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Short communication

Isolation of a tyrosinase inhibitor from unripe grapes juice: A spectrophotometric study

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ABSTRACT

Grapes are known to contain high quantity of polyphenolic compounds, including caffeic, coumaric and ferulic acids esterified with tartaric acid, to yield caftaric, coutaric and fertaric acids, respectively. These acids are more abundant in unripe grapes, which can be processed into verjuice, a product that shows intrinsic resistance against microbial growth and significant antioxidant activity. In the present work, the isolation of hydroxycinnamoyl tartaric acids from unripe grape juice by chromatographic techniques was described. Moreover, the capability of caftaric acid to inhibit tyrosinase activity was evaluated by spectrophotometric assays. According to the kinetics parameters calculated, caftaric acid was shown to be a competitive inhibitor of tyrosinase, more potent than the related caffeic and chlorogenic acids, suggesting that it can be used in cosmetic and food industries for the development of natural skin whitening formulations and as an agent able to counteract the enzymatic browning of food.

1. Introduction

Grapes contain high quantity of polyphenolic compounds and this content depends on many factors such as variety, climatic conditions and ripeness stage. The most important class of grape polyphenols in terms of quantity and enological importance is the flavonoids, whose concentration shows a fast increase after the veraison stage, i. e. the onset of ripening. Besides, grapes contain also moderate quantities of non-flavonoid compounds, including phenolic acids and stilbenes (Lago-Vanzela et al., 2014). Many studies have shown the positive effects of these polyphenols on human health (Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010). The phenolic acids class includes caffeic, coumaric and ferulic acids (hydroxycinnamic acids), which in grapes are present as esters with tartaric acid, the main organic acid present in the grape berries (Fig. 1). These hydroxycinnamyl tartaric (HCT) acids are present in few other plants such as chicory and *Echinacea purpurea* (Bauer & Foster, 1991; Innocenti et al., 2005). The concentration of caftaric acid in grapes is about 170 mg/kg, whereas the contents of the coumaric and ferulic acid derivatives are much lower, 20 and 5 mg/kg, respectively (Ong & Nagel, 1978; Singleton, Zaya, & Trousdale, 1986). The synthesis of HCT acids in grapes starts before veraison and their concentrations decrease during the ripening, due to the dilution effect related to the increase of berry size and, probably, also to their involvement in the biosynthesis of other phenolic compounds (Romeyer, Macheix, Goiffon, Reminiac, & Sapis, 1983). In grape juice and wine the concentration of HCT acids is even lower, as these molecules are very fast oxidized by endogenous tyrosinase after the grapes are crushed.

However, a commercial product made by pressing unripe grapes, called verjuice, should contain a higher quantity of HCT acids. This product showed an intrinsic resistance against microbial growth by virtue of its high ascorbic acid concentration (about 20 mg/L), and a significant antioxidant activity due to its high polyphenol content in terms of gallic, caffeic, *p*-coumaric and ferulic acid, and in terms of catechins, quercetin, and resveratrol (Hayoglu, Kola, Kaya, Özer, & Turkoglu, 2009; Pour Nikfardjam, 2008).

For these reasons a wide range of uses can be depicted for verjuice: as an antioxidant agent in the chemical-pharmaceutical industry (Ahmadi & Romney, 2014), an acidifying agent in food (Dupas de Matos et al., 2019) and wine industries (Kontoudakis, Esteruelas, Fort, Canals, & Zamora, 2011), as well as an acidic food ingredient instead of vinegar in many food preparations such as salads, marinades, and cocktails (Dupas de Matos et al., 2018). Recently, an inhibitory activity upon tyrosinase (E.C.1.14.18.1), an enzyme that catalyzes the produc-

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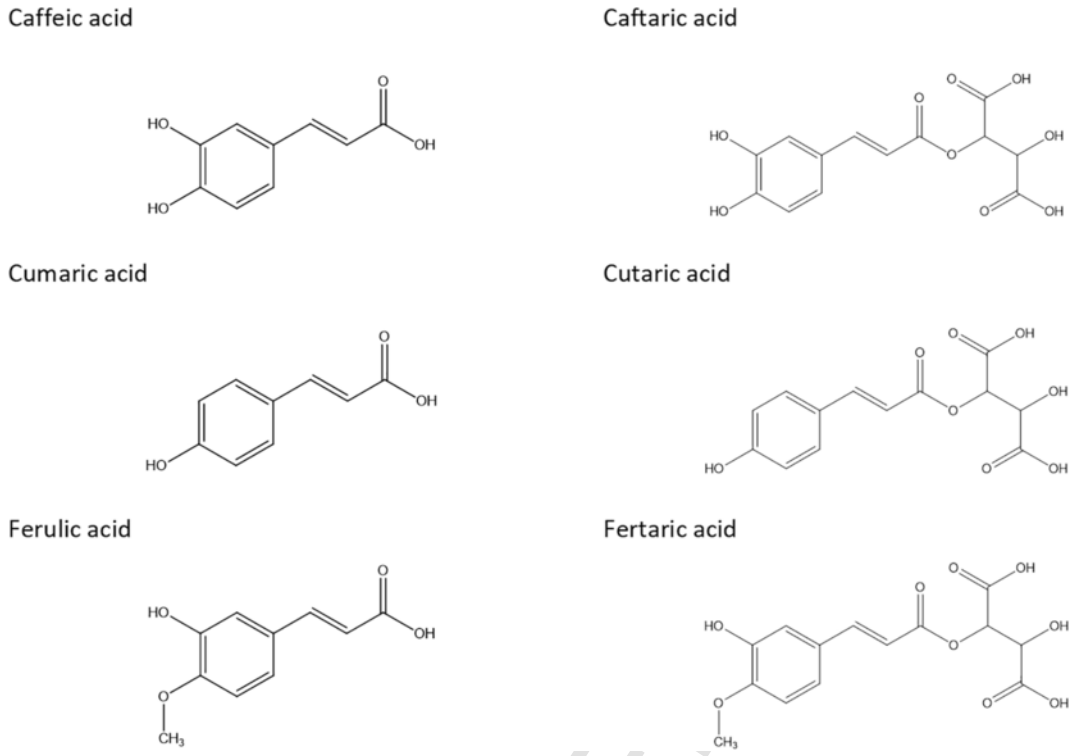

Fig. 1. Chemical structures of most common hydroxycinnamic acids and their tartaric acid esters.

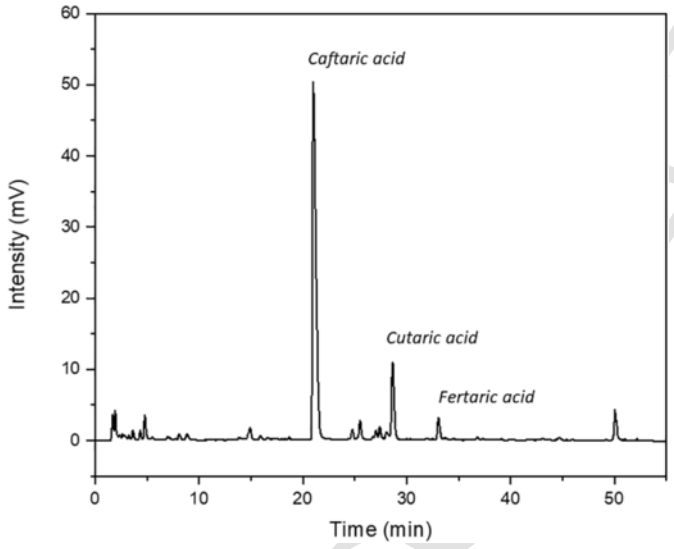

Fig. 2. Analytical HPLC of unripe grape juice. HPLC was performed on an LC-10AD Shimadzu HPLC system equipped with a Kinetex C18, 100 Å, 5 μ, 150 × 4.6 mm column equilibrated and eluted with a binary solvent system: **A** (0.05% TFA in H2O) and **B** (0.05% TFA in 9:1 v/v MeCN/H2O); λ detector: 320 nm; flow: 1 mL/min; elution program: isocratic elution to 3% **B** for 5 min and then linear gradient from 3 to 15% **B** in 45 min. Chromatogram was acquired and processed using ChromNav software.

tion of melanin from tyrosine oxidation and which is involved in the browning phenomenon of fruits and vegetables, has been described for the juice obtained from thinned unripe grapes (Tinello & Lante, 2017).

In the active site of tyrosinase, six conserved residues of histidine bind a pair of copper ions, which interact with both molecular oxygen and the phenolic substrate (Seo, Sharma, & Sharma, 2003), catalyzing two distinct reactions: i) the hydroxylation of aminophenols (monophenolase or creolase activity), where the enzyme passes through four different states (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}M}$, and $E_{met\text{-}D}$); and ii) the oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity), that involves five enzyme states (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}D}$, E_{met}, and $E_{met\text{-}D}$) (Seo et al., 2003). The *o*-quinones are generally reactive and can sustain 1,4-addition to the benzene ring (Jimenez & Garcia-Carmona, 1996).

Considering the important role that tyrosinase plays in processing of fruit and vegetables and during the storage of processed foods as well as in the hyperpigmentation of the skin, with melisma and age spots, its inhibition is attractive in the cosmetic, medicinal and food industries. For this purpose, many natural and synthetic inhibitors have been developed (Zolghadri et al., 2019) with the aim to obtain new safe and efficient anti-tyrosinase agents for the prevention of browning in plant-derived foods, seafood and hyperpigmentation treatments.

In the present work, the isolation of HTC acids and, in particular, of caftaric acid from an unripe grape juice, was described. Moreover, the evaluation of its capability to inhibit tyrosinase activity was carried out by spectrophotometric assays.

2. Material and methods

2.1. Materials and general remarks

Acetonitrile (chromatographic grade), methanol, ethanol, tyrosinase from mushroom lyophilized powder (4187 units/mg), and L-tyrosine were purchased from Sigma Aldrich S.r.l (Milano, Italia). Potassium phosphate mono- and di-basic were obtained from Carlo Erba (Milano, Italia).

Water used in this study was obtained by a MilliQ (Millipore, Burlington, MA) filtration system.

ESI-MS spectra were registered on a Xevo G2-S quadrupole time-of-flight mass spectrometer from Waters Corporation (Milford, MA).

NMR spectra were recorded on a Bruker (Billerica, MA) Avance DRX 400 MHz spectrometer at 20 °C in D_2O. Chemical shifts are given in ppm and coupling constants in hertz.

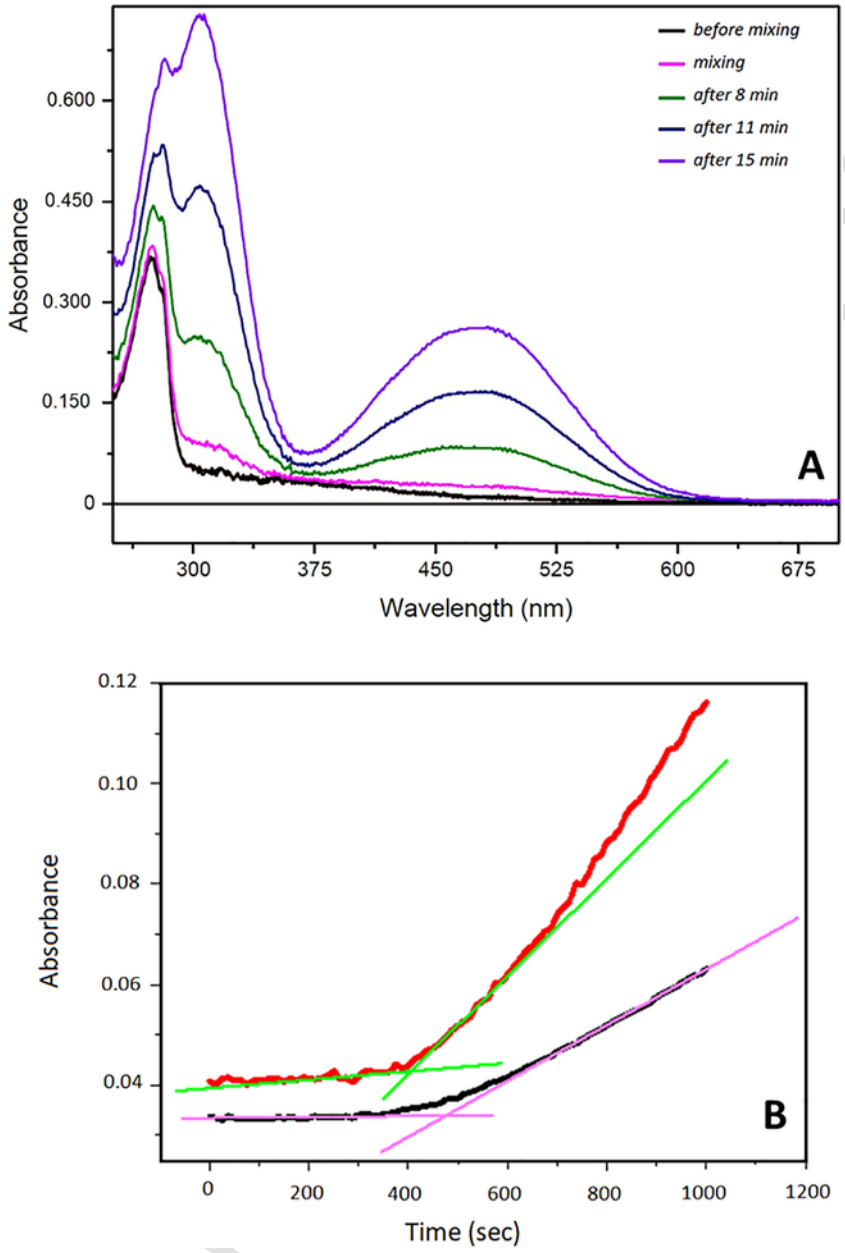

Fig. 3. Spectroscopic evaluation of tyrosinase activity. (**A**) UV spectra of Tyr (0.3 mM, in 20 mM phosphate buffer at pH 6.8) before and after mixing with tyrosinase 1000 U/mL (indicated). (**B**) Time courses of tyrosinase activity monitored at both 304 (red line) and 475 nm (black line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Kinetic parameters of tyrosinase activity. Experiments were carried out in 20 mM phosphate buffer at pH 6.8; tyrosine was 0.3 mM and tyrosinase 1000 U/mL.

	K_m (mM)	V_{max} (Abs·s^{-1})	K_{cat} (s^{-1})	Efficiency (mM^{-1} s^{-1})
Tyr	0.31	$1.85 * 10^{-4}$	96.4	310.8
Tyr + caftaric acid 5.7 µM	0.62	$1.87 * 10^{-4}$	97.3	157.1
Tyr + caftaric acid 14.2 µM	0.79	$1.69 * 10^{-4}$	88.0	111.4

2.2. Unripe grape juice extraction

The unripe grape juice was made by crushing several grape clusters of cultivar Riesling harvested in July 2011. The crushing was performed in the presence of 200 mg/kg potassium metabisulfite to avoid polyphenol oxidation. From 1 kg of grapes 470 mL of juice were obtained, with a titratable acidity of 34.1 g/L (expressed as tartaric acid) and a sugar content of 10 g/L. The juice was vacuum filtered at 1.6 µm (GF/A, Whatman), portioned in tubes of 50 mL and stored at −80 °C until used. Before use the juice was thawed and filtered again to remove the precipitated salts.

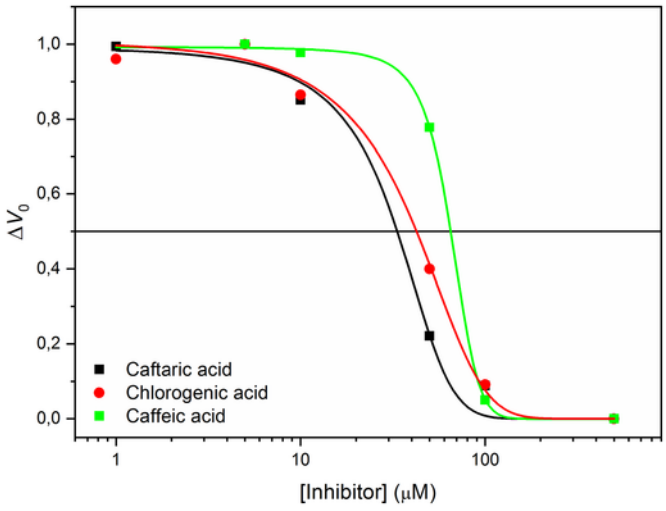

Fig. 4. Dose-response graphs of caftaric, chlorogenic and caffeic acids (indicated). The determined IC_{50} values are 30, 42 and 65 μM, respectively. Experiments were carried out in 20 mM phosphate buffer at pH 6.8, tyrosine was 0.5 mM and tyrosinase 1300 U/mL.

2.3. Purification

The analytical HPLC were performed on a Shimadzu HPLC system (Kyoto, Japan) composed of LC-10AD pump, SLC-10A controller and Gastor 154 degasser and equipped with a Kinetex C18, 100 Å, 5 μ, 150 × 4.6 mm column (Phenomenex, Torrance, CA) equilibrated and eluted with a binary solvent system: **A** (0.05% TFA in H_2O) and **B** (0.05%TFA in 9:1 v/v MeCN/H_2O); λ detector: 280–320 nm; flow: 1 mL/min; elution program: isocratic elution to 3% **B** for 5 min and then linear gradient from 3 to 15% **B** in 45 min. Chromatograms were acquired and processed using the ChromNav software (Jasco, Tokyo, Japan).

The separation was performed on a preparative HPLC system (Shimadzu, Kyoto, Japan) composed of two LC-8A pumps, an SCL-8A controller and an SPD-6A spectrophotometric detector. Solvents were degassed by an ERMA ERC-3562 degasser. The column used for separation was a Kinetex C18, 100 Å, 5 μm, 150 × 21.2 mm (Phenomenex), eluted with the same solvent system used for analytical purposes; λ detector: 280 nm; flow: 12 mL/min; elution program: isocratic elution to 8% **B** for 5 min and then linear gradient from 8 to 20% **B** in 45 min.

2.4. Spectrophotometric assays

Kinetic assays were carried out by measuring the appearance of the product of tyrosine oxidation in the reaction medium at both 304 and 475 nm in a Shimadzu UV-2501 spectrophotometer equipped with UV-Probe software according to previously described methods (Ruzza et al., 2009; Ruzza et al., 2017). Kinetic data were recorded using the kinetic application implemented in the UV-Probe software and a tandem quartz cuvette (Hellma, Milan, Italy) with a 2 × 4.375 mm path length. Briefly: one side of the dual chamber cuvette was filled with 920 μL of a 2000 units/mL of mushroom tyrosinase solution in 20 mM phosphate buffer, pH 6.8. The other chamber was loaded with 900 μL of a 0.6 mM tyrosine solution in 20 mM phosphate buffer (pH 6.8) added with either 20 μL of buffer (control) or 20 μL of a 5.25 mM solution of tested inhibitors. A background spectrum in the 250–700 nm range was acquired with a 2.0-nm slit and fast speed before starting the reaction. Thereafter, the solutions in the two chambers were mixed by inversion for several times and the kinetic data were acquired at 304 and 475 nm. Alternatively, UV–Vis spectra were acquired at different time points after mixing. Data processing was performed with UV Probe and OriginPro 2019 (v. 9.60) software.

Purchased mushroom tyrosinase was used without further purification. Both L-tyrosine and tyrosinase solutions were filtered through a 0.45-μm syringe filter, aliquoted in Eppendorf tubes, and stored in the freezer. The relative concentration was determined spectrophotometrically (ε_{Tyr} at 274.6 nm = 1.420; $\varepsilon_{tyrosinase}$ at 280 nm = 1.426).

K_m and V_{max} values were calculated from triplicate measurements of the steady state rate (V_{ss}) for each initial substrate concentration. Non-linear regression fitting of V_{ss} *vs* [S] was carried out by using the Michaelis-Menten algorithm implemented in the Origin 2019 (v. 9.60) software.

3. Results and discussion

3.1. Purification and characterization of hydroxycinnamic acid

The presence of hydroxycinnamates in the unripe grape juice is confirmed by a strong absorbance in the 300–320 nm region of the UV spectra (Fig. 1S in the Supplementary material). Indeed, the spectral determination of total hydroxycinnamates can be made at 320 nm without significant interference from both flavonoid (λ_{max} at 280 nm) and non-phenolic (λ_{max} at about 265 nm) constituents (Somers & Ziemelis, 1985). The analytical HPLC of unripe grape juice (Fig. 2) did not show the complexity of hydroxycinnamate composition found in wines where up to ten of such components were found (Somers, Vérette, & Pocock, 1987), and the LC–MS analysis of crude unripe grape juice identified the presence of three most abundant hydroxycinnamic esters that showed *m/z* values at 313.1, 149.2 as $[M + 2H]^{2+}$ and 327.2 (Fig. 2S in the Supplementary material) attributable to the tartaric acid esters of caffeic, p-coumaric and ferulic acids, respectively (Fig. 1).

The preparative separation of hydroxycinnamates was performed using a Kinetex C18 column eluted with a linear gradient from 8 to 20% **B** in 45 min. Initially, 50 mL of unripe grape juice filtered through 0.45-μm membranes were purified, loading ten aliquots of 5 mL each of grape juice. Successively, to optimize the operation time, preparative HPLC was performed on a 0.45-μm filtered aqueous solution of the residue obtained by lyophilization of 50 mL of unripe grape juice. This last procedure showed better results in the productivity of the preparative HPLC purification (an example of preparative purification is reported in Fig. 3S of the Supplementary material).

Overall, 10.74 mg of caftaric acid of satisfying purity (> 99%) were obtained, while only a small amount of both coutaric (1.31 mg) and fertaric (0.98 mg) acids were isolated. The purity of these two last hydroxycinnamates was not acceptable for the successive enzymatic studies. Isolated esters were characterized by ESI–MS and NMR (Figs. 4S and 5S, and Table 1S in the Supplementary material). The *cis*- and *trans*-isomerism of hydroxycinnamic derivatives was assigned measuring the $^3J_{HH}$ coupling constant between the vicinal hydrogens of the double bond. In this study, the *trans* (*E*) configuration of all isolated compounds was exclusively indicated by the $^3J_{HH}$ values of about 16 Hz (Table 1S) (Bishop & Musher, 1963). This is in agreement with previous research, showing that in grape, caftaric, *p*-coutaric and fertaric acids are mainly found in the *trans* form, only *p*-coutaric acid showing a not negligible fraction of *cis* form (Vanzo et al., 2007).

3.2. Tyrosinase inhibition

The capability of caftaric acid to inhibit the tyrosinase activity was evaluated using the natural substrate tyrosine by UV–Vis spectroscopy as previously described (Ruzza et al., 2017). The tyrosinase oxidation of Tyr induces the appearance of two absorption bands in the near-UV spectrum of tyrosine at 304 and 475 nm attributable to the *o*-diphenol

and the *o*-quinone, respectively (Fig. 3A), corresponding to the monophenolase and diphenolase activities of tyrosinase.

The time course of Tyr oxidation monitored at both 304 and 475 nm (Fig. 3B) displays the characteristic lag period. This period is the time required by the enzyme to accumulate a quantity of *o*-diphenol in the reaction medium. Only when a sufficient amount of *o*-diphenol produced by the small amount of the *oxy* form present in the enzyme preparation is available, the *met* form of tyrosinase binds to *o*-diphenol transforming it to *o*-quinone and the enzyme is reduced to the active *deoxy* form. This form has a very high affinity for molecular oxygen generating the *oxy* form (Chang, 2009; Sánchez-Ferrer, Neptuno Rodríguez-López, García-Cánovas, & García-Carmona, 1995; Zolghadri et al., 2019). The non-linear regression analysis of the kinetic data correlated to the product of the monophenolasic and diphenolasic tyrosinase activity (L-dopa and L-dopaquinone; Fig. 6S in the Supplementary material), using the Michaelis-Menten algorithm implemented in the Origin software, allows the determination of K_m (0.14 mM and 0.10 mM for monophenolasic and diphenolasic activity, respectively) and the V_{max} (1.8×10^{-4} Abs·s^{-1} and 5.4×10^{-5} Abs·s^{-1}, respectively) values. The observed difference in the kinetic parameters determined analyzing the appearance of either L-dopa or L-dopaquinone reflects the complexity of the tyrosinase catalyzed reaction, in particular when a phenol substrate is used. Indeed, in addition to the presence of three different forms of the enzyme involved in the oxidation of the tyrosine substrate, the two chemical reactions occur at the same time, and the *o*-quinone end product spontaneously reacts with nucleophiles and/or with each other.

Successively the K_m and V_{max} values of tyrosine oxidation by tyrosinase are determined in the presence of caftaric acid (Figs. 7S and 8S in the Supplementary material), and the kinetic parameters are reported in Table 1.

As shown in Table 1, the V_{max} values determined for the tyrosinase catalyzed oxidation of tyrosine analyzing the data at 304 nm are similar in the absence or presence of low amount of caftaric acid. On the other hand, on increasing the concentration of inhibitor a decrease in the V_{max} value was observed. On the contrary, the K_m values increased in the presence of the hydroxycinnamic ester. Overall, our results clearly show that caftaric acid has different inhibition mechanisms as a function of its concentration. While displaying a competitive inhibition mode at low concentration, caftaric acid acts differently when its concentration is increased. This dual mode of inhibition is evident analyzing the Lineweaver-Burk plot of the experimental data reported in Fig. 8S.

This atypical behavior has been described in different research papers on derivatives of caffeic acid. Indeed, a recent study on *n*-nonyl caffeate demonstrated that this compound is a reversible competitive inhibitor of tyrosinase at low concentration, and an irreversible (suicide) inhibitor at high concentration (Jia et al., 2016). A similar behavior has been found also for caffeic acid, a substrate of tyrosinase, that at low concentration acts as a competitive inhibitor and at high concentration acts as a suicide inhibitor, due to the generated unstable *o*-caffeoquinone (Garcia-Jimenez et al., 2018).

The IC_{50} values, representing the concentration at which tyrosinase activity was reduced by 50%, were determined from the dose–response curves reported in Figs. 4 and 9S.

Caftaric acid activity was initially compared to that of caffeic acid and chlorogenic acid, the ester of caffeic acid with L-quinic acid, two naturally occurring tyrosinase inhibitors (Garcia-Jimenez et al., 2018; Li, Habasi, Xie, & Aisa, 2014). Moreover, the activity of caftaric acid was also compared to that of 4-(3,4-dihydroxyphenyl)-3-buten-2-one (Ohmura, Miyase, & Ueno, 1989; Yoshikawa, Kunioka, Kokudo, & Hashimoto, 2010), an efficient tyrosinase inhibitor with antimelanogenesis properties under study (personal communication). The dose–response curves showed that caftaric acid is the more active inhibitor of the tyrosinase activity with an IC_{50} of 30 μM, while the determined IC_{50} values for chlorogenic and caffeic acids are 42 and 65 μM, respectively. Moreover, caftaric acid is more active than 4-(3,4-dihydroxyphenyl)-3-buten-2-one, which has an IC_{50} 1.5 times greater (Fig. 9S in Supplementary material).

4. Conclusions

Cinnamic acid derivatives having a hydroxyl group or a methoxy group at the 4-position were good inhibitors of the tyrosinase enzyme (Billaud, Lecornu, & Nicolas, 1996). Among the analogs of cinnamic acid that possess one of these two groups, caffeic, coumaric and ferulic acids are present in grapes, usually esterified to the hydroxyl moiety of tartaric acid.

This study showed that caftaric acid can be easily isolated from unripe grape juice with a good yield and that the same procedure allows the isolation of coutaric and fertaric acids, in lower yields. Moreover, the enzymatic study demonstrated that caftaric acid successfully inhibits tyrosinase, with an inhibitory activity rated as competitive, and an IC_{50} value lower than those of the related compounds caffeic and chlorogenic acids.

Collectively these results suggest that caftaric acid alone can be used for the development of skin whitening formulations and as a natural agent able to counteract the enzymatic browning of fruits, vegetables, and seafood products. Furthermore, unripe grape juice could be a low-cost natural source of this molecule as it is obtained from thinned grapes normally discarded during viticulture.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

UV spectrum of the unripe grape juice; LC-MS analysis of unripe grape juice; preparative HPLC of unripe grape juice; ESI-(-) and ^{1}H NMR spectra mass spectra of the three isolated hydroxycinnamyl tartaric acids; schematic representation of the reactions catalyzed by tyrosinase; Michaelis-Menten plot of the reaction velocity (V0) against tyrosine concentration of tyrosinase catalyzed reaction in the presence of different caftaric acid concentrations; Lineweaver-Burk plot for tyrosinase enzyme inhibition by caftaric acid; dose-response plot of enzyme activity as a function of caftaric acid or 4-(3,4-dihydroxyphenyl)-3-buten-2-one concentrations. Supplementary data associated with this article can be found in the online version, at doi: https://doi.org/10.1016/j.foodchem.2019.125506.

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