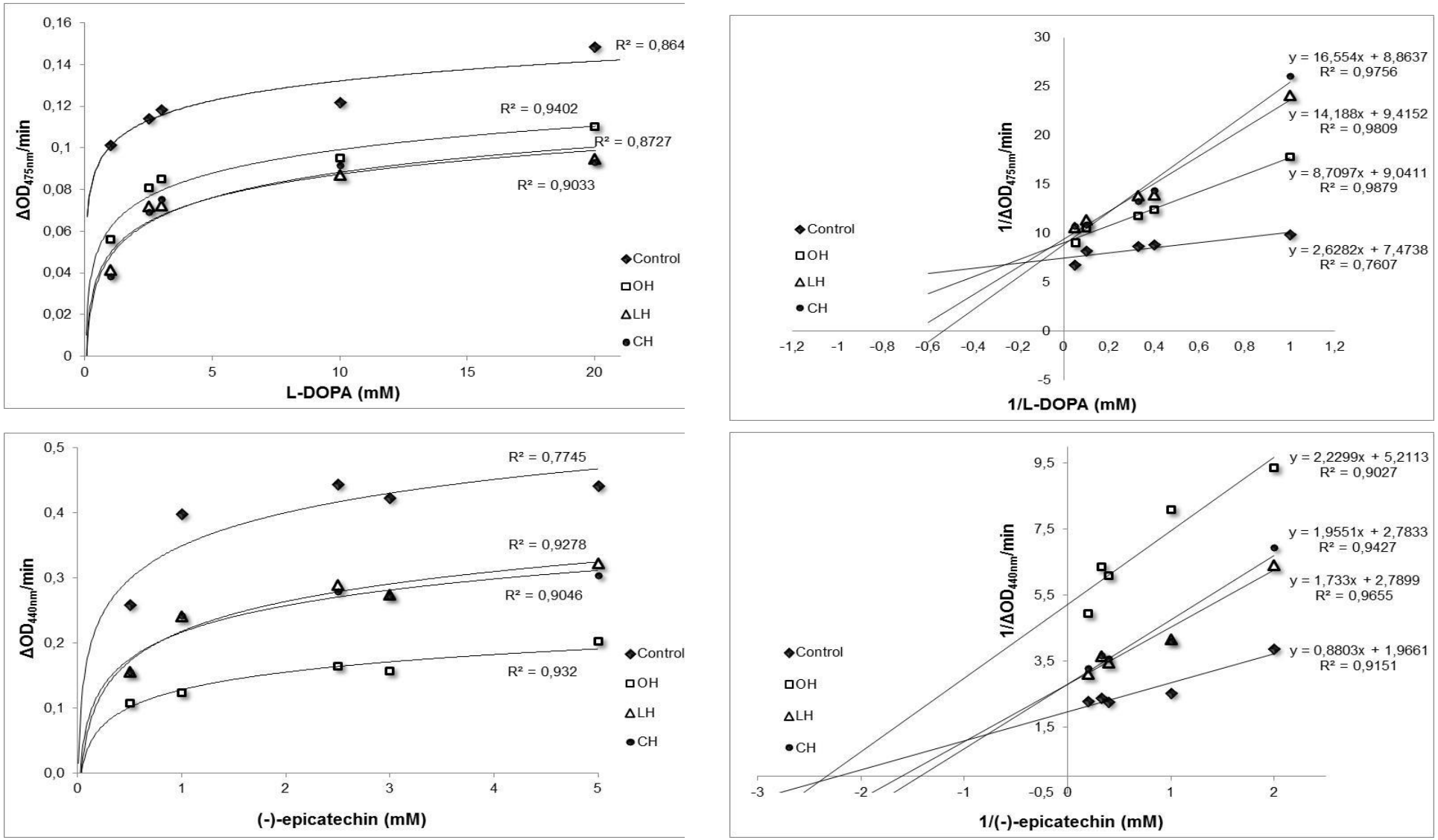

Figure 4.1 TYR activity (ΔOD/min) corresponding to the initial velocity of enzymatic reaction (V_0) *vs.* substrate concentration (L-DOPA, top plots; (−)-epicatechin, bottom plots) in the absence and presence of CIHs and corresponding Lineweaver–Burk plots.

Table 4.3 Effect of CIHs on the kinetic constants (K_M and V_{max}) of commercial TYR.

Inhibitors[1]	L-DOPA		(-)-epicatechin		Inhibition type
	V_{max} (ΔOD_{475}/min)	K_M (mM)	V_{max} (ΔOD_{440}/min)	K_M (mM)	
C	0.133	0.35	0.508	0.45	–
OH	0.110	0.96	0.191	0.43	Mixed
LH	0.106	1.51	0.358	0.62	Mixed
CH	0.112	1.87	0.359	0.70	Mixed

[1] C: control without inhibitors. CH: citron hydrosol. LH: lemon hydrosol. OH: orange hydrosol.

4.3.2 Determination of quinone inhibition by CIHs

To determine if CIHs act simply as antioxidants involved in non-enzymatic browning reactions or are truly enzyme inhibitors, *o*-quinone production was monitored. The production of *o*-quinone occurs upon exposure of naturally occurring catechols to oxygen in the presence of plant-derived polyphenol oxidases and does not involve TYR activity (Waite, 1976). CIHs did not decreased the absorbance values of the control (A_{400} = 0.165), thus showing no inhibitory effect on *o*-quinone production. These results confirmed that CIHs were not simply serving as antioxidants and their mechanism of anti-browning effects included the inhibition of enzymatic activity.

4.3.3 GC analysis of the terpene contents in CIHs

To further characterize the inhibitory activity of the three kinds of CIHs, we searched their characteristic volatile constituents for compounds known to have TYR inhibitory activity. Inhibitors discussed in this paragraph, therefore, are known inhibitors of the diphenolase activity of mushroom TYR. Nakatsu *et al.* (2000) reported that acyclic terpenoids show strong inhibitory effects on TYR activity. Monoterpene alcohols such as citronellol were stronger inhibitors than higher acyclic terpene alcohols such as phytol. This indicates that the length of the carbon chain of the molecule is an important factor in suppressing TYR activity. The isoprenyl group in citronellol appears to enhance the inhibitory activity when compared to tetrahydrogeraniol. In addition primary alcohols such as citronellol, geraniol and farnesol more strongly inhibited TYR than tertiary alcohols such as linalool and nerolidol (Nakatsu *et al.,* 2000). Matsuura *et al.* (2006) also reported that aromatic or aliphatic aldehydes such as anisaldehyde, cuminaldehyde, and (2E)-alkenal are considered to be TYR inhibitors. Moreover, citrus essential oils contain a number of aliphatic aldehydes such as citral (geranial and neral), myrcene and sabinene. These aldehydes such as citral are protein reactive compounds and are known to react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. The aldehyde compound can react with the primary amino group in TYR to form a Schiff base. A comparison

of TYR inhibitory activity by various aldehydes showed that the stability of the Schiff base formed between TYR and an aldehyde was significantly correlated with activity (Kubo & Kinst-Hori, 1999). Some of the known TYR inhibitors, such as citral, myrcene, sabinene and geraniol, were detected and quantified in CIHs using GC analysis (Figure 4.2).

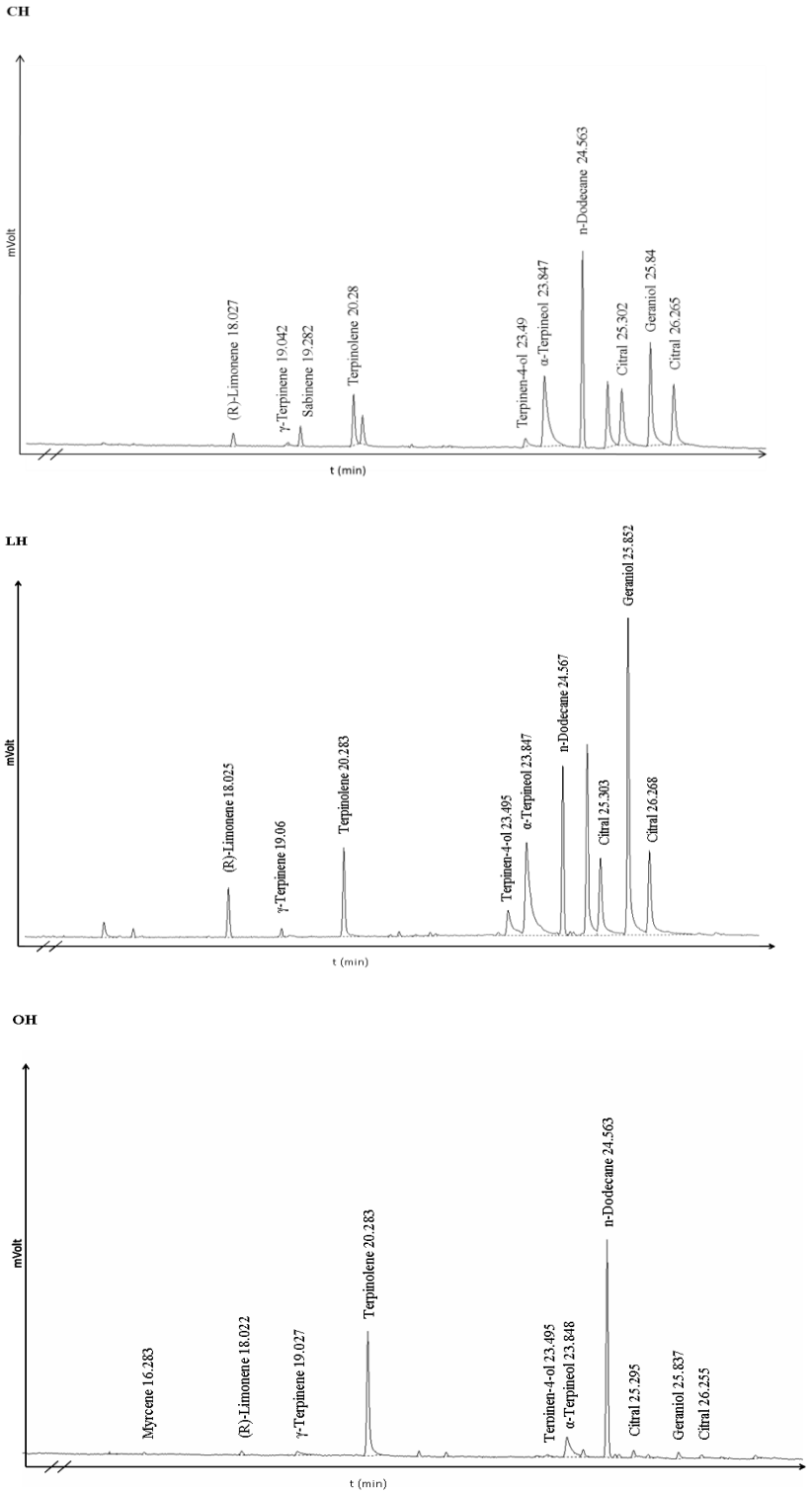

Figure 4.2 GC chromatograms of CIHs.

Table 4.4 shows that the terpene content and inhibitor content of CIHs were significantly different in the three distillates ($P \leq 0.05$). A comparison of the amounts of the inhibitors present in the various CIHs revealed that LH had the highest levels of geraniol and citral, whereas OH had the lowest levels of geraniol and citral. Myrcene was present only in OH and sabinene only in CH. However, further investigations to establish how components interact to provide the TYR inhibitory activity are required. Studies should also be extended to evaluating more replicates and the use of compounds identified by GC pure to assess their inhibitory effect.

Results from the study of enzyme kinetics had shown that the hydrosol of citron can significantly lower the K_M of the enzyme (Table 4.3). It is interesting to note that the terpene concentration of OH was the lowest (≈20 μg/ml) due to the low content of oxygenated monoterpenes, but its anti-TYR activity, using (−)-epicatechin as a substrate, was the highest. The significant TYR inhibition showed by all of the tested CIHs can thus likely be attributed to the presence of monoterpenic hydrocarbons and oxygenated monoterpenes. However, it is known that the compositions of hydrosols depend on the profile of essential oils; for this reason an extraction method is the first critical step to produce extracts of defined quality. Going back in the production chain, the best results are also dependent on different citrus types, varieties, agronomic practices, and climatic and storage conditions of raw material, so further work will be required to address these issues.

Table 4.4 GC analysis of terpenes contents in CIHs.

Terpenes (µg/ml)	**CH**	**LH**	**OH**
Monoterpenic hydrocarbons			
α-Pinene	n.d.[§]	n.d.	n.d.
Myrcene	n.d.	n.d.	0.18 ± 0.03
(R)-Limonene	1.22^{c} ± 0.02	4.36^{a} ± 0.07	0.34^{b} ± 0.02
α-Terpinene	n.d.	n.d.	n.d.
γ-Terpinene	0.40^{b} ± 0.05	0.85^{a} ± 0.03	0.61^{ab} ± 0.19
Terpinolene	5.58^{c} ± 0.21	9.78^{a} ± 0.49	12.41^{b} ± 0.25
Sabinene	1.83 ± 0.04	n.d.	n.d.
Oxygenated monoterpenes			
Terpinen-4-ol	1.57^{c} ± 0.58	0.28^{b} ± 0.05	7.22^{a} ± 0.39
α-Terpineol	16.81^{c} ± 2.01	4.41^{b} ± 0.08	29.98^{a} ± 0.20
β-Citronellol	n.d.	n.d.	n.d.
Geraniol	15.35^{c} ± 1.06	0.80^{b} ± 0.06	48.27^{a} ± 0.88
Citral (*cis*- and *trans*-isomers*)*	17.4^{c} ± 1.02	0.61^{b} ± 0.07	28.84^{a} ± 0.93
Total terpenes	60.16	128.59	19.64

[§] Not detectable.
[a, b, c] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

4.4 Conclusions

The main results of the second contribution can be summarised as follows.

- The spectrophotometric assays allowed investigating the inhibition type and degree of CIHs, which are co-produced during the distillation of citron, lemon, and orange peels, by analysing the enzymatic kinetics of TYR, in the presence of (+)-epicatechin and L-DOPA as the model phenolic substrates of plant enzymatic browning and skin melanogenesis.
- All of the CIHs showed a mixed-type inhibition at varying levels in the 21.8–68.9 % range, depending on substrate type and concentration and inhibitor type and concentration. In details, OH exhibited the greatest inhibitory effects with (+)-epicatechin, while CH and LH showed the best TYR inhibition with L-DOPA.
- Because of CIHs did not inhibit the *o*-quinone production, they did not act as reducing agents such as ascorbic acid as the reference inhibitor.
- The anti-browning effectiveness of CIHs was related mainly to the type and concentration of CIHs.

- The GC analysis of their terpene contents indicated that some known TYR inhibitors including myrcene, sabinene, geraniol and citral were present in CIHs.

CIHs exerted significant anti-TYR activity due to their high terpene contents. Thus, the hydrosols recovered from discarded citrus peels can be used as a source of natural TYR inhibitors in food additives or cosmetic and medicinal products since they are not only easy and inexpensive to produce but also without any known hazards for human consumption or contact. Moreover the recovery of these hydrosols could represent an ecofriendly strategy to extend the life cycle of various agro-food products.

5 Third contribution:

Vineyard pruning residues

SCIENTIFIC PUBBLICATION IN ISI-INDEXED JOURNAL

Title: Anti-browning and antioxidant potential in vineyard pruning residues

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Journal: Under review in *Food chemistry*

5.1 Introduction

Enzymatic browning in plant products is associated with most of the qualitative and economic losses in the agro-food industry. The main compound responsible is polyphenol oxidase or tyrosinase (PPO; EC 1.14.18.1), a copper-containing oxidoreductase that catalyses two different reactions involving the oxidation of phenolic compounds and subsequent production of quinones that polymerize to dark pigments known as melanins (Sanchez-Ferrer *et al.*, 1995; Seo *et al.*, 2003). The degree of browning is related to the type and concentration of endogenous phenolic compounds; presence of oxygen, reducing substances and metallic ions; pH; and temperature that affect the PPO activity (Nicolas *et al.*, 1994). The enzymatic reaction in agro-food products leads not only to colour alteration but also to a reduction in the nutritional and organoleptic quality as a consequence of quinone condensation with amino acids and proteins (Rapeanu *et al.*, 2006) and the degradation of polyphenolic substrates recognized for their health benefits as antioxidants (Quideau *et al.*, 2011; Mihaylova *et al.*, 2014).

Most strategies for controlling the enzymatic 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 and its derivatives as well as sulphites (Queiroz *et al.*, 2011), which have critical points related not only to the organoleptic quality but also to nutritional and health claims (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). Therefore, agro industrial wastes and by-products might offer a valid source. They are rich in bioactive compounds (Schieber *et al.*, 2001) not only with anti-browning (Zocca *et al.*, 2010; Lante & Tinello, 2015) but also with strong antioxidant potentials (Moure *et al.*, 2001).

Grapes (*Vitis vinifera*) are the world's largest fruit crop with more than 77 million tons produced in the year 2013. After winemaking, an amount of grape marc between 3 and 6 million tons per year is produced as reported by FAOSTAT data (2015) in the 2000-2013 period, and all of the wine industry by-products, including skins, seeds, stems and dregs, are very rich in phenolic antioxidants (Negro *et al.*, 2003; Rockenbach *et al.*, 2011).

Additionally, vineyards, which cover a large area worldwide, (approximately 7 million Ha in 2013 as reported by FAOSTAT, 2015) annually generate a huge amount of waste. In this

regard, Spinelli *et al.* (2012) suggested an innovative application of the ligno-cellulosic biomass of vineyard pruning residues for achieving industrial bio-fuel.

To date, there are no studies concerning the recovery of bioactive compounds from vineyard waste. Hence, the present study is focused on investigating the anti-browning and antioxidant potentials of berries collected from the pruning residues of two red grape cultivars for the first time in order to find a possible recycling use of these agro-food wastes.

5.2 Materials and methods

5.2.1 Reagents

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), sodium dodecyl sulfate (SDS), 4-tert-butyl catechol (t-BC), 2,4,6-tripyridyls-triazine (TPTZ) and HPLC standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Coomassie Plus Protein Assay Reagent and bovine serum albumin (BSA) were purchased from Pierce (Rockford, USA). NatureSeal® AS1 was obtained from AgriCoat NatureSeal, Berkshire, England.

5.2.2 Preparation of the vineyard pruning residues(VPRs)

Berries were collected from the VPRs of two red grape (*Vitis vinifera*) cultivars, 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, Italia) that usually removed 3-4 bunches per plant producing approximately 2,000 kg of waste per ha annually. Merlot berries at different harvest times in the 2013 season (July corresponding to M1, August, September, and October) were also used for some analyses.

All grape samples were subjected to cold pressing with a small scale centrifuge. After measuring the pH value, the grape juice was centrifuged at 5,000 rpm for 15 min at 4 °C, filtered using Millipore 0.45-μm filter membranes (MA, USA) and stored at −20 °C in dark conditions.

5.2.3 PPO sources

Commercial mushroom tyrosinase (TYR; 3,130 U/ml) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Apples (*Malus domestica* cv. Fuji, Golden Delicious), pears (*Pyrus communis* cv. Abate Fétel, Decana) and potato tubers (*Solanum tuberosum* cv. Bintje) were purchased at commercial maturity from a local market and stored at 4 °C. All fruits and vegetables 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 contents of buffer dilutions of lyophilized PPO were determined by the Bradford (1976) assay using BSA as a protein standard.

5.2.4 Catechol gel diffusion assay

The inhibitory effectiveness of Barbera and Merlot VPRs in the 2013 season (B1 and M1, respectively) at 100% and 50% v/v on TYR activity was evaluated in comparison with 0.05% w/v ascorbic acid as the reference inhibitor, using a test on Petri dishes (Zocca *et al*., 2008). Gel was obtained by dissolving with a microwave oven 2% (w/v) agarose in 0.1 M phosphate citrate buffer (0.2 M di-sodium hydrogen phosphate/0.1 M citric acid) at pH 6.5. Subsequently the solution was cooled to 70°C and 10 mM catechol was added. A volume of 20 ml of this solution was placed in each plate, cooled at room temperature and stored at 4°C in dark condition. Wells of 3 mm diameter were obtained in order to create a volume per well of approximately 20 µl. After that, 2 µl of TYR, previously solubilized in 0.1 M sodium citrate buffer at pH 6.0 to a final concentration of 9,390 U/ml, and 18 µl of VPR were added. The control wells (C) were loaded with the same amount of enzyme, previously diluted with distilled water. The TYR inhibition was evaluated by monitoring the presence/absence of dark rings around the wells, as index of enzymatic browning, up to 24 h of incubation at 25°C, under lightless conditions. The plate images were captured using scanner.

5.2.5 TYR activity inhibition

The inhibitory effects of VPR from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars on the activity of TYR, previously solubilized in 0.1 M sodium citrate buffer (0.1 M trisodium citrate dihydrate/0.1 M citric acid) at a pH = 6.0 to a final concentration of 9,390 U/ml, was spectrophotometrically quantified in accordance with Zocca *et al.* (2011) at 400 nm and 25 °C with 10 mM catechol as the substrate and 0.05% w/v ascorbic acid (AA) as the reference inhibitor. The control reaction mixture included 1.0 ml of 10 mM catechol, 200 µl of 0.1 M sodium citrate buffer at pH 6.0 and 5 µl of TYR. The inhibitor reaction mixture was obtained by substituting the sodium citrate buffer with respectively AA and vineyard wastes. The same reaction mixtures without enzyme were used as blank. The TYR kinetic was monitored at 25°C for 5 min by measuring the absorbance value (A) at 420 nm with a Varian Carry 50 Bio UV/Vis spectrophotometer. The enzymatic activity was calculated as absorbance variation per minute (ΔA/min) considering the linear part of kinetic curve and after the lag phase only in the presence of AA (Alam *et al*., 2011). One unit of enzyme activity corresponded to the amount of enzyme that caused an increase of 0.001 in absorbance per minute at 420 nm and at 25°C.

The percent inhibition of TYR activity was calculated as follows (Baurin *et al.*, 2002):

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

where $\Delta A_{control}$ = absorbance variation per minute at 400 nm without inhibitor and $\Delta A_{inhibitor}$ = absorbance variation per minute at 400 nm with inhibitor.

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

The inhibitory kinetics of all of the vineyard wastes were analysed using Lineweaver-Burk plots at different concentrations of catechol substrate in order to calculate the kinetic constants (K_M and V_{max}) and define the inhibition type as described by Lante & Tinello (2015). The Lineweaver-Burk plots were obtained from the Michaelis-Menten curves by plotting the reciprocal of initial velocity of enzymatic reaction corresponding to TYR activity ($1/V_0$ equal to 1/U/min) on the Y-axis against the reciprocal of substrate concentration (1/[S]) on the X-axis. The Michaelis-Menten constant (K_M) and maximum reaction velocity (V_{max}) were determined respectively from the slope and intersect of the straight lines.

5.2.6 Non-reducing SDS-PAGE electrophoresis and PPO zymography

The inhibitory potentials of VPRs (B1, B2, M1, and M2) and 0.05% v/v AA as a reference inhibitor were also assessed on the isoforms isolated from commercial mushroom TYR and some plant PPOs. Non-reducing SDS-PAGE and zymographic techniques with the L-DOPA/MBTH complex were performed in a Mini Protean II (Bio- Rad, Milano, Italy) at room temperature, with a 12% polyacrylamide gel at 100 V, following the procedure of Zocca *et al.* (2011). Initially, the commercial TYR (1 mg) and the freeze-dried powders of apples, pears and potatoes (400, 200, and 20 mg, respectively) were solubilized with 700 µl of distilled water and 300 µl of Laemmli buffer (1.33 M Tris pH = 7.4, 40% v/v glycerol, 8% w/v SDS; Laemmli, 1970) and centrifuged at 14,000 rpm for 2 min. Each gel well was loaded as follows: 5 µl of TYR solution (3,130 U/ml) and 15 µl of other plant PPOs. The running buffer was composed of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. After electrophoresis, the gels were exhaustively washed for 15 min in 0.2 M sodium acetate buffer (0.2 M sodium acetate/0.2 M acetic acid) at pH 5.0 and cut in several lanes. A distilled water volume of 4 ml was added to control lane (C) while the other lanes were treated with 3.5 ml of distilled water and 0.5 ml of VPR and 0.05% v/v AA. After waiting 15 min, each gel lane was added with 4 ml of sodium acetate buffer at pH 5.0 containing 5 mM L-DOPA and 3 mM MBTH and was incubated at 25°C for 30 min (Núñez-

Delicado *et al*., 2005). The presence of deep pink bands in treated gel lanes was associated to PPO activity. The images of zymograms were acquired by a scanner.

5.2.7 Anti-browning effect on fresh-cut fruits and vegetables

The anti-browning potentials of VPRs from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars were evaluated *in vivo* (Zocca *et al.* 2011) on several fresh-cut apples (*Malus domestica* cv. Fuji, Golden Delicious), pears (*Pyrus communis* cv. Abate Fétel, Decana), potatoes (*Solanum tuberosum* cv. Bintje) and eggplants (*Solanum melongena*) in comparison with 0.05% w/v ascorbic acid (AA) as a reference PPO inhibitor. Moreover, the anti-browning effects of Merlot pruning residues in the 2013 and 2014 seasons (M1 and M2, respectively) on Golden Delicious apple slices were compared with reference anti-browning formulations including an aqueous solution of 1% (w/v) ascorbic acid and 0.5% w/v calcium chloride (AAC) as well as 6% (w/v) NatureSeal® AS1.

All fruits and vegetables were purchased at commercial maturity from a local market and stored at 4 °C. Each plant 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. A control slice (C) was sprayed on the surface with 1 ml of distilled water using a syringe, and the other one was treated with 1 ml of anti-browning formulation. After waiting 15 min at 25 °C, the surface of each slice was wiped and treated with 1 ml of 10 mM catechol as a PPO substrate. Browning was observed before and after chemical treatment, and after catechol application at 10, 30 and 60 min, by acquiring the images with a digital camera and by measuring the colour surface using a Tristimolus colorimeter (Chroma Meter CR-410, Konica-Minolta, Milan, Italy) in the CIE 1976 (L*, a*, b*) colour space.

The anti-browning effect was expressed as the colour change (ΔE) according to the following equation (Ozoglu & Bayindirli, 2002):

$$\Delta E = \sqrt{(\Delta L^2 + \Delta \alpha^2 + \Delta b^2)^2} = \sqrt{(L_t - L_{t0})^2 + (\alpha_t - \alpha_{t0})^2 + (b_t - b_{t0})^2}$$

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 in colour change (%RΔE) was also calculated as follows (Ozoglu & Bayindirli, 2002):

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

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

5.2.8 Anti-browning effect on dried apples

The anti-browning potentials of VPRs from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars were evaluated *in vivo* (Zocca *et al.* 2011) on Golden Delicious apple slices subjected to the subsequent drying processing, in comparison with reference anti-browning formulations including an aqueous solution of 1% (w/v) ascorbic acid and 0.5% w/v calcium chloride (AAC) as well as 6% (w/v) NatureSeal® AS1. Apple slices, which were pre-treated which each anti-browning formulation for 15 min at 25 °C (I15), were dried using a Biosec De Luxe B6 dryer (Tauro Essiccatori, Camisano Vicentino, VI, Italy) for 18 hours at 45°C (DR1080). After that, all dried slices were covered with 1 ml of 10 mM catechol as the substrate to accelerate the enzymatic browning. Browning was monitored before and after chemical pre-treatment, and after drying and catechol application at 10, 30, and 60 min, by acquiring the images with a digital camera and by measuring ΔE and %RΔE as previously described (see 8.2.7 section).

The water activity of untreated and treated apple slices was also determined before and after drying using LabMaster-aw instrument (Novasina AG, Lachen Switzerland).

5.2.9 Determination of quinone inhibition

The level of 4-tert-butyl-o-benzoquinones after the chemical oxidation of t-BC using $NaIO_4$ was detected spectrophotometrically at 400 nm and 25 °C in accordance with Lante & Tinello (2015). The reaction mixture contained 50 µl of 2 mM $NaIO_4$ and 1.0 ml of 1 mM t-BC in the absence and presence of inhibitors (200 µl). The VPRs from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars were tested in comparison to ascorbic and citric acids at the concentrations corresponding to 50% (AA50 = 0.0019 mM and AC50 = 0.29 mM, respectively) and 100% of TYR inhibition (AA100 = 0.0039 mM and AC100 = 0.57 mM, respectively).

5.2.10 Whitening effect

The whitening effect was also evaluated by spectrophotometrically measuring the accumulation of dark compounds formed after the chemical oxidation of a catechol solution at 400 nm and 25 °C. The reaction mixture contained 50 µl of 4 mM NaOH and 1.0 ml of 10 mM catechol in the absence and presence of inhibitors (200 µl). The VPRs from Barbera (B1 and B2)

and Merlot (M1 and M2) cultivars were tested in comparison to ascorbic and citric acids at the concentrations corresponding to 50% (AA50 = 0.0019 mM and AC50 = 0.29 mM, respectively) and 100% of TYR inhibition (AA100 = 0.0039 mM and AC100 = 0.57 mM, respectively).

5.2.11 Antioxidant activities

The antioxidant activities of VPRs (B1, B2, M1, and M2) and Merlot berries at different harvest times in the 2013 season (July corresponding to M1, August, September, and October) were estimated using the two following spectrophotometric assays based on electron transfer (Huang *et al.*, 2005). The antioxidant activity was expressed as Trolox equivalents (mg TE) per ml of sample previously diluted in ethanol (EtOH).

DPPH assay

The 2, 2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) assay was carried out according to the slightly modified method of Miliauskas *et al.* (2004). Initially, an ethanolic solution of DPPH (0.2 mM) was daily prepared and covered with aluminum foil. A DPPH volume of 500 µl was mixed with 500 µl of sample previously diluted in EtOH. A control solution was also prepared by substituting the sample with the same EtOH volume. After waiting 30 min at room temperature, under dark conditions, the absorbance value of each mixture was measured at 515 nm and at 25 °C with a Varian Carry 50 Bio UV/Vis spectrophotometer using a 1 ml volume of EtOH as blank.

The radical scavenging activity was calculated as follows:

$$\%DPPH = ((A_{control} - A_{sample})/A_{control}) \times 100$$

where $A_{control}$ = absorbance at 515 nm without antioxidant sample and A_{sample} = absorbance at 515 nm with antioxidant sample.

FRAP assay

The ferric ion reducing antioxidant power (FRAP) assay was carried out according to Stratil *et al*. (2006). The FRAP reagent was daily prepared by mixing a solution of 0.01 M TPTZ in 40 mM HCl with the same volume of an aqueous solution of 0.02 M $FeCl_3$ and 10 times higher volume of 0.2 M sodium acetate buffer (0.2 M sodium acetate/0.2 M acetic acid). After that, it was wrapped with aluminum foil and incubated at 37 °C for 5 minutes. A FRAP volume of 900 µl was mixed with 100 µl of sample previously diluted in EtOH and incubated at 37 °C for 40, min under dark conditions. A blank solution was also prepared by substituting the sample with the same EtOH volume. The absorbance value of each mixture was measured at 593 nm and at 25°C with a Varian Carry 50 Bio UV/Vis spectrophotometer.

5.2.12 Total phenolic content

The total phenolic contents of VPRs (B1, B2, M1, and M2) and Merlot berries at different harvest times in the 2013 season (July corresponding to M1, August, September, and October) were quantified by the Folin–Ciocalteu method in accordance to Azuma *et al*., (1999). A 1 ml volume of sample properly diluted in ethanol was mixed with 0.5 ml of Folin-Ciocalteu reagent previously diluted two times in distilled water and 5 ml of 10% w/v Na_2CO_3 containing 1 M NaOH. A blank solution was also prepared by substituting the sample with the same volume of ethanol. After waiting 30 min at room temperature under dark conditions, the absorbance value of each mixture previously filtered using Millipore 0.22 μm filter membranes (MA, USA) was measured at 650 nm and at 25°C with a Varian Carry 50 Bio UV/Vis spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (mg GAE) per ml of sample previously diluted in EtOH.

5.2.13 HPLC analysis

The bioactive compounds of VPRs (B1, B2, M1, and M2) and Merlot berries at different harvest times in the 2013 season (July, August, September, and October) were characterized by HPLC using a Thermo Finnigan SpectraSystem UV6000LP HPLC system (Thermo Finnigan, San Jose, CA, USA) with diode-array detection (DAD). The identification of bioactive compounds was performed by comparing their retention times with those of commercial standards. Before their injection into the column, samples were filtered through 0.22 μm cellulose acetate filters (Millipore, USA).

Organic acids (citric, fumaric, L-malic, oxalic, succinic and tartaric) were quantified using a Aminex HPX-87H column (Bio-Rad, CA, USA) according to the method proposed by Nardi *et al.* (2003). The mobile phase consisted of 0.0025 N sulfuric acid. The HPLC analysis was carried out at 60 °C with a run time of 60 min and a flow rate of 0.6 ml/min.

The phenolic compounds (caffeic acid, (+)–catechin, chlorogenic acid, (-)–epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and gallic acid) were quantified using a Supelcosil™ LC-18 column (Sigma-Aldrich, MO, USA) in accordance with the method described by Zocca *et al.* (2011). The mobile phase included a mixture of water acidified with sulfuric acid (pH = 2.5) and methanol at different gradient elutions and flow rates. The HPLC analysis was carried out at 40 °C with a run time of 100 min and a DAD wavelength in the 200–600 nm range.

5.2.14 Statistical analysis

Statistical analysis was performed by subjecting all of the data obtained from three replicates to one-way analysis of variance (ANOVA) using R software (3.1.2 version) after

verifying a normal distribution and homogeneity of variance. Significant differences were determined by Tukey's multiple range test ($P \leq 0.05$).

5.3 Results and discussion

5.3.1 Evaluation in vitro of the anti-browning potential in VPRs

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

An initial screening to assess the inhibition of TYR by Barbera and Merlot wastes in the 2013 season (B1 and M1, respectively) was performed, using catechol gel diffusion (Figure 5.1). The VPRs better limited the appearance over time of the dark rings around the wells than the control (C) and reference inhibitor (AA).

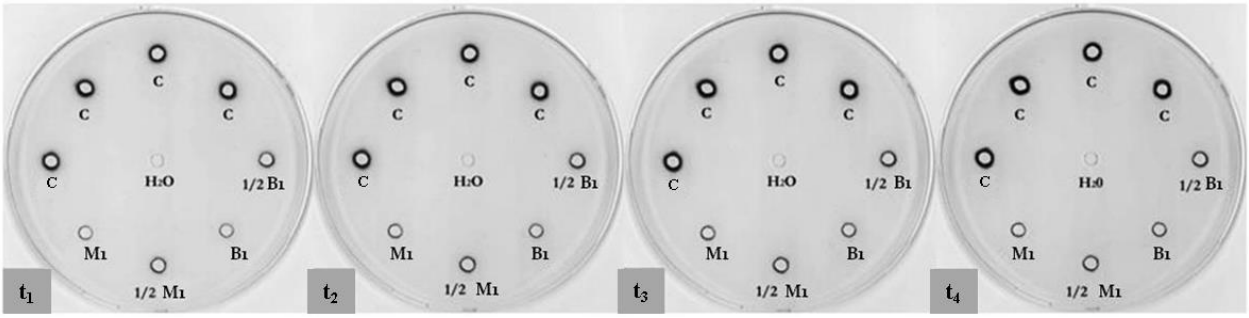

Figure 5.1 Inhibition of TYR (2 µl, 18 U; C) on 10 mM catechol agarose plate after incubation for 30 min (t1), 1h (t2), 2h (t3) and 24 h (t4) at 25°C under dark conditions. C: control. B1 and M1: Barbera and Merlot wastes in the 2013 season at 100% v/v. ½ B1 and ½ M1: Barbera and Merlot wastes in the 2013 season at 50% v/v.

The inhibitory effects of VPRs on a TYR were also quantified spectrophotometrically in comparison with 0.05% ascorbic acid (AA) as a reference anti-browning compound, using 10 mM catechol as the phenolic substrate (Figure 5.2).

All of the inhibitors tested significantly decreased ($P \leq 0.001$) the enzymatic activity with a TYR inhibition of more than 50% compared to the control (C). Although AA exhibited the best anti-TYR performance (85.7%), all VPRs showed stronger TYR inhibition than pomegranate extract (27.5%; Zocca *et al.,* 2011) and *Brassicacaea* processing water (23.2%; Zocca *et al.,* 2010). Among the vineyard wastes, M1 and M2 (68.2% and 67.8% TYR inhibition, respectively) better limited the enzymatic browning than B1 and B2 (56.3% and 58.8% TYR inhibition, respectively). Because the spectrophotometric results achieved from the VPRs of the 2013 season were confirmed by those from the 2014 season, the anti-browning effectiveness was related only to grape cultivar.

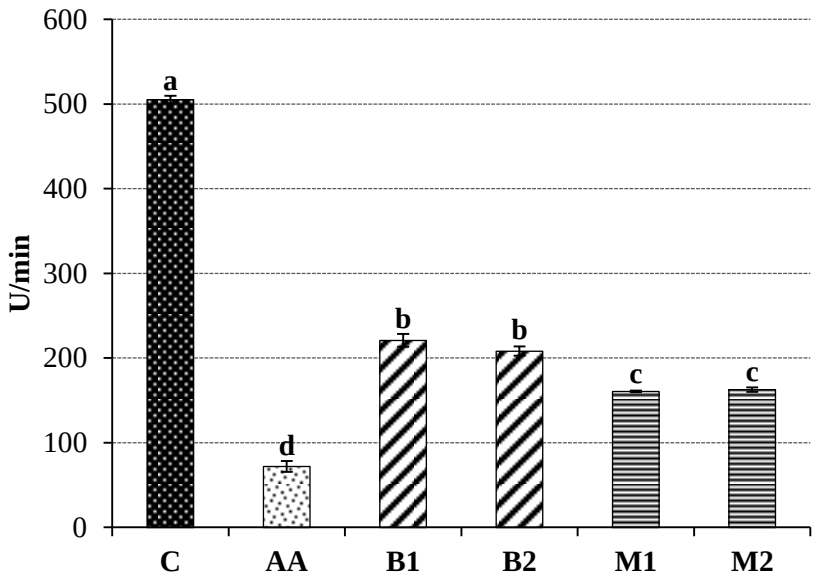

Figure 5.2 The enzymatic activity of TYR in the absence (C) and presence of 0.05 % w/v ascorbic acid (AA) and VPRs from Barbera and Merlot cultivars in the 2013 and 2014 seasons (B1, B2, M1, and M2, respectively) using 10 mM catechol as the substrate.

Moreover, all of the vineyard wastes did not show a LAG phase, which was a typical characteristic of reducing agents such as AA (20 seconds) that indirectly inhibited TYR activity by reducing o-quinones to o-diphenols and thus slowing the biosynthesis of dark compounds (Ros *et al.,* 1993). In this regard, the level of quinone inhibition was investigated spectrophotometrically in order to determine if the VPRs from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars acted as real TYR inhibitors in comparison with ascorbic and citric acids at the concentrations corresponding to 50% (AA50 and CA50) and 100% (AA100 and CA100) of TYR inhibition. The o-quinones can also be formed after the exposition of catechols to oxygen without involving enzyme activity. On the basis of spectrophotometric results (Figure 5.3), ascorbic acid at both anti-TYR concentrations (AA50 and AA100) significantly reduced ($P \leq 0.001$) the absorbance value at 400 nm of the control mixture (without inhibitors), confirming its indirect inhibition of enzyme activity. Instead, citric acid at both anti-TYR concentrations (CA50 and CA100) and all of the vineyard wastes did not limit the *o*-quinone production, thus confirming their direct inhibition.

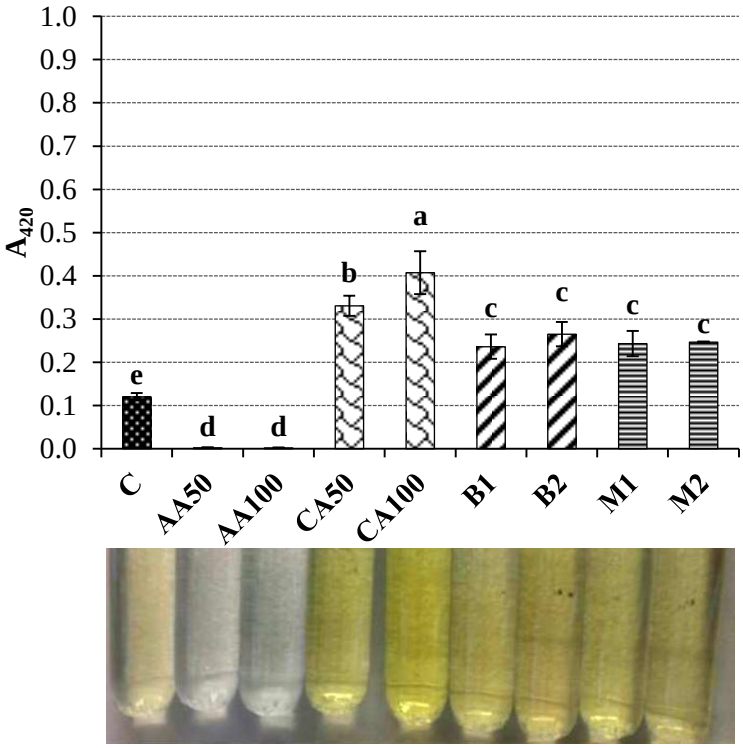

Figure 5.3 The effect of PPO inhibitors on *o*-quinone production, using 1 mM t-BC. C: control without inhibitors. AA50 and AA100: ascorbic acid at the concentration corresponding to 50% and 100% of TYR inhibition. CA50 and CA100: citric acid at the concentration corresponding to 50% and 100% of TYR inhibition. B1 and B2: VPRs from Barbera cultivar in the 2013 and 2014 seasons. M1 and M2: VPRs from Merlot cultivar in 2013 and 2014 seasons.

The mechanism of TYR inhibition was defined by spectrophotometrically measuring the enzymatic kinetic constants using the Lineweaver–Burk plots at different catechol concentrations in the absence and presence of VPRs (Figure 5.4).

The double-reciprocal plots described a family of lines that intersected the vertical axis at different points. In particular, the lines of VPRs were almost parallel to the control line. As a consequence, V_{max} and K_M values of the entire vineyard wastes decreased in comparison to those of the control (C), confirming an uncompetitive inhibition where the reversible inhibitor reacted only with enzyme-substrate complex.

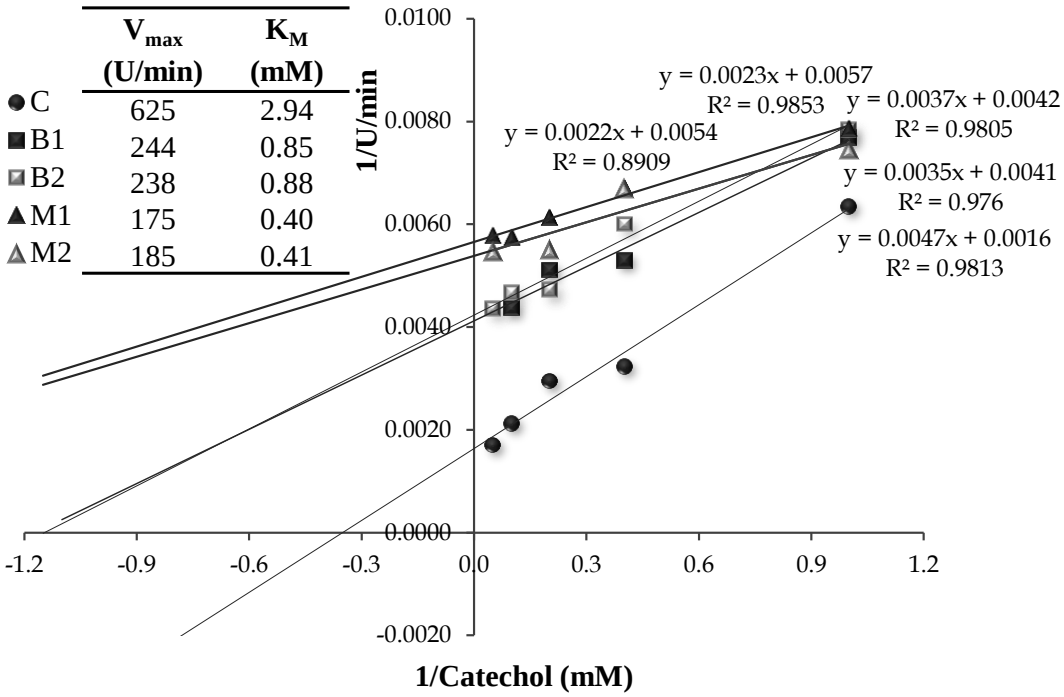

Figure 5.4 Lineweaver-Burk plots and corresponding kinetic constant values of TYR in the absence (C) and presence of VPRs from Barbera and Merlot cultivars in the 2013 and 2014 seasons (B1, B2, M1, and M2, respectively) using catechol as the substrate.

Next, the anti-browning effects of VPRs from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars and 0.05% w/v ascorbic acid (AA) were tested by carrying out electrophoretic assays on a commercial TYR and some plant PPOs in order to isolate the corresponding isoforms (Figure 5.5). The zymographic technique was a useful tool to visualize the activity of PPO isoforms with/without inhibitors by monitoring the appearance of bands in the gel (Martinez-Alvarez *et al.*, 2008).

All vineyard wastes showed a greater inhibitory effect on the activity of the one isoform of TYR and potato PPO than AA by completely reducing the colour intensity of the corresponding bands (Figure 5.5A Figure 5.5B, respectively). 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 noncyclizable (catechol) or cyclizable (L-DOPA) diphenolic substrates (Sanchez-Ferrer *et al.*, 1995). The TYR enzyme exhibited a different stereo specificity among phenolic substrates with a greater affinity for dihydroxyphenols, especially for L-isomers (Seo *et al.*, 2003). In the case of potato PPO, the zymographic results were confirmed by *in vivo* trials on fresh-cut potatoes as the colour changes of slices treated with Merlot and Barbera pruning residues from the 2013 season (M1 and B1, respectively) were preserved after 10 min of the application of 10 mM catechol (Figure 5.6).

A lower inhibitory effectiveness by vineyard wastes was achieved on the one isoform of Golden Delicious apple PPO and four isoforms of Fuji apple PPO (Figure 5.5C and D, respectively). Additionally, the *in vivo* assay on fresh-cut apples confirmed the good anti-browning performance of VPRs, especially from the Merlot cultivar (Figure 5.8Figure 5.9, respectively).

Additionally, any evident anti-browning effect was observed on the zymograms of Abate Fétel and Decana pear PPOs (Figure 5.5E) as confirmed by *in vivo* trials on fresh-cut pears. The different anti-browning performance among plant PPOs confirmed that the inhibitory effectiveness was mainly related to the enzyme source, as spectrophotometrically demonstrated by Zocca *et al.* (2010 and 2011).

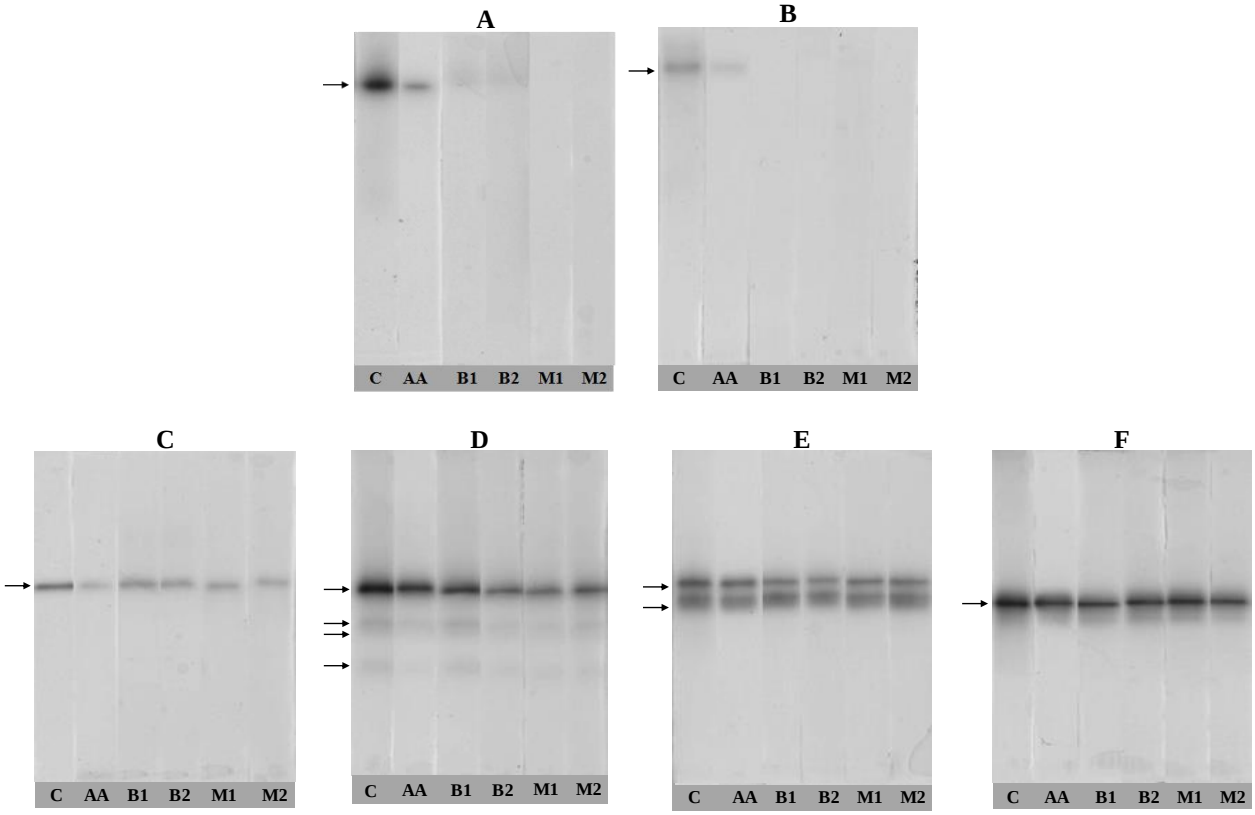

Figure 5.5 Zymograms of TYR and PPOs activity in the absence (C) and presence of 0.05 % w/v ascorbic acid (AA) and VPRs from Barbera and Merlot cultivars in the 2013 and 2014 seasons (B1, B5, M1, and M2, respectively). A: TYR (16 U per lane). B: potato PPO (14.62 μg of protein per lane). C and D: Golden Delicious and Fuji apple PPOs (14.62 and μg 4.84 of protein per lane, respectively). E and F: Abate Fétel and Decana pear PPOs (12.76 and 2.17 μg of protein per lane, respectively).

5.3.2 Evaluation in vivo of the anti-browning potential in VPRs

The anti-browning potentials of VPRs from Barbera and Merlot cultivars in the 2013 season (B1 and M1, respectively) were widely evaluated *in vivo*, after different times of catechol application at 25°C, on fresh-cut potatoes (Bintje, Figure 5.6), eggplants (Figure 5.7), apples

(Golden Delicious, Figure 5.8; Fuji, Figure 5.9; Granny Smith, Figure 5.10), and pears (Abate Fétel, Figure 5.11).

The VPRs, especially from Merlot cultivar, showed greater anti-browning performances than untreated control (C) and even 0.05% ascorbic acid as the reference inhibitor (AA) on the most of fresh-cut fruits and vegetables. Among the fresh-cut vegetables, they were more effective in potato slices (Figure 5.6c), whose colour surface was still clear at 60 min, rather than in eggplant slices (Figure 5.7c). Also in fresh-cut apples, especially from Golden Delicious and Fuji cultivars, the treatment with VPRs effectively limited over time the colour alterations, with strong inhibitory capacities with 10 minutes of catechol application (Figure 5.8a Figure 5.9b, respectively). Any anti-browning effect was observed towards the PPO activity of Abate Fétel pear slices (Figure 5.11).

The *in vivo* results, which confirmed those of PPO zymograms (Figure 5.5), demonstrated that the anti-browning effectiveness was related mainly to the type and cultivar of fruits and vegetables.

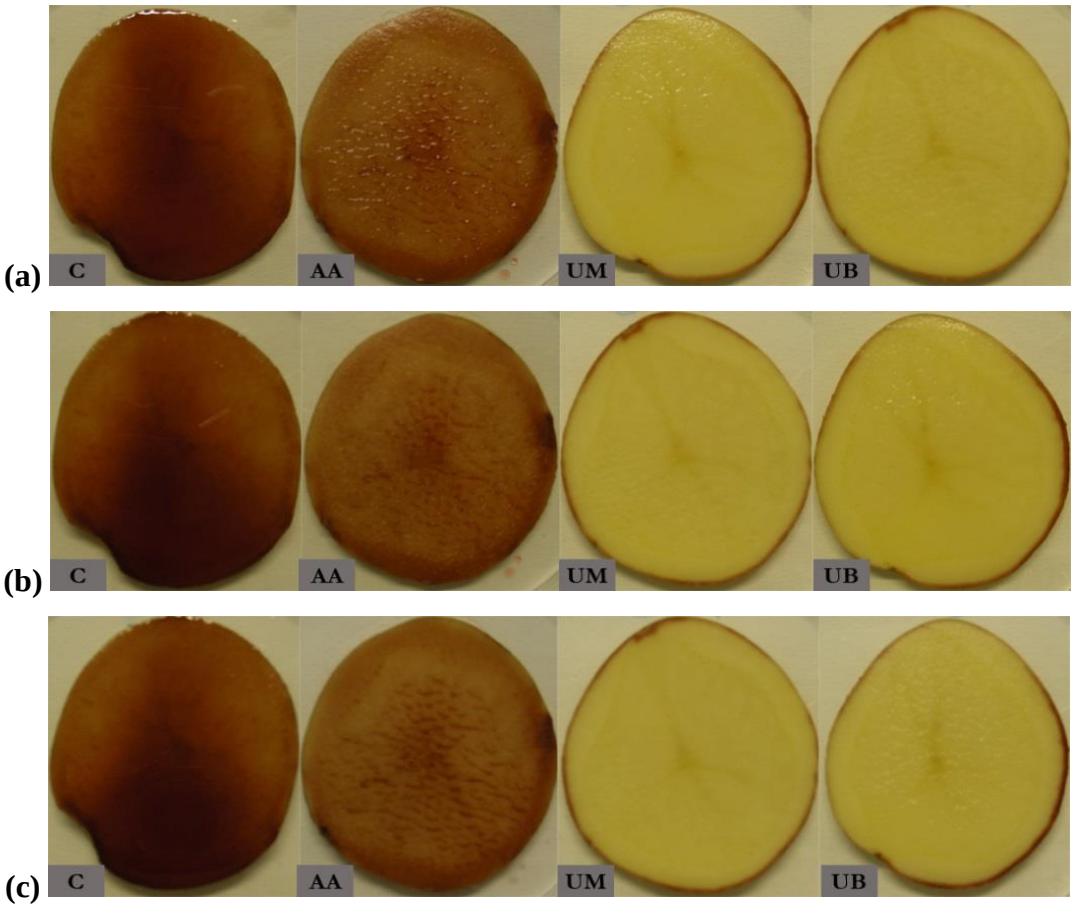

Figure 5.6 The anti-browning effects of some formulations on Bintje potato slices after 10 (a), 30 (b), and 60 min (c) of 10 mM catechol application at 25 °C. C: control without inhibitors. AA: 0.05 % w/v ascorbic acid. UM = M1: VPRs from Merlot cultivar in the 2013 season. UB = B1: VPRs from Barbera cultivar in the 2013 season.

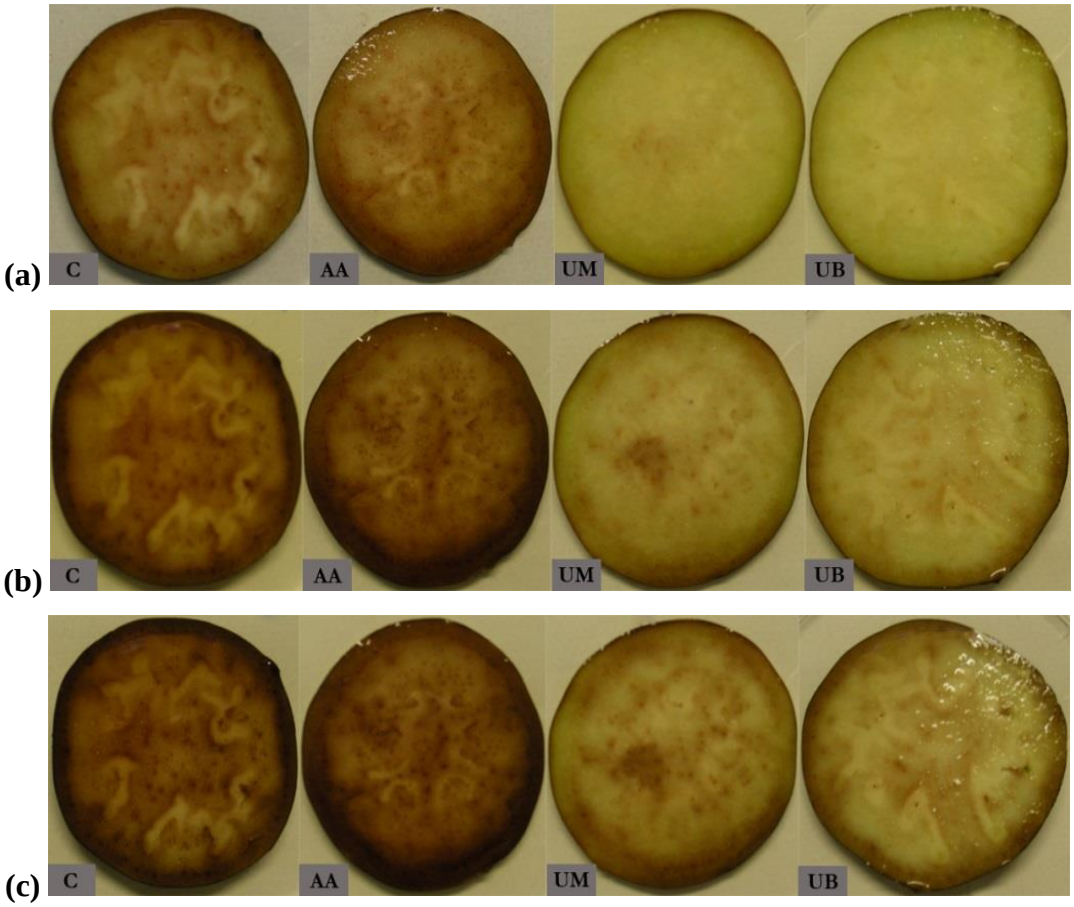

Figure 5.7 The anti-browning effects of some formulations on eggplant slices after 10 (a), 30 (b), and 60 min (c) of 10 mM catechol application at 25 °C. C: control without inhibitors. AA: 0.05 % w/v ascorbic acid. UM = M1: VPRs from Merlot cultivar in the 2013 season. UB = B1: VPRs from Barbera cultivar in the 2013 season.

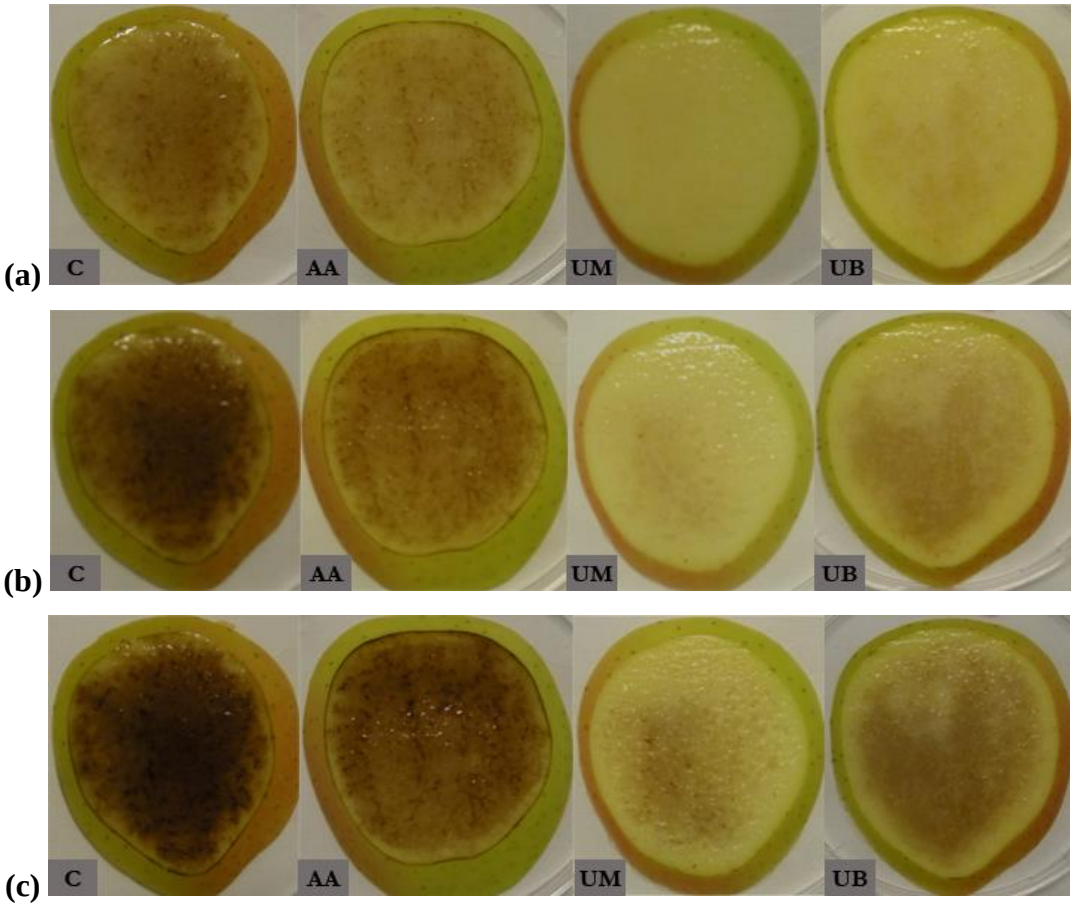

Figure 5.8 The anti-browning effects of some formulations on Golden Delicious apple slices after 10 (a), 30 (b), and 60 min (c) of 10 mM catechol application at 25 °C. C: control without inhibitors. AA: 0.05 % w/v ascorbic acid. UM = M1: VPRs from Merlot cultivar in the 2013 season. UB = B1: VPRs from Barbera cultivar in the 2013 season.

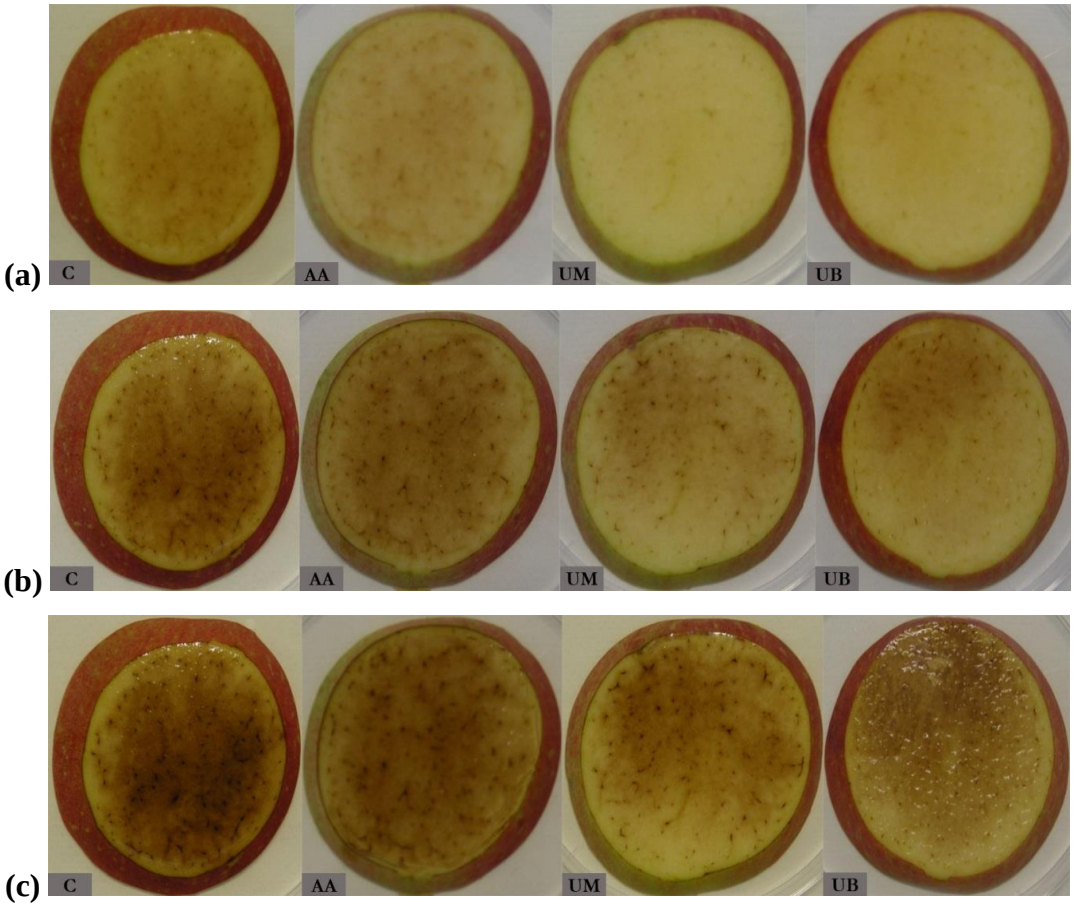

Figure 5.9 The anti-browning effects of some formulations on Fuji apple slices after 10 (a), 30 (b), and 60 min (c) of 10 mM catechol application at 25 °C. C: control without inhibitors. AA: 0.05 % w/v ascorbic acid. UM = M1: VPRs from Merlot cultivar in the 2013 season. UB = B1: VPRs from Barbera cultivar in the 2013 season.

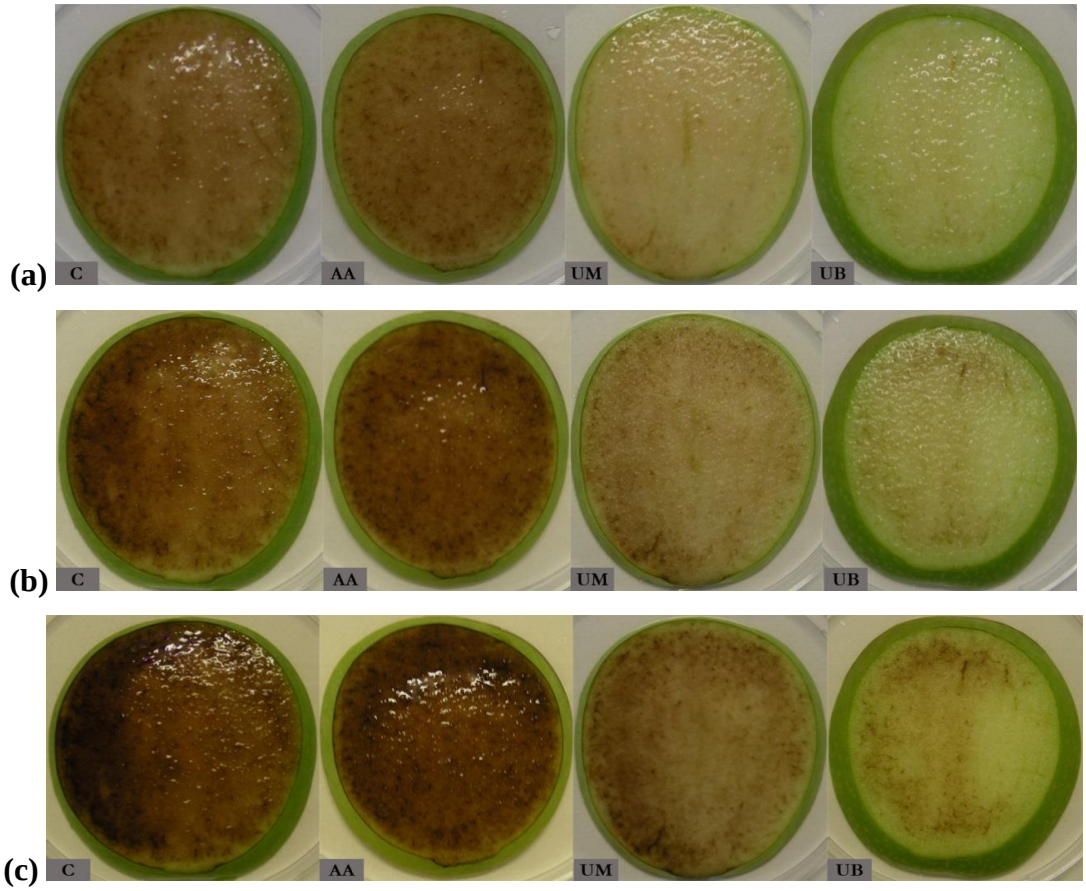

Figure 5.10 The anti-browning effects of some formulations on Granny Smith apple slices after 10 (a), 30 (b), and 60 min (c) of 10 mM catechol application at 25 °C. C: control without inhibitors. AA: 0.05 % w/v ascorbic acid. UM = M1: VPRs from Merlot cultivar in the 2013 season. UB = B1: VPRs from Barbera cultivar in the 2013 season.

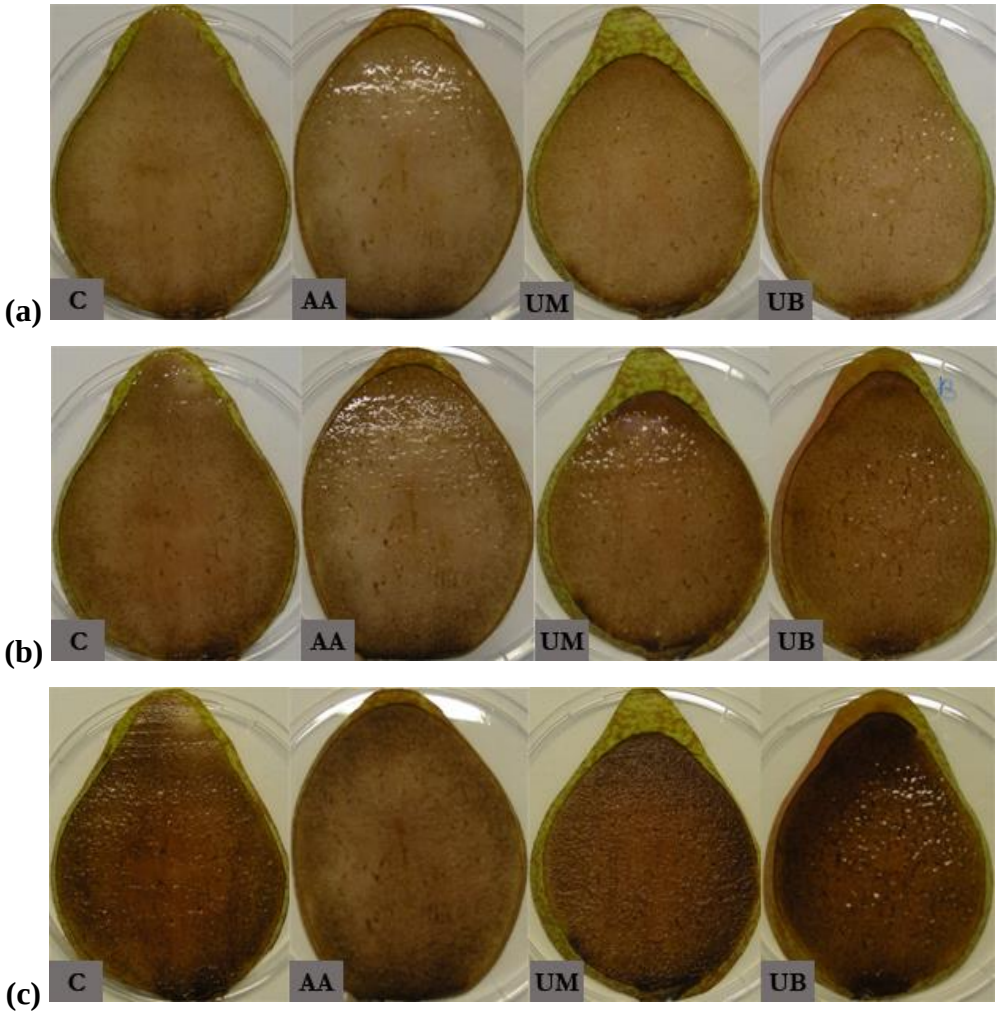

Figure 5.11 The anti-browning effects of some formulations on Abate Fétel pear slices after 10 (a), 30 (b), and 60 min (c) of 10 mM catechol application at 25 °C. C: control without inhibitors. AA: 0.05 % w/v ascorbic acid. UM = M1: VPRs from Merlot cultivar in the 2013 season. UB = B1: VPRs from Barbera cultivar in the 2013 season.

Merlot pruning residues (M1 and M2), which showed better *in vitro* performance than the Barbera residues, were also applied for 15 min at 25 °C on Golden Delicious apple slices in order to compare their effectiveness in controlling enzymatic browning *in vivo* with an aqueous solution of 1% w/v ascorbic acid and 0.5% w/v calcium chloride (AAC) as well as 6% w/v NatureSeal® (AS1) as reference anti-browning formulations. The colour change (ΔE) on apple slice surface was monitored over time by digital camera (Figure 5.13) and was quantified by colorimetric analysis (Table 5.1) immediately after the chemical treatment with each anti-browning formulation and different times of catechol application used for accelerating enzymatic browning. As reported in Table 5.1, the application of all anti-browning formulations resulted significantly effective ($P \leq 0.001$) in controlling ΔE of fresh-cut apples, compared to untreated samples (C). In details, the ΔE reduction of M1 and M2 wastes (42.3% and 47.3%, respectively) resulted lower that of AAC (72.3%) and AS1 (56.6%), after 15 min of chemical application. The

anti-browning effect of formulations applied was more evident after the addition of phenolic substrate (Figure 5.13). In this case, the surface colour of apple slices treated with AAC and AS1 was unaltered up to 60 min catechol application (Figure 1.1e) showing a RΔE of approximately 96% for both reference inhibitors (Table 5.1). Meanwhile, brown spots appeared in the presence of Merlot pruning residues after 30 min of catechol addition (Figure 5.13d) as confirmed by the higher ΔE values measured (Table 5.1). As regards 10 min of catechol treatment (Figure 5.13a), the application of all formulations significantly preserved ($P \leq 0.001$) the colour change (ΔE) of fresh-cut apples compared to untreated samples (C). Although AAC and AS1 were more effective than the vineyard wastes, with a reduction in colour change (RΔE) of 95.7% and 93.7%, respectively, M1 and M2 showed strong anti-browning potentials with RΔE values of 79.4% and 85.8%, respectively.

Table 5.1 The effect of anti-browning formulations on the colour change of Golden Delicious apple slices subjected to chemical treatment and catechol application at different times.

	Anti-browning formulations[1]					
Treatment times[2]	**C**	**AAC**	**AS1**	**M1**	**M2**	***P*-value**[3]
I15						
ΔE	$3.00^{a} \pm 0.37$	$0.83^{d} \pm 0.15$	$1.30^{cd} \pm 0.03$	$1.73^{b} \pm 0.12$	$1.58^{bc} \pm 0.43$	***
RΔE (%)	–	72.3	56.6	42.3	47.3	
CAT10						
ΔE	$21.03^{a} \pm 1.5$	$0.90^{d} \pm 0.35$	$1.32^{cd} \pm 0.19$	$4.33^{b} \pm 0.43$	$2.98^{bc} \pm 0.17$	***
RΔE (%)	–	95.7	93.7	79.4	85.8	
CAT30						
ΔE	$33.16^{a} \pm 1.59$	$1.22^{d} \pm 0.25$	$1.56^{d} \pm 0.23$	$10.88^{b} \pm 0.02$	$5.14^{c} \pm 0.41$	***
RΔE (%)	–	96.3	95.3	67.2	84.5	
CAT60						
ΔE	$42.47^{a} \pm 0.70$	$1.87^{d} \pm 0.66$	$1.65^{d} \pm 0.10$	$20.58^{b} \pm 0.27$	$10.36^{c} \pm 1.05$	***
RΔE (%)	–	95.6	96.1	51.5	75.6	

[1] C: control. AAC: 1% w/v ascorbic acid and 0.5% w/v $CaCl_2$. AS1: 6% w/v NatureSeal®. M1 and M2: Merlot pruning residues in the 2013 and 2014 seasons, respectively.

[2] I15: chemical treatment with anti-browning formulation at 25°C for 15 min. CAT10, CAT30, CAT60: application of 10 mM catechol at 25°C for 10, 30, and 60 min, respectively.

[3] '***' $P \leq 0.001$.

[a, b, c, d] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

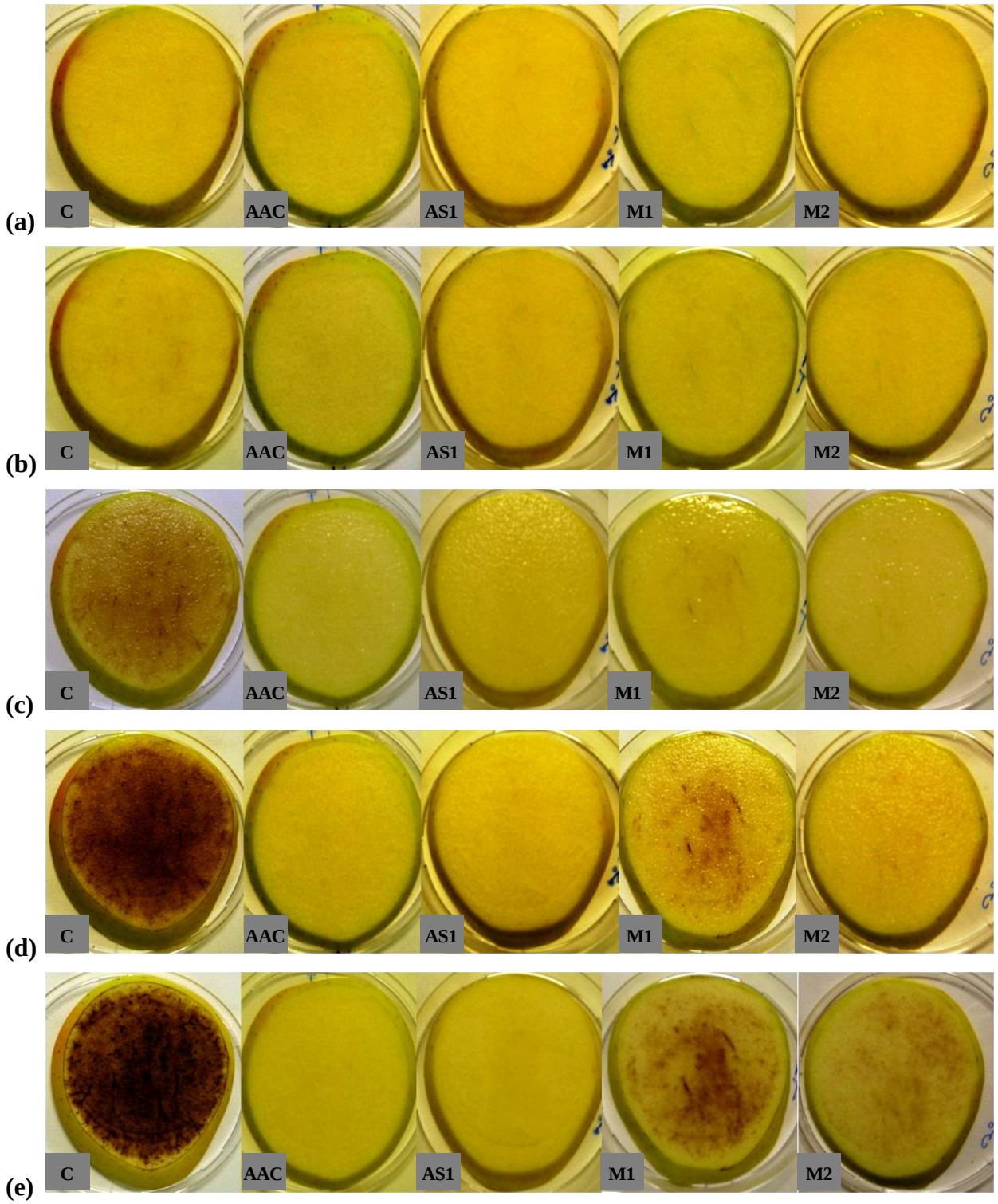

Figure 5.12 The anti-browning effect of anti-browning formulations on fresh cut Golden delicious apples before (a) and after 15 min of chemical treatment at 25°C (b), and after 10, 30, 60 min of 10 mM catechol application at 25°C (c, d, and e, respectively).

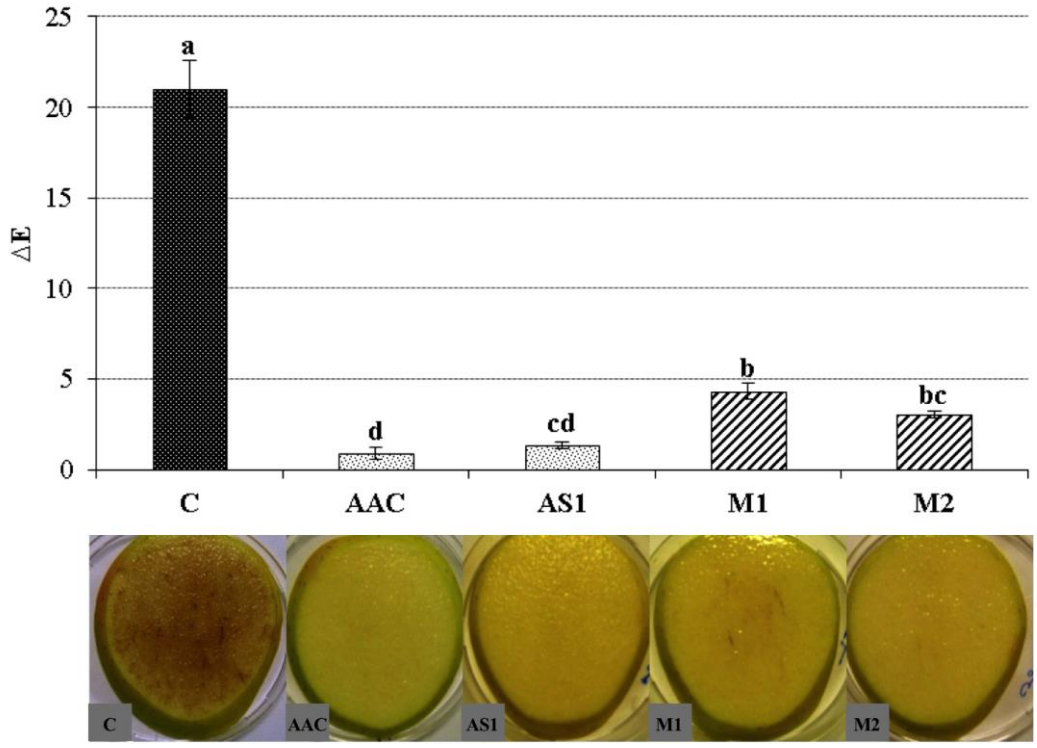

Figure 5.13 The anti-browning effects of some formulations on Golden Delicious apple slices after 10 min of 10 mM catechol application at 25 °C. C: control without inhibitors. AAC: 1% w/v ascorbic acid and 0.5% w/v calcium chloride. AS1: 6% w/v NatureSeal®. M1 and M2: vineyard pruning residues from the Merlot cultivar in the 2013 and 2014 seasons.

The VPRs from Merlot cultivar in the 2013 and 2014 seasons (M1 and M2, respectively) were also sprayed on Golden Delicious apple slices subjected then to drying processing for 18 hours at 45°C, in order to evaluate their anti-browning potential in comparison to AAC and AS1 as the reference PPO inhibitors. The colour change (ΔE) on apple slice surface was monitored over time by digital camera (Figure 5.14) and was quantified by colorimetric analysis (Table 5.2) before and after chemical pre-treatment (I15), and after drying (DR1080) and catechol application at 10, 30, and 60 min (CAT10, CAT30, and CAT60, respectively). As reported in Table 5.2, all of the anti-browning formulations resulted significantly effective ($P \leq 0.001$) in controlling ΔE of fresh-cut apples, compared to untreated samples (C). In particular, M1 and M2 wastes that reduced ΔE around 72% after 15 min of their application showed an inhibitory effect similar to AS1 and AAC with %RΔE of 73.6% and 59.8% respectively. The anti-browning differences were more relevant after the addition of phenolic substrate on dried slices (Figure 5.14d, e, and f). In this case, the surface colour of apple slices treated with AS1 was unaltered up to 60 min catechol application (Figure 5.14f) showing a 91.5% ΔE reduction (Table 5.2). Instead brown spots appeared in the presence of M1 and M2 after 10 min of catechol addition (Figure 5.14d), thus indicating a lower stability over time compared to AS1. However, both Merlot

pruning residues exhibited greater effectiveness in limiting the colour alterations in dried apple slices than AAC and the controls (C) after each catechol application. (Table 5.2).

Table 5.2 The effect of the chemical pre-treatment with some anti-browning formulations on the colour change of Golden Delicious apple slices subjected to drying processing.

	Anti-browning formulations[1]					
Treatment times[2]	**C**	**AAC**	**AS1**	**M1**	**M2**	***P*-value**[3]
I15						
ΔE	$2.39^{a} \pm 0.31$	$0.96^{b} \pm 0.14$	$0.63^{b} \pm 0.15$	$0.77^{b} \pm 0.18$	$0.59^{b} \pm 0.06$	***
RΔE (%)	–	59.8	73.6	67.8	75.3	
DR1080						
ΔE	$5.34^{a} \pm 1.16$	$3.38^{b} \pm 0.56$	$2.73^{b} \pm 0.10$	$3.96^{ab} \pm 0.26$	$3.71^{ab} \pm 0.77$	***
RΔE (%)	–	36.7	48.9	25.8	30.5	
CAT10						
ΔE	$38.19^{a} \pm 0.30$	$16.83^{b} \pm 0.29$	$4.84^{d} \pm 0.24$	$6.45^{d} \pm 1.82$	$9.86^{c} \pm 0.87$	***
RΔE (%)	–	55.9	87.3	83.1	74.2	
CAT30						
ΔE	$41.72^{a} \pm 1.33$	$32.56^{b} \pm 4.61$	$4.32^{d} \pm 0.12$	$16.81^{c} \pm 1.11$	$21.36^{c} \pm 1.50$	***
RΔE (%)	–	21.9	89.6	59.7	48.8	
CAT60						
ΔE	$42.70^{a} \pm 1.22$	$34.30^{b} \pm 4.31$	$3.62^{d} \pm 0.94$	$18.90^{c} \pm 1.24$	$23.45^{c} \pm 0.95$	***
RΔE (%)	–	19.7	91.5	55.7	45.1	

[1] C: control. AAC: 1% w/v ascorbic acid and 0.5% w/v $CaCl_2$. AS1: 6% w/v NatureSeal®. M1 and M2: Merlot pruning residues in the 2013 and 2014 seasons, respectively.

[2] I15: chemical pre-treatment with anti-browning formulation at 25°C for 15 min. DR1080: drying processing at 45°C for 18 hours. CAT10, CAT30, CAT60: application of 10 mM catechol at 25°C for 10, 30, and 60 min, respectively.

[3] '*' $P \leq 0.05$; '***' $P \leq 0.001$.

[a, b, c, d] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

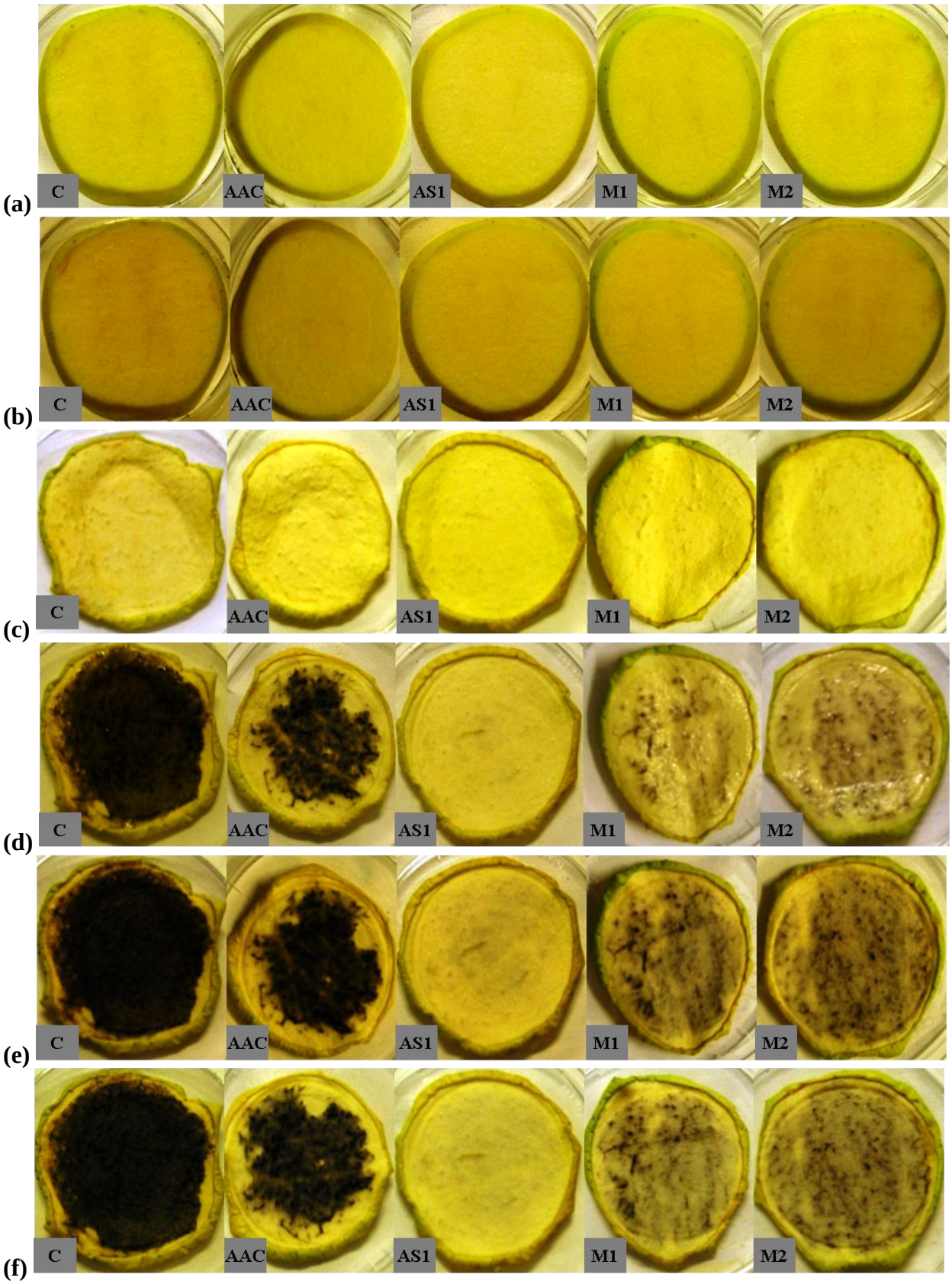

Figure 5.14 The effect of the chemical pre-treatment with some anti-browning formulations on the colour change of Golden Delicious apple slices subjected to drying processing. The colour change on apple slice surface was detected before (a) and after chemical pre-treatment for 15 min at 25°C (b), and immediately after 18 h of drying at 45 °C (c), and 10, 30, 60 min of 10 mM catechol application at 25°C (c, d, e, respectively).

The effect of chemical pre-treatment with some anti-browning formulations on the colour change of Golden Delicious apple slices subjected to drying processing.

The water activity (A_w) was also measured on the surface of dried Golden Delicious apple slices before (I0) and after (I15) chemical pre-treatment, and at the end of drying processing, for each anti-browning formulation. The A_w of slices pre-treated with anti-browning formulations (AAC, AS1, M1, and M2) was similar ($P > 0.05$) to that of untreated slices (C). Moreover, the A_w values decreased from 0.98 to 0.42 on average after drying (Table 5.3). A low A_w can be another parameter for controlling not only the shelf-life of dried products but also the enzymatic browning. In this regards, Labuza & Tannenbaum (1972) and Villamiel (2006) reported that the enzymatic browning was strong only in the 0.5-0.8 range.

Table 5.3 The effect of the chemical pre-treatment with some anti-browning formulations on the A_w of Golden Delicious apple slices subjected to drying processing.

Treatment times[2]	**Anti-browning formulations**[1]					***P*-value**[3]
	C	**AAC**	**AS1**	**M1**	**M2**	
I0	0.98 ± 0.01	097 ± 0.03	0.98 ± 0.02	0.97 ± 0.01	0.97 ± 0.02	NS
I15	0.98 ± 0.0	0.98 ± 0.01	0.98 ± 0.01	0.98 ± 0.01	0.98 ± 0.01	NS
DR1080	0.41 ± 0.01	0.44 ± 0.04	0.41 ± 0.04	0.42 ± 0.06	0.41 ± 0.05	NS

[1] C: control. AAC: 1% w/v ascorbic acid and 0.5% w/v $CaCl_2$. AS1: 6% w/v NatureSeal®. M1 and M2: Merlot pruning residues in the 2013 and 2014 seasons, respectively.

[2] I0: before chemical pre-treatment. I15: chemical pre-treatment for 15 min at 25°C. DR1080: drying processing for 18 hours at 45°C.

[3] 'NS' $P > 0.05$.

5.3.3 Evaluation of the whitening potential in VPRs

The whitening effects of VPRs from Barbera (B1 and B2) and Merlot (M1 and M2) cultivars were also evaluated in comparison with ascorbic and citric acids at the concentrations corresponding to 50% (AA50 and AC50) and 100% (AA100 and AC100) of TYR inhibition by spectrophotometrically measuring the dark compounds formed after the chemical oxidation of a catechol solution (Figure 5.15). The presence of citric acid and all vineyard wastes significantly reduced ($P \leq 0.001$) the absorbance value at 400 nm of a control mixture (C) by more than 50%. Although both inhibitory concentrations of citric acid showed the best whitening effect (74% and 76% for CA50 and CA100, respectively) by making the corresponding test tube solutions very clear, in comparison to the control, Barbera and Merlot wastes exhibited good whitening performance (58% and 62% for B1 and B2, 57% and 64% from M1 and M2, respectively), thus improving their anti-browning effectiveness. The whitening potential could be explained by the

low correlation between spectrophotometric and *in vivo* results by further increasing the anti-browning effect of VPRs on fresh-cut fruits and vegetables compared to the 0.05% w/v AA reference inhibitor.

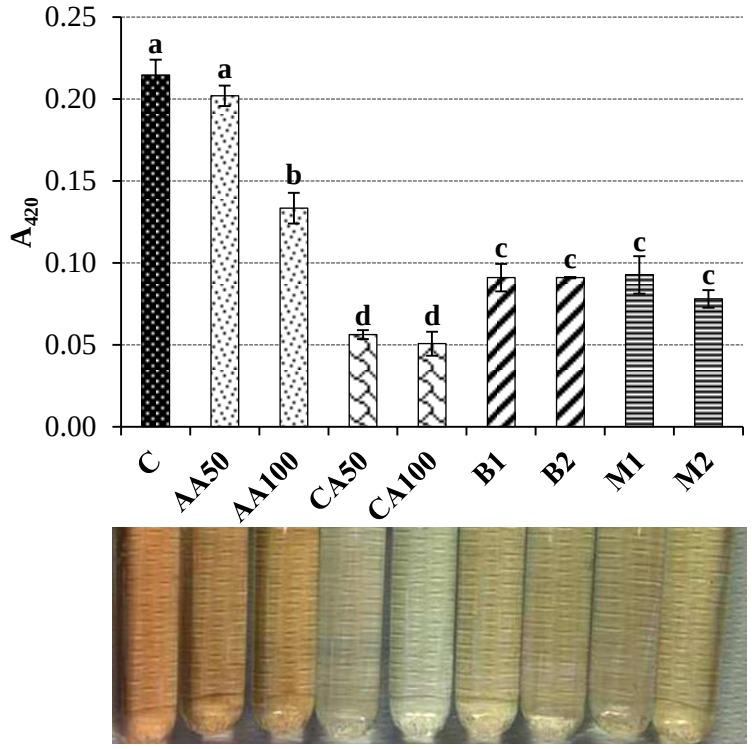

Figure 5.15 The whitening effect of PPO inhibitors on a 10 mM catechol solution. C: control without inhibitors. AA50 and AA100: ascorbic acid at the concentrations corresponding to 50% and 100% of TYR inhibition. CA50 and CA100: citric acid at the concentrations corresponding to 50% and 100% of TYR inhibition. B1 and B2: VPRs from the Barbera cultivar in the 2013 and 2014 seasons. M1 and M2: VPRs from the Merlot cultivar in the 2013 and 2014 seasons.

5.3.4 Evaluation of the antioxidant potential in VPRs

The antioxidant activities of VPRs from Barbera (B1 and B2) and Merlot cultivars (M1 and M2) were detected using two different 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 confirmed the same results (Figure 5.16).

As shown in Figure 5.16a, the antioxidant activities of all of the vineyard wastes were statistically relevant ($P \leq 0.001$). In particular, the Merlot cultivar (M1 and M2) had an antioxidant activity twice that of the Barbera cultivar (B1 and B2) in both DPPH and FRAP assays.

The antioxidant effectiveness of grape juices depended not only on the different phenolic compound and organic acid compositions of cultivars (Lima *et al.*, 2014) but also on the ripening stage of the berries. In this regard, the antioxidant potential of Merlot berries collected from the

VPRs at the end of July in the 2013 season (M1) was compared with 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 5.16b).

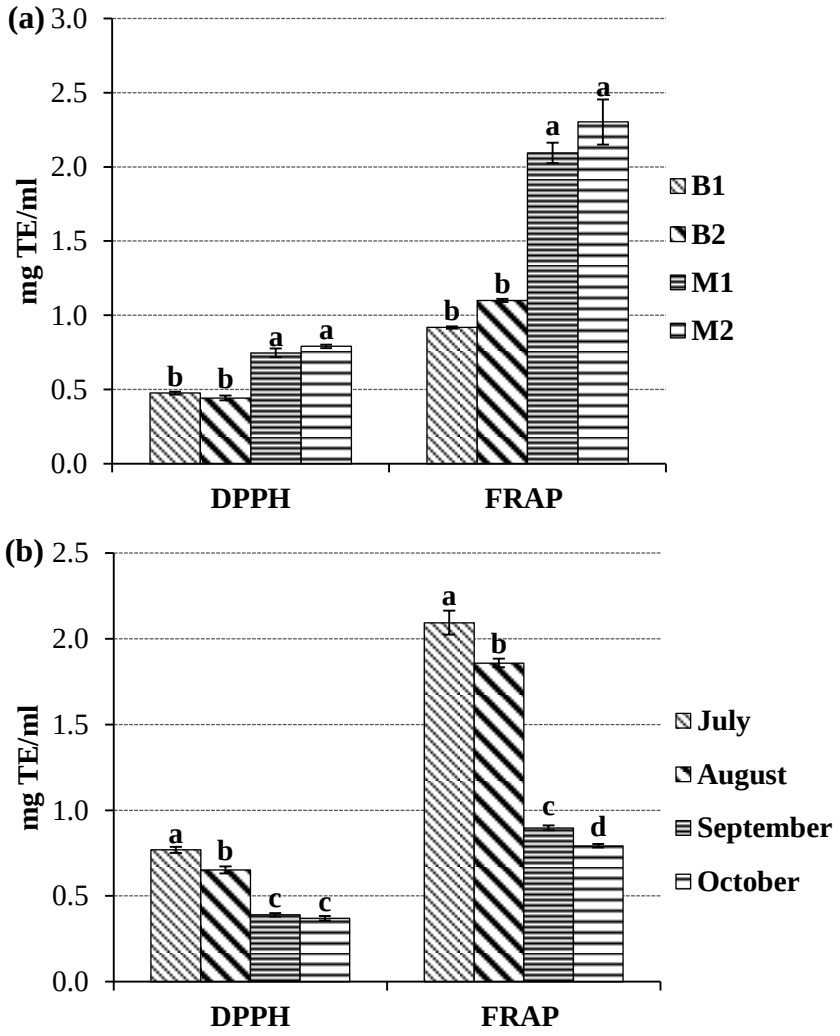

Figure 5.16 The antioxidant activity detected by DPPH and FRAP assays of (a) Barbera and Merlot pruning residues in the 2013 and 2014 seasons (B1, B2, M1, and M2, respectively) and (b) Merlot berries at different harvest times in the 2013 season.

5.3.5 Biochemical characterization of VPRs

The anti-browning and antioxidant potential of VPRs were related mainly to their composition in organic acids and phenolic compounds that was detected by HPLC analysis.

The anti-browning and antioxidant potentials of VPRs were mainly related to their organic acid (Table 5.4) and phenolic compound (Table 5.6) compositions that were detected by HPLC analysis.

The total amount of organic acids (citric, fumaric, malic, oxalic, succinic and tartaric) resulted in statistically relevant ($P \leq 0.01$) differences among the vineyard wastes (Table 5.4). In particular, the Merlot cultivar had an organic acid concentration higher than Barbera (328.44 ± 15.82 and 320.55 ± 0.28 mM for M1 and M2 *vs.* 278.22 ± 0.72 and 290.57 ± 0.76 mM for B1 and B2, respectively). Moreover, the organic acid contents of Merlot berries decreased significantly ($P \leq 0.01$) at increasing harvest times from July (328.44 ± 15.82 mM) to October (208.35 ± 10.81 mM) in the 2013 season according to the stage of ripeness (Table 5.5).

The high concentration of organic acids, especially malic, tartaric, and citric acids, contributed to the lower pH values of Barbera (2.22 and 2.24 for B1 and B2, respectively) and Merlot (2.24 and 2.18 for M1 and M2, respectively) pruning residues. The acidic pH could represent an important factor for the control of enzymatic browning by reducing the activity below the optimum pH that varied depending on the enzyme source (Rapeanu *et al.*, 2006).

Moreover, the organic acids analysed included some known PPO inhibitors. Son *et al.*, (2000) reported a strong anti-browning effectiveness of oxalic acid defining a competitive inhibition on a catechol-mushroom PPO system with a K_i value of 2.0 mM. The oxalic acid contents of VPRs were greater than its calculated IC_{50} value (1.5 mM), contrary to those of other organic acids (Table 5.4). Merlot cultivar had an oxalic acid concentration six times higher than that of Barbera (14.72 ± 0.12 mM and 16.84 ± 0.05 mM for M1 and M2 *vs.* 2.50 ± 0.03 mM and 3.16 ± 0.00 mM for B1 and B2, respectively). Moreover, the oxalic contents of M1 decreased at increased significantly ($P \leq 0.01$) at increasing harvest times from July (14.72 ± 0.12 mM) to October (5.52 ± 0.96 mM) in the 2013 season according to the stage of ripeness (Table 5.5). In addition, oxalic acid could also contribute to the antioxidant potential of vineyard wastes (Kayashima & Katayama, 2002). Son *et al.* (2001), studying the anti-browning performances 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 were mostly found in the VPRs, especially in Merlot cultivar (Table 5.4) and decreased at the ripening stages of grape berries (Table 5.5). Meanwhile, fumaric acid, whose content in Barbera and Merlot wastes was very low (Table 5.4), and succinic acid were less effective in controlling enzymatic browning as shown by Son *et al.* (2001). Citric acid, whose concentrations in M1 (78.14 ± 10.79 mM) and M2 (73.58 ± 0.40 mM) were higher than those in B1 (56.49 ± 0.009 mM) and B2 (62.88 ± 0.45 mM), is the main acidulant widely used in the agro-food industry. The inhibition of citric acid was mainly attributed to its capability of unfolding the conformation of enzyme structure (Liu *et al*., 2013) and consequently decreasing enzyme activity (Queiroz *et al*., 2011; Sun *et al*., 2012).

Table 5.4 Organic acids contents in VPRs from Barbera and Merlot cultivars in the 2013 and 2014 seasons (B1, B2, M1, and M2, respectively).

		Vineyard pruning residues				
Organic acids (mM)	**IC_{50}[1]**	**B1**	**B2**	**M1**	**M2**	***P*-value[2]**
Citric acid	289.87	$56.49^{a} \pm 0.09$	$62.88^{a} \pm 0.45$	$78.14^{b} \pm 10.79$	$73.58^{b} \pm 0.40$	*
Fumaric acid	n.d.[§]	0.13 ± 0.00	0.13 ± 0.00	0.16 ± 0.02	0.15 ± 0.00	NS
Malic acid	163.79	$126.56^{a} \pm 0.32$	$126.12^{a} \pm 0.05$	$129.17^{b} \pm 1.08$	$128.34^{b} \pm 0.05$	*
Oxalic acid	1.55	$2.50^{a} \pm 0.03$	$3.16^{a} \pm 0.00$	$14.72^{b} \pm 0.12$	$16.84^{b} \pm 0.05$	***
Succinic acid	536.74	$4.60^{a} \pm 0.16$	$5.25^{a} \pm 0.18$	$12.10^{b} \pm 2.47$	$8.46^{b} \pm 0.08$	***
Tartaric acid	293.09	87.91 ± 0.11	93.02 ± 0.43	94.14 ± 6.99	93.16 ± 0.19	NS
Total organic acids		$278.22^{a} \pm 0.72$	$290.57^{a} \pm 0.76$	$328.44^{b} \pm 15.82$	$320.55^{b} \pm 0.28$	**

[§] not detected.

[1] IC_{50}: the inhibitor concentration (mM) that reduces the enzyme activity by 50%.

[2] 'NS' $P > 0.05$, '*' $P \leq 0.05$, '**' $P \leq 0.01$,'***' $P \leq 0.001$.

[a, b] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

Table 5.5 Organic acids contents in VPRs from Merlot cultivar in the 2013 season (M1) at different harvest times.

		Harvest times				
Organic acids (mM)	**IC_{50}[1]**	**July**	**August**	**September**	**October**	***P*-value[2]**
Citric acid	289.87	$78.14^{a} \pm 10.79$	$62.72^{b} \pm 0.16$	$21.03^{c} \pm 0.14$	$19.33^{c} \pm 2.75$	***
Fumaric acid	n.d.[§]	$0.16^{a} \pm 0.02$	$0.11^{b} \pm 0.00$	$0.09^{bc} \pm 0.00$	$0.05^{c} \pm 0.01$	***
Malic acid	163.79	$129.17^{a} \pm 1.08$	$127.40^{b} \pm 1.59$	$125.8^{c} \pm 1.59$	$127.29^{bc} \pm 0.90$	***
Oxalic acid	1.55	$14.72^{a} \pm 0.12$	$10.22^{b} \pm 0.07$	$5.63^{c} \pm 0.06$	$5.52^{c} \pm 0.96$	***
Succinic acid	536.74	$12.10^{a} \pm 2.47$	$6.64^{b} \pm 0.31$	$5.49^{b} \pm 0.61$	$7.03^{b} \pm 1.59$	***
Tartaric acid	293.09	$94.14^{a} \pm 6.99$	$79.59^{b} \pm 9.72$	$51.3^{c} \pm 9.89$	$49.14^{c} \pm 9.78$	***
Total organic acids		$328.44^{a} \pm 15.82$	$286.67^{b} \pm 10.92$	$209.35^{c} \pm 11.81$	$208.35^{c} \pm 10.81$	***

[§] not detected.

[1] IC50: the inhibitor concentration (mM) that reduces the enzyme activity by 50%.

[2] 'NS' $P > 0.05$, '*' $P \leq 0.05$, '**' $P \leq 0.01$,'***' $P \leq 0.001$.

[a, b, c] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

The VPRs were also rich in polyphenols, which included the main antioxidants (Tabart *et al.*, 2009) and PPO inhibitors (Loizzo *et al.*, 2012). The total phenolic contents detected by the Folin-Ciocalteu assay among vineyard wastes (Figure 5.17a) were significantly different ($P \leq$

0.01), with greater concentrations in the Merlot cultivar (1.62 ± 0.11 and 1.51 ± 0.12 mg GAE/ml for M1 and M2, respectively) than in the Barbera cultivar (1.00 ± 0.01 and 1.15 ± 0.06 mg GAE/ml for B1 and B2, respectively). The phenolic concentration in the Merlot berries increased significantly ($P \leq 0.01$) at increasing harvest times from July (1.62 ± 0.11 mg GAE/ml) to October (2.59 ± 0.14 mg GAE/ml) in the 2013 season, according to the stage of ripeness (Figure 5.17b).

Among the flavanols (catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate) and phenolic acids (caffeic, chlorogenic, and gallic acids) detected by HPLC, epigallocatechin gallate (EGCG) was the main phenolic compound widely found in VPRs (Table 5.6). In particular, M1 (1,491 ± 15.98 µM) and M2 (1,440 ± 13.02 µM) had higher EGCG concentrations than B1 (463.88 ± 3.02 µM) and B2 (521.22 ± 6.78 µM), and they were at least three times the calculated IC_{50} value (421.12 µM). The EGCG content in Merlot grapes increased significantly ($P \leq 0.01$) at increasing harvest times from July (1,491 ± 15.98 µM) to October (255.65 ± 3.70 µM) in the 2013 season (Table 5.7), contrary to the normal phenolic ripeness (Figure 5.17b). Moreover, all VPRs showed a greater EGCG content than pomegranate extract (0.7 mM; Zocca *et al.* 2011) and green tea infusion (130 and 200 µM after 3 and 20 min of infusion time, respectively; Bronner & Beecher, 1998). Green tea has been widely recognized for its strong antioxidant capacity related mainly to its high catechin content. In particular, EGCG (El-Shahawi *et al.*, 2012) showed the best antioxidant performance among several phenolic compounds (Tabart *et al.*, 2009). Moreover, EGCG behaved as strong competitive inhibitor toward tyrosinase, thus confirming its anti-browning 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 VPRs.

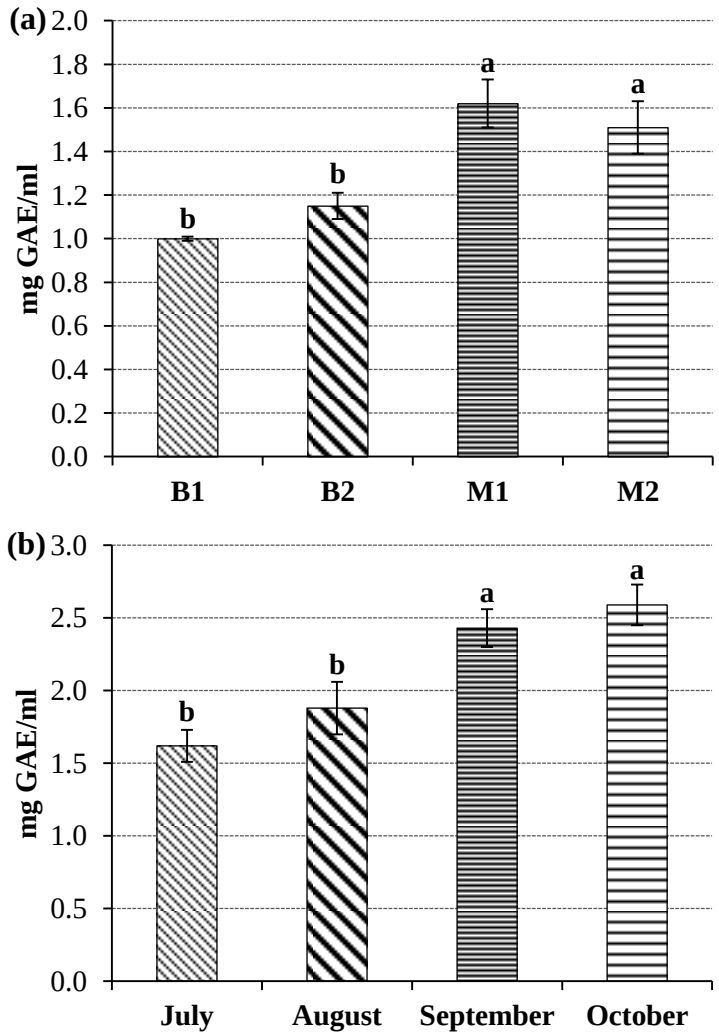

Figure 5.17 The total phenolic content detected by Folin-Ciocalteu assay of (a) Barbera and Merlot pruning residues in the 2013 and 2014 seasons (B1, B2, M1, and M2, respectively) and (b) Merlot berries at different harvest times in 2013 season.

Table 5.6 Polyphenols contents in VPRs from Barbera and Merlot cultivars in the 2013 and 2014 seasons (B1, B2, M1, and M2, respectively).

Polyphenols (µM)	**IC_{50}**[1]	**Vineyard pruning residues**				***P*-value**[2]
		B1	**B2**	**M1**	**M2**	
Caffeic acid	955.70	$119.98^{a} \pm 0.18$	$104.50^{b} \pm 0.61$	$80.72^{c} \pm 02.47$	$85.32^{c} \pm 1.75$	***
Catechin	n.d.[§]	$17.74^{a} \pm 0.24$	$14.83^{a} \pm 0.58$	$32.17^{b} \pm 0.83$	$41.61^{c} \pm 0.50$	***
Chlorogenic acid	n.d.	$61.74^{a} \pm 0.18$	$53.51^{a} \pm 0.22$	$30.13^{b} \pm 0.55$	$26.18^{b} \pm 0.17$	***
Gallic acid	59.24	2.72 ± 0.17	3.82 ± 0.06	2.69 ± 0.21	2.74 ± 0.11	NS
Epicatechin	n.d.	$9.50^{a} \pm 0.14$	$6.95^{b} \pm 0.08$	$18.86^{c} \pm 1.57$	$18.21^{c} \pm 0.74$	***
Epicatechin gallate	383.17	$45.57^{a} \pm 0.08$	$70.70^{b} \pm 0.15$	$52.72^{c} \pm 0.86$	$176.59^{d} \pm 0.07$	***
Epigallocatechin	615.75	$30.13^{a} \pm 0.35$	$37.15^{a} \pm 0.53$	$118.77^{b} \pm 3.45$	$169.60^{c} \pm 1.32$	***
Epigallocatechin gallate	421.12	$463.88^{a} \pm 3.02$	$521.22^{b} \pm 6.78$	$1{,}491^{c} \pm 15.98$	$1{,}440^{c} \pm 13.02$	***
Total polyphenols		$751.27^{a} \pm 2.80$	$812.68^{b} \pm 7.00$	$1{,}827^{c} \pm 17.34$	$1{,}961^{c} \pm 16.04$	***

[§] not detected.

[1] IC_{50}: the inhibitor concentration (mM) that reduces the enzyme activity by 50%.

[2] 'NS' $P > 0.05$, '*' $P \leq 0.05$, '**' $P \leq 0.01$, '***' $P \leq 0.001$.

[a, b, c, d] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

Table 5.7 Polyphenols contents in VPRs from Merlot cultivar in the 2013 (M1), at different harvest times.

Polyphenols (µM)	IC_{50} [1]	Harvest times				*P*-value [2]
		July	August	September	October	
Caffeic acid	955.70	$80.72^{a} \pm 2.47$	$42.18^{b} \pm 1.94$	$0.00^{c} \pm 0.00$	$0.00^{c} \pm 0.00$	***
Catechin	n.d. [§]	$32.17^{a} \pm 0.83$	$31.74^{b} \pm 0.90$	$5.58^{c} \pm 0.15$	$6.35^{c} \pm 0.07$	***
Chlorogenic acid	n.d.	$30.13^{a} \pm 0.55$	$17.93^{b} \pm 0.72$	$0.00^{c} \pm 0.00$	$0.00^{c} \pm 0.00$	***
Gallic acid	59.24	$2.69^{a} \pm 0.21$	$2.49^{a} \pm 0.25$	$1.16^{b} \pm 0.05$	$0.81^{c} \pm 0.01$	***
Epicatechin	n.d.	$18.86^{a} \pm 1.57$	$18.83^{a} \pm 0.78$	$5.56^{b} \pm 0.04$	$5.57^{b} \pm 0.18$	***
Epicatechin gallate	383.17	$52.72^{a} \pm 0.86$	$51.19^{a} \pm 0.36$	$45.93^{b} \pm 0.68$	$52.36^{a} \pm 1.78$	**
Epigallocatechin	615.75	$118.77^{a} \pm 3.45$	$51.50^{b} \pm 0.54$	$26.81^{c} \pm 0.51$	$33.83^{c} \pm 0.48$	***
Epigallocatechin gallate	421.12	$1{,}491^{a} \pm 15.98$	$962.02^{b} \pm 14.65$	$249.41^{c} \pm 8.22$	$255.62^{c} \pm 3.70$	***
Total polyphenols		$1{,}827^{a} \pm 17.74$	$1{,}178^{b} \pm 12.96$	$334.45^{c} \pm 8.86$	$354.55^{c} \pm 5.43$	***

[§] not detected.

[1] IC_{50}: the inhibitor concentration (mM) that reduces the enzyme activity by 50%.

[2] 'NS' $P > 0.05$, '*' $P \leq 0.05$, '**' $P \leq 0.01$, '***' $P \leq 0.001$.

[a, b, c] Means within each row and with different superscript letters are statistically different ($P \leq 0.05$).

5.4 Conclusions

The main results of the third contribution can be summarised as follows.

- The development of new inhibitors needs a multidisciplinary approach by combining in vitro with in vivo assays.
- The catechol gel diffusion test, which has been used for an initial screening, showed the strong anti-browning potentials of the grape juices obtained by cold-pressing the berries collected from the VPRs of Barbera (B) and Merlot (M) cultivars during 2013 (1) and 2014 (2) seasons.
- The spectrophotometric assays allowed investigating the inhibition type and degree of VPRs by analysing the enzymatic kinetics of TYR, in the presence of catechol as the phenolic substrate. The Lineweaver-burk plots defined an uncompetitive inhibition of VPRs. Moreover, all of these vineyard wastes did not behave as reducing agents such as ascorbic acid as the reference inhibitor. Although 0.05% ascorbic acid exhibited the best anti-TYR performance (85.7%), the VPRs significantly decreased the enzymatic activity with a TYR inhibition of more than 50% compared to the control. Moreover, the inhibitory effectiveness was related mainly to the grape cultivar. In details, Merlot wastes better inhibited TYR activity than those of Barbera (68.2% and 67.8% for M1 and M2, respectively; 56.3% and 58.8% for B1 and B2, respectively).
- The zymographic results showed the strongest inhibitory effects of VPRs on the one isoform of TYR and potato PPO. A good inhibitory effectiveness was achieved on the one isoform and four isoforms of Golden Delicious and Fuji apple PPOs. Any evident inhibitory capacity was observed on the zymograms of Abate Fétel and Decana pear PPOs.
- The *in vivo* trials confirmed the strong anti-browning effectiveness of Merlot pruning residues on fresh-cut apples, especially from Fuji and Golden Delicious cultivars, and on some fresh-cut vegetables such as Bintje potatoes and eggplants. Any anti-browning effectiveness was observed on fresh-cut pears from Abate and Decana cultivars. Moreover, Merlot wastes were very effective in limiting the colour change of fresh (79.4% and 85.8% for M1 and M2 *vs.* 95.7% and 93.7% for AAC and AS1 as the reference anti-browning formulations, after 10 min of catechol application) and dried slices (83.1% and 74.2% for M1 and M2 *vs.* 55.9% and 87.3% for AAC and AS1 as the reference anti-browning formulations, after 10 min of catechol application) of Golden Delicious apples.

- The *in vitro* assays as well as in vivo trials demonstrated that the anti-browning effectiveness was mainly related to the PPO source and grape cultivar. In details, Merlot cultivar showed the best inhibitory properties and pear PPO resulted less sensitive to anti-browning treatment.
- The additional whitening effect contributed to improving the anti-browning performances of VPRs.
- The VPRs, especially from Merlot cultivar, had also antioxidant potentials as simultaneously confirmed by two different spectrophotometric assays based on electron transfer (DPPH and FRAP). Their antioxidant capacities were related to the ripening stages of grape berries.
- The strong anti-browning, antioxidant and whitening performances mainly achieved by the Merlot pruning residues have been associated with their greater organic acids and EGCG contents according to the ripening stage of the grape berries, as detected by HPLC analysis. In details, the concentration in organic acids, which contributed to lowering the pH of VPRs below the optimal values of PPO, decreased with increasing harvest times. Also EGCG followed this pattern, contrary to the normal phenolic ripeness.

The VPRs, especially from Merlot cultivar, are rich in bioactive compounds, including organic acids as well as polyphenols e.g. ECGC, with strong anti-browning and antioxidant potentials, as confirmed by several in vitro and in vivo assays, for a possible application in the agro-food industry. These results are preliminary and deserve further investigations with additional focus on the cosmetic field. However, the recovery of bioactive compounds from natural wastes and by-products without any solvents may be an eco-friendly and inexpensive strategy to extend the life cycle of several agro-food products.

6 General conclusions and Future perspectives

Innovative non-thermal technologies and new natural PPO inhibitors are eco-friendly, anti-browning alternatives to conventional thermal treatments and traditional additives, which have some drawbacks including low stability, alterations of organoleptic and nutritional properties in agro-food products and potential hazards for human health.

Their effectiveness in inhibiting PPO activity and thus preventing the enzymatic browning depends mainly on enzyme source. In fact, the catalytic activity of PPO widely varies according to enzyme origin (animal, bacterial, fungal, or plant), species, and also plant varieties and cultivars. Hence, the PPO inhibition by chemical and/or physical systems may be evaluated on a large scale, using a multidisciplinary approach. Among the qualitative *in vitro* assays, the plate test is a simple and inexpensive approach for an initial screening, while zymographic technique is a useful tool to specifically investigate the inhibitory effects on PPO isoforms and latent forms. The spectrophotometric assays allow defining the inhibition type and degree by analyzing the enzymatic kinetics. Finally, the *in vivo* trials, which can be carried out not only on plant but also on animal tissues, are suitable to confirm the anti-browning performances in real conditions. Moreover, the colorimetric parameter ΔE, which gives an overall evaluation of total colour change including L*, a*, b* components, can be considered as a valid, indirect anti-browning index for estimating PPO inhibition during the *in vivo* assays.

The UV-A LED technology may be an easy, safe, and inexpensive solution for processing fresh-cut products. In particular, the anti-browning potential of UV treatment is a function of several operational conditions e.g. wavelength, irradiance, and exposure time, and other external factors e.g. fruit type and cultivar. In this regards, UV irradiation has been more effective on fresh-cut apples, especially from Fuji and Golden cultivars, rather than fresh-cut pears.

Although UV-A light is less powerful than UV-C, which is the most used preservative technology in food industry, it can effectively control enzymatic browning without compromising the organoleptic and nutritional qualities of agro-food products. In fact, literature reports some adverse effects, including the alteration of sensory and nutritional properties as well as antioxidant capacities and the formation of furan recognized by WHO as a potential human carcinogen, in food subjected to UV-C exposure.

Finally, the use of LED light sources leads to many advantages such as energy savings, device durability, low environmental impacts, high luminous efficiency and, negligible thermal effects.

Citrus hydrosols (CIHs) and vineyard pruning residues (VPRs) represent an eco-friendly and inexpensive source of natural PPO inhibitors, thus allowing the recycle of these agro-food wastes and by-products into food additives and cosmetic formulations, which may be used against the plant enzymatic browning, melanogenesis and skin hyperpigmentation. Because their preparation is carried out by "mild extraction", without using any chemical solvent, they are also safe for human health after topical, oral or parenteral exposure.

The effectiveness of these natural extracts in modulating PPO activity is attributed not only to enzyme source but also to their bioactive compounds contents according to the plant cultivar and ripening stage. In this regards, terpenes in CIHs, and organic acids as well as polyphenols, especially epigallocatechin gallate, in VPRs contribute to enhancing anti-browning and even antioxidant potentials.

The VPRs as well as UV-A LED treatments, which have been very effective in controlling the colour changes in fresh and dried slices of Golden Delicious apples, may be proposed in the processing of fresh-cut products and snacks.

On the basis of these considerations, the UV-A LED technology, CIHs, and VPRs are able to improve both the organoleptic and nutritional qualities of agro-food products by effectively controlling enzymatic browning, thus reducing the colour alterations and the degradation of polyphenolic substrates recognized for their health benefits as antioxidants. In this way, qualitative and economical losses in post-harvest can be limited.

However, could these new anti-browning proposals be an alternative to reduce SO_2 in foods and drinks? An implementation of this preliminary research must be performed for answering it. To date, SO_2, whose application has been restricted because of adverse clinical effects in sensitive individuals, is still one of the most versatile and efficient additives used in agro-food industry thanks to its antioxidant and antimicrobial properties. Hence, the results previously reported and discussed deserve further investigations with additional focus on the microbiological aspects to improve food preservation.

Also the sensory quality of agro-food products subjected to physical or chemical treatments should be taken into account in terms not only of colour and appearance but also texture, flavour, and taste in order to meet consumers' preferences.

In the present study, the *in vivo* trials on fresh-cut fruits and vegetables, have been performed in conditions of accelerated browning, therefore, a future study of shelf-life may be useful to check the stability over time of these new anti-browning systems. Further investigations

on the packaging of fresh-cut products may be also carried out to discuss the effect of environmental conditions such as relative humidity, temperature and inert gas on enzymatic browning.

The anti-browning treatments with UV-A LED, CIHs, and VPRs, which have been mainly evaluated on fresh-cut products, may be extended to other agro-food products such as juices, smoothies, purees, nectar, dehydrated and canned fruits and vegetables as well as fish and meet.

It is clear that cosmetic application of these innovative systems for controlling melanogenesis and skin disorders need further research by *in vitro* assays on human tyrosinase and *in vivo* trials.

Last but not least, new trials will be performed to evaluate a possible synergistic anti-browning effect by combining UV-A LED technology with the bioactive compounds recovered from agro-food by-products and wastes.

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Enjoy!!!

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