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Inhibition of Apple Polyphenol Oxidase Activity by Procyanidins and Polyphenol Oxidation Products

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The rate of consumption of dissolved oxygen by apple polyphenol oxidase in cider apple juices did not correlate with polyphenol oxidase activity in the fruits and decreased faster than could be explained by the decrease of its polyphenolic substrates. The kinetics parameters of a crude polyphenol oxidase extract, prepared from apple (*Braeburn* cultivar), were determined using caffeoylquinic acid as a substrate. Three apple procyanidin fractions of $\overline{DP}$n 80, 10.5, and 4 were purified from the parenchyma of cider apples of various cultivars. Procyanidins, caffeoylquinic acid, (−)-epicatechin, and a mixture of caffeoylquinic acid and (−)-epicatechin were oxidized by reaction with caffeoylquinic acid *o*-quinone in order to form oxidation products. All the fractions were evaluated for their inhibitory effect on PPO activity. Native procyanidins inhibited polyphenol oxidase activity, the inhibition intensity increasing with $\overline{DP}$n. The polyphenol oxidase activity decreased by 50% for 0.026 g/L of the fraction of $\overline{DP}$n 80, 0.17 g/L of the fraction of $\overline{DP}$n 10.5, and 1 g/L of the fraction of $\overline{DP}$n 4. The inhibitory effect of oxidized procyanidins was twice that of native procyanidins. Oxidation products of caffeoylquinic acid and (−)-epicatechin also inhibited polyphenol oxidase.

KEYWORDS: Cider; browning; tannin; polyphenols; enzymatic inhibition

INTRODUCTION

The oxidative browning due to oxidation of polyphenols is quite important in the food industry. Polyphenol oxidation takes place after physical injuries inflicted to fruits and vegetables during harvesting and storage or during technological operations. For fresh fruits and vegetables, this phenomenon is often harmful because the yellow and brown pigments impart an undesirable appearance (*1*, *2*) to the end products. However, in some cases this coloring effect can be desired, for example, during the harvest of dates or for some beverages such as tea or cider.

The first step of the browning reaction is the oxidation of *o*-diphenols to their corresponding highly reactive *o*-quinones. This reaction uses oxygen as a second substrate and is catalyzed by polyphenol oxidase (PPO) (*3*, *4*). Then *o*-quinones react following different pathways according to their electrophilic and/or oxidant character to produce secondary products. A lot of previous works deal with characterization of the PPO enzymes, determination of their kinetics parameters (*5*, *6*), and their inhibition (*3*, *7*). Numerous PPO inhibitors were tested and proposed to prevent enzymatic browning.

Tannins and polyphenols are known to interact with proteins, leading to inhibition of enzymes (*8*−*13*) and decreased protein digestibility (*14*). However, none of these studies were made on enzymes of which polyphenols are substrates, and very little data is available about inhibition of PPO activity by phenolic compounds occurring naturally in the medium and known for their tanning effect, such as procyanidins or their oxidation products, formed by coupled oxidation between procyanidins and the *o*-quinones produced in the medium (*15*).

The phenolic composition of apple must and ciders depends on the initial composition of the fruit and the extraction and solubilization of the phenols during pressing. This pool is further modified during the process because of the oxidation of *o*-diphenols to their corresponding *o*-quinones which then react leading to the appearance of brown color. We studied the polyphenols of cider apple varieties representative of the french orchard, showing (*16*, *17*) total polyphenol concentrations up to 7 g/L and very variable depending on the cultivar. Proanthocyanidins were the most abundant class in all cultivars (between 44% and 89%, expressed on a weight basis), followed either by monomeric catechins or hydroxycinnamic acids; dihydrochalcones and flavonols were present as minor components. The degree of polymerization modulates the physicochemical properties of procyanidins (*18*). In apples, the average degree of polymerization ($\overline{DP}$n) of the procyanidins was characteristic of the cultivar (*17*). It varied usually between 4.2 and 7.5 with an exception for varieties *Avrolles* and *Guillevic*, which showed procyanidins with $\overline{DP}$n 40 and 50, respectively. In cider, the same four groups of polyphenol have been identified (*19*): hydroxycinnamic acids, phloretin derivatives, catechins, and procyanidins. Various oligomeric and polymeric

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procyanidins were also characterized such as dimers B-1, B-2, and B-5 (*19*).

The kinetic parameters of apple PPO have been studied in detail (*2*), in particular by Janovitz-Klapp et al. (*6*). Among the polyphenols present in apple, caffeoylquinic acid was the preferential substrate, followed by the monomeric catechins.

Some of our recent observations (unpublished data) have shown that the rate of oxygen consumption of apple juices did not correlate with the PPO activity of apple pulp as might have been expected. Moreover, the reaction rate decreased faster than the concentration of phenolic substrates, suggesting that some native phenolic compounds and some oxidation products could have an inhibitory action on PPO, thus limiting the oxidation process. This fact might have major consequences on the fermentation process used to convert juice into cider: for instance, the biochemical system PPO/polyphenol could compete with microorganisms toward oxygen; the oxygen depletion of the medium could limit the growth of some *Saccharomyces* spp, which need small amounts of oxygen to grow. Besides, this depletion can prevent aerobes such as moulds and acetic acid bacteria from developing. Inactivation of the PPO would lift the competition with the microorganisms and limit the production of oxidized polyphenols, which contribute to organoleptic properties of cider such as color.

To substantiate the hypothesis of an inhibition of PPO by phenolic compounds, procyanidins and chemically oxidized products have been prepared and tested for their effects on the polyphenoloxidase activity.

MATERIALS AND METHODS

Plant Material. Apple fruits (*Malus domestica* Borkh) from the Avrolles, Kermerrien, and Jeanne Renard cultivars were harvested at maturity during the 1999 season in the experimental orchard of the CTPC (Centre Technique des Productions Cidricoles; Sées, Orne, France). Fruits of the Braeburn variety were purchased at the local market.

For must preparation and preparation of the PPO extract, fresh plant material was used. For extraction of polyphenols, fruits were mechanically peeled and cored as already described (*16*) and cortex tissues were freeze-dried.

Controlled Oxidation of Apple Must. A 4 kg amount of cider apples (*Malus domestica*, var. Jeanne Renard) was crushed with cold ice. The milled pulp was collected in a cheesecloth and pressed under nitrogen. The must was collected in a flask and maintained under bubbling of nitrogen for 20 min. This prevented contact of the must with oxygen.

A 500 mL amount of this must was transferred to a closed vessel, equipped with a Clark electrode (model 3650, Orbisphere Laboratories, Neuchatel, Geneva, Switzerland), and an interface for gas diffusion. The must was kept under constant stirring at 25 °C. Controlled oxygenation was carried out by successive injections of oxygen in the must by diffusion of pure oxygen through an interface for 2 min. Oxygen rapidly diffused to reach up to 280 μM. The level then decreased as it was used by the PPO of the must. Oxygen addition was repeated when the oxygen level fell below the detection limit and until the time needed to decrease from 280 μM to nondetectable level was longer than 3 h.

Aliquots of 500 μL of must were taken before the reaction, at the end of each oxygenation, and at the end of the experiment and put in a Eppendorf containing NaF at a final concentration of 24 μM to stop PPO activity.

Preparation of a Crude Apple PPO Extract. Three apples (*Malus domestica*, var. Braeburn) were rapidly and mechanically peeled and cored at 4 °C. The flesh was crushed using a Waring Blendor with 1.5 L of malic acid/malate buffer (pH 3.8, 20 mM) containing ascorbic acid (20 mM). The mixture was sieved through a coarse filter (50 μm) to separate the liquid phase from the solid crude material, and dry ice was added to keep the mixture cold and to limit oxidation. The pomace was rinsed out twice with 500 mL of cold malic buffer containing ascorbic acid. The particles bigger than 50 μm were then eliminated. Filtrates were pooled to constitute the crude extract (2.5 L), which was dialyzed through a tangential Millipore filter with a 0.45 μm PTFE membrane, rinsed out with malic acid/malate buffer pH 3.8 (3 L containing ascorbic acid and then 3 L without ascorbic acid), and stored at 4 °C until used.

Extraction and Purification of Native Apple Procyanidins. Methanolic and aqueous acetone extracts of apple polyphenols were obtained by successive solvent extractions of the freeze-dried pulp material of three French cider apple varieties (*Malus domestica*, var. Jeanne Renard, Kermerrien, and Avrolles) according to Guyot et al. (*20*). The Kermerrien (Pro KE) and the Avrolles (Pro AV) aqueous acetone extracts were purified as described (*20*). The Jeanne Renard (Pro JR) methanol extract was purified according to the Reversed-Phase HPLC Purification of Oxidized Fractions of Pro JR section.

Procyanidin B-2, prepared as described in ref *20*, was a gift of Mr. S. Bernillon.

Chemical Oxidation of Caffeoylquinic Acid and (−)-Epicatechin. Caffeoylquinic acid and (−)-epicatechin were obtained from Sigma Chemical Co. (St. Louis, MO). They were oxidized using periodate immobilized on a solid matrix, according to a method adapted from Fulcrand et al. (*21*).

Caffeoylquinic acid (10 mM) or (−)-epicatechin (10 mM) in pure anhydrous methanol (100 mL) was stirred with the periodate form of Amberlite GC 400 type 1 (30 mM equiv of oxidant) for 10 min (or 2 min for (−)-epicatechin) at 30 °C, under ambient atmosphere. The resin was filtered off through a PVDF 0.45 μm membrane, and the *o*-quinone solution was cooled by dry ice.

The caffeoylquinic acid *o*-quinone solution (100 mL, 8 mM) was diluted with 900 mL of malic acid buffer (pH 3.8, 20 mM) containing either (−)-epicatechin (0.88 mM) or the Pro JR fraction (0.88 mM) or buffer alone, giving molarities of 0.8 mM for both *o*-quinone and flavan-3-ol, expressed in (−)-epicatechin units. The fractions were incubated at 30 °C for 2 h. Methanol was then removed by evaporation under vacuum. These samples were noted ECQ Ox, Pro JR Ox, and CQ Ox, respectively.

The (−)-epicatechin−quinone solution (100 mL, 8 mM), diluted with 900 mL of malic acid buffer (pH 3.8, 20 mM), was incubated at 30 °C for 2 h before removing methanol and noted EC Ox.

Reversed-Phase HPLC Purification of Oxidized Fractions and of Pro JR. The oxidized and native samples were purified on a semipreparative HPLC system (Rainin, Instrument Co. Inc.; Woburn, MA): two high-pressure pumps (Dynamax SD 300, Rainin) equipped with 100 WTI pump heads, a manual injection valve (Rheodyne L. P.; Rhonert Park, CA) equipped with a 5 mL injection loop, a UV-detector (Dynamax UV1, Rainin), and a EZ-chrom software (Scientific Software, Inc.; Pleasanton, CA) for data collecting and processing. The semipreparative column system (Waters) was a PrepLC 25-mm radial compression module equipped with a Prepnova-Pak HR-C18 Silica cartridge (25 × 100 mm, 6 μm, 60 Å) and a Guard NovaPak cartridge (25 × 10 mm). The purification was performed at a flow rate of 30 mL min^{-1} in a multistep elution. Conditions were solvent A (aqueous acetic acid, 2.5% v/v) and solvent B (acetonitrile); initial 99% A; 0−5 min, 97% A linear; 5−30 min, 70% A linear; 30−32 min, 50% A linear; 32−37 min, 10% A linear; 37−40 min, 99% A linear. For fractions CQ Ox, EC Ox, and ECQ Ox, the remaining caffeoylquinic acid and (−)-epicatechin were removed; the rest of the eluates were pooled. For the fraction Pro JR, the hydroxycinnamic acids, dihydrochalcones, (+)-catechin, and (−)-epicatechin were removed and the procyanidins collected as a single fraction.

Three HPLC runs were performed; the fractions were pooled and concentrated to remove organic solvents and freeze-dried.

Determination of Kinetic Constants of PPO. Kinetic constants of PPO were obtained using caffeoylquinic acid as substrate in a malic acid buffer solution (pH 3.8, 2 mL, and 20 °C). PPO activity (20 μL of the extract prepared as given in the Preparation of a Crude Apple PPO Extract section) was assayed by polarography with a Clark electrode (Oxymeter Oroboros, Innsbruck, Austria) using air-saturated substrate solutions at 20 °C ([O_2] = 280 μM). The caffeoylquinic acid

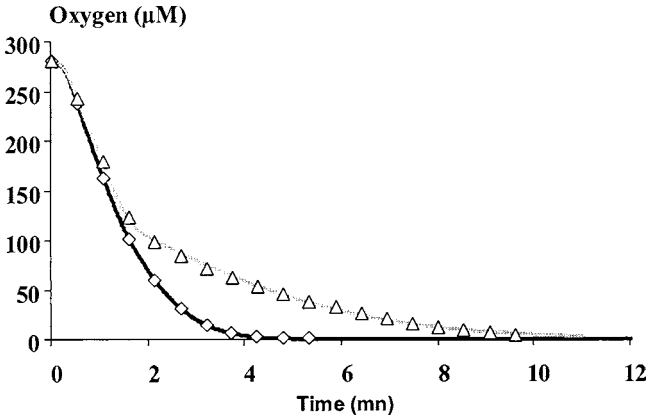

Figure 1. Experimental and calculated use of oxygen without or with addition of inhibitor: (◇) experimental data without inhibitor, (black line) model curve according to eq 1 without inhibitor, (△) experimental data with inhibitor, (gray line) model curve according to eq 1 with inhibitor.

concentration varied from 0.5 to 15 mM (0.5, 1, 2, 5, 10, 15 mM). A series of incubations was also performed with various oxygen concentrations. The latter were adjusted by bubbling either oxygen or nitrogen, and the medium was equilibrated 10 min before addition of the enzyme. Five oxygen concentrations were tested (50, 100, 200, 280, 350 μM). Initial oxygen concentrations were determined with the Clark electrode. All assays were performed in duplicate.

Enzymatic Oxidations. Enzymatic oxidations were carried out as described above for the determination of kinetic parameters using 2 mM caffeoylquinic acid in a malic acid buffer at pH 3.8. When the oxygen concentration reached 120 μM, 20 μL of the solution to be tested was added. The registered O_2 concentrations were fitted to a model to calculate the consumption rate reduction (as a percentage of the initial rate), which represented the inhibiting effect. Aliquots were filtered through 0.45 μm membranes and analyzed by HPLC to determine the stoichiometry of the reaction with respect to O_2 and caffeoylquinic acid. All assays were duplicated.

Determination of an Integration Model. Experimental results have been adjusted with a Michaelis model with two substrates (eq 1)

$$V = \frac{[\Phi]\,[O_2]\,V_{max}}{(Ki_{O2}Km_{\Phi}) + (Km_{O2}[\Phi]) + (Km_{\Phi}[O_2]) + ([\Phi][O_2])}$$

where V = rate of the reaction, [Φ] = phenolic substrate concentration, [O_2] = oxygen concentration, V_{max} = maximal rate, Ki_{O2} = dissociation constant of E−O_2 complex, Km_{O2} = affinity constant for oxygen, and Km_{ϕ} = affinity constant for the phenolic substrate.

Knowing the kinetics parameters, the rate of the reaction was calculated as a function of the concentration of oxygen and phenolic compounds, but a numeric integration must be done to fit the oxygen evolution curves. The stoichiometric rate we used to link the evolution of phenolic compounds and that of oxygen was a mean value. It was determined from the HPLC analysis of an aliquot of the solution after reaction (we assumed this rate to be constant during the assay). This model was applied to our results (**Figure 1**) and fitted by minimizing the sum of the square of the difference between observed and calculated values. In our conditions (consumption of 280 μM of oxygen to oxidize added chlorogenic acid in a pH 3.8 malic buffer, at 25 °C), the model could be made to fit correctly the experimental values by optimizing a single parameter, the V_{max} value. This good fit led us to assume that the oxidation products which appeared during the assay did not have enough effect to perturb the measurement.

When inhibitory compounds were added, the two parts of the curve were fitted with two different V_{max} and the inhibiting effect was obtained as the ratio between the calculated V_{max} before and after addition of the inhibitor (**Figure 1**). If the curves reflecting the inhibition are fitted by changing only V_{max}, this means that the inhibition is of noncompetitive type. This method was chosen to avoid the imprecision that would arise from minute differences in enzyme concentrations and volumes added between experiments.

Thioacidolysis Degradation. Toluene-α-thiol was purchased from Merck (Darmstadt, Germany), and organic solvents of HPLC-grade were purchased from Biosolve LTD (Nertherlands).

Thioacidolysis conditions of the must were the same as previously described (*20*). The freeze-dried juice (200 μL) was dissolved with 160 μL of dried methanol acidified by concentrated HCl (3.3% v/v), and 320 μL of toluene-α-thiol solution (5% v/v in dried methanol) was added. The reaction was carried out at 40 °C for 30 min. After filtration (PTFE, 0.45 μm), the reaction medium was directly injected (10 μL) into the HPLC system.

Thioacidolysis of procyanidins fractions (oxidized or not): a 4 g/L solution of each dry fraction was prepared by sonicating the fraction in methanol. In a 250 μL glass insert (02CTVG, Chromacol, Trumbull, CT), 50 μL of the solution was mixed together with 50 μL of methanol acidified by concentrated HCl (3.3% v/v) and 100 μL of toluene−α-thiol solution (5% v/v in dried methanol) was added. After sealing with an inert cap (8AC-CBT1, Chromacol), the reaction was carried out at 40 °C for 30 min. After filtration (PTFE, 0.45 μm), the reaction medium was directly injected (10 μL) into the HPLC system.

HPLC Conditions. HPLC analyses without thiolysis were carried out: the freeze-dried juice (200 μL) was dissolved in 480 μL of methanol acidified by acetic acid (1% v/v) and sonicated for 15 min. All samples were filtered through a 0.45 μm filter (PTFE, Interchim, France), and 10 μL of each filtrate was injected into the HPLC system.

HPLC analyses of the enzymatic reaction media in the presence of inhibitor were performed by filtering the media (0.45 μm filter, Interchim, France) and injecting 10 μL of each filtrate into the HPLC system.

The HPLC apparatus was a Waters (Milford, MA) system 717 plus autosampler equipped with a cooling module set at 4 °C, a 600 E multisolvent system, a 996 photodiode array detector, and a Millenium 2010 Manager system). The column was a Purospher RP18 endcapped, 5 μm (Merck, Darmstadt, Germany). The solvent system was a gradient of solvent A (aqueous acetic acid, 2.5% v/v) and solvent B (acetonitrile): initial, 3% B; 0−5 min, 9% B linear; 5−15 min, 16% B linear; 15−45, 50% B linear, followed by washing and reconditioning the column.

HPLC Characterization and Quantification of Phenolic Compounds. Phenolic compounds, for which standards were available, were identified by their retention time and their UV−vis spectra as already described by Guyot et al. (*16*). Flavan-3-ols and dihydrochalcones were quantified at 280 nm and hydroxycinnamic acids at 320 nm.

Quantification was performed by reporting the integration area in the calibration equation of the corresponding standard. Flavan-3-ols (i.e., (−)-epicatechin, (+)-catechin, and (−)-epicatechin−benzylthioether) were quantified as (−)-epicatechin equivalent and dihydrochalcones (i.e., phloridzin and phloretin xyloglucoside) as phloridzin equivalent. For caffeoylquinic acid and *p*-coumaroylquinic acid, we used their own response factor.

Calculation of Standard Errors for Effects Using Replicated Runs. First, one calculates the variance for each analysis and then considers the original variance of handling by calculating the average of our variances balanced by the degree of freedom

$$S^2 = \frac{\sum S_i^2 \upsilon_i}{\sum \upsilon_i}$$

with υ_i degrees of freedom, which is equal to

$$\upsilon_i = n_i - 1$$

and n corresponds to the number of repetitions for each analysis. The standard error σ is calculated in this way

$$\sigma = \frac{\sqrt{S^2}}{\sqrt{n}}$$

The precision of the manipulation P is obtained in this way

$$P = \sigma t$$

Table 1. Initial Composition (mM) of "Jeanne Renard" Apple Must and Final Composition (mM) of "Jeanne Renard" Apple Must after 600 min of Oxidation[a]

conc.	B2	CAT	EC	CA	PL	XPL
initial (mM)	1.30	0.08	2.82	1.60	0.04	0.09
final (mM)	0.95	0.04	2.14	1.35	0.03	0.06

[a] B2, procyanidins B2; CAT, (+)-catechin; EC, (–)-epicatechin; CA, caffeoylquinic acid; PL, phloridzin; XPL, phloretin xyloglucoside.

where t corresponds to the value of the table of Student for a probability of 95% and a degree of freedom v equal to the sum of the degrees of freedom of each analysis which is

$$v = \sum v_i$$

RESULTS AND DISCUSSION.

Oxidation of Apple Must. Reverse-phase HPLC was used to characterize and quantitate the phenolic compounds (*16*, *20*).

The initial and final phenolic compositions of the anaerobically prepared apple must are presented in **Table 1**. The different groups of phenolic compounds displayed differences in sensitivity to oxidation**.** The concentrations of dihydrochalcones, i.e., phloridzin and phloretin xyloglucoside, were not modified, while the concentrations of caffeoylquinic acid and flavan-3-ols decreased. Among these compounds Janovitz-Klapp et al. (*6*) showed that caffeoylquinic acid and catechin were substrates of PPO. However, caffeoylquinic acid disappeared less than expected, while the concentration of procyanidin B2, which is a poor substrate of polyphenoloxidase (*22*), decreased. We verified that isolated B2 as well as our fractions were not substrates for our apple PPO preparation. This decrease was due to coupled oxidation mechanisms between caffeoylquinic acid and procyanidin B2, which lead to reduction of the *o*-quinone of caffeoylquinic acid back to caffeoylquinic acid. (*15*).

This apple must was submitted to successive oxygenations. Evolution of the oxygen concentrations is reported on **Figure 2A**. The fast increase of the concentration represented diffusion of oxygen from the membrane into the body of the liquid. This was followed by a decrease, due to the PPO activity. Though the total oxygen input at each oxygenation was nearly the same, the first peaks were often lower. This observation was ascribed to the relative rates of oxygen diffusion and consumption by PPO: when the oxygen concentration rose, the rate of the enzymatic reaction increased and could actually exceed the oxygen diffusion rate.

These curves were geometrically derivatized to calculate the rate curves (**Figure 2A**). The rate decreased both with the oxygen concentration (as expected) and with successive oxygenations. A reason for this second decrease could be the consumption of the phenolic substrates (**Figure 2B**), the concentrations of which were below the Km. However (**Figure 3**), the rate of oxygen evolution was divided by 25 while the substrate concentration was divided at most by 1.5 (for (−)-epicatechin). Therefore, the decrease of the phenolic substrate could not be the only reason to explain the reduction of the PPO activity.

On the same time scale, inactivation of PPO in a model solution presenting the same ionic and temperature conditions was not significant (results not shown). This implied an inhibition of this enzyme by its reaction products or by procyanidins, which are known for their tanning effect.

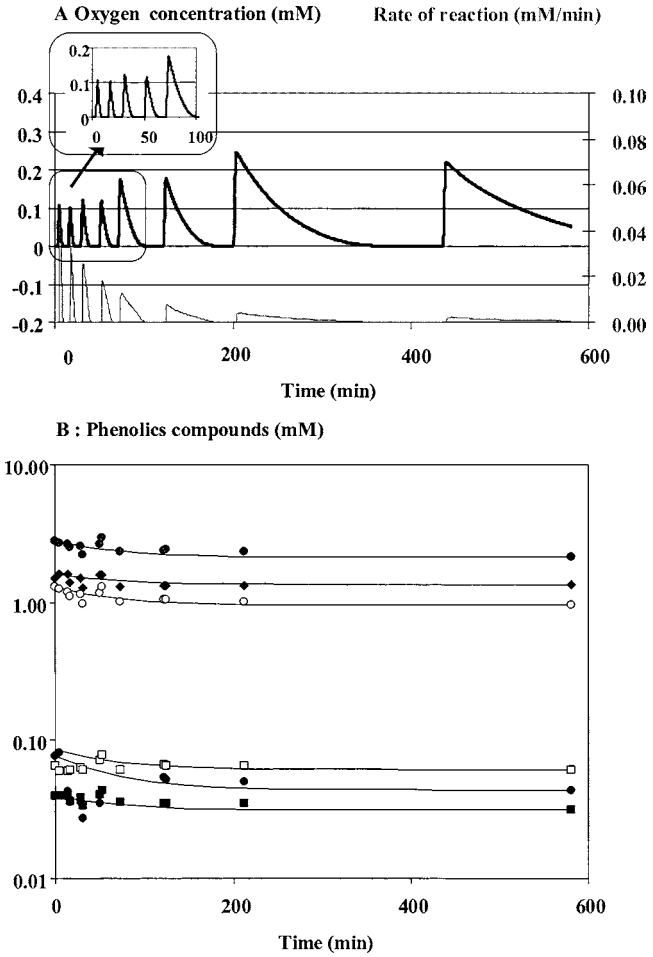

Figure 2. (A) Evolution of the concentration of oxygen and of the rate of the reaction as a function of successive oxygenations in "Jeanne Renard" apple must: (black line) oxygen concentration (mM), (gray line) rate of reaction (mM/min). (B) Evolution of phenolic compounds concentration as a function of successive oxygenations in "Jeanne Renard" apple must: (◆) caffeoylquinic acid, (◇) para coumaroylquinic acid, (●) (+)-catechin, (gray dots) (−)-epicatechin, (○) procyanidin B2, (■) phloridzin, and (□) phloretin xyloglucoside.

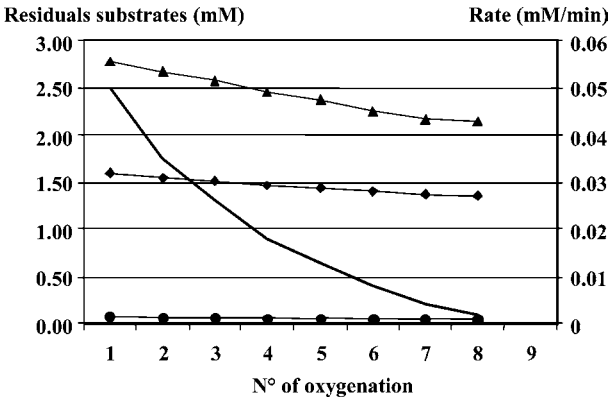

Figure 3. Evolution of the rate of the reaction and of the phenolic substrates of PPO as a function of successive oxygenations in "Jeanne Renard" apple must: (▲) (–)-epicatechin, (◆) caffeoylquinic acid, (●) (+)-catechin, (–) rate of the reaction.

Characterization of the Crude PPO Extract. To verify this hypothesis, PPO was extracted from apple; procyanidins and chemically oxidized products were also prepared and tested for their effects on the polyphenoloxidase activity. PPOs catalyze

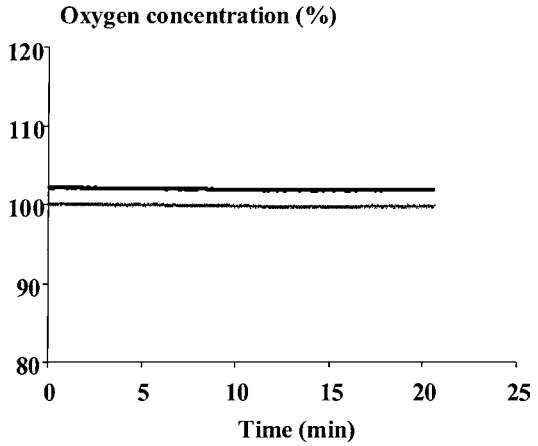

Figure 4. Oxygen consumption in the absence of enzyme (i.e., acid malic buffer pH 3.8 containing 2 mM caffeoylquinic acid) and in the presence of NaF: (–) oxygen consumption in the absence of enzyme and (—) oxygen consumption in the presence of NaF, enzyme, and caffeoylquinic acid 2 mM (acid malic buffer pH 3.8).

Table 2. Kinetic Parameters of Apple PPO with Caffeoylquinic Acid as Substrate in Air-Saturated Solutions at 20 °C and pH 3.8 Compared to Those Obtained by Janovitz-Klapp (1990) with Caffeoylquinic Acid as Substrate in Air-Saturated Solutions at 30 °C and pH 4.5[a]

kinetic parameters	experimental values (mM)	Janovitz-Klapp values (1990) (mM)
Km_{φ}	3.44	4.2
KmO_2	0.25	0.28
KiO_2	0.01	0.29

[a] Km_{φ}, affinity constant for the phenolic substrate; KmO_2, affinity constant for oxygen; KiO_2, dissociation constant of $E–O_2$ complex.

two types of reactions, cresolase and catecholase, but only catecholase activity has been considered in the present work.

The experiments need to carry out at least two important negative controls: oxygen measurements in the absence of enzyme (i.e., adding buffer but no PPO preparation) and measurements in the presence of a known PPO inhibitor, e.g., NaF, insofar as the PPO produced is a crude preparation, containing a large number of small molecules as well as other enzymes. The oxygen consumption could be derived in part from chemical (nonenzymatic) oxidation of the polyphenols as well as other enzymes. It is therefore important to demonstrate that the results are really due to PPO.

No oxygen consumption in the absence of enzyme or in the presence of NaF was observed (**Figure 4**). By these measurements we can say with certainty that the results observed in this work are really due to PPO and not to another enzyme.

Janovitz-Klapp et al. (*6*) determined the kinetic parameters of apple PPO, KmO_2, Km_{φ}, and KiO_2 (related to a Michaelian model for two substrates and two products). In the present work, we used a crude PPO preparation to mime the conditions of fresh apple juice and measured the oxygen concentration to determine the parameters (**Table 2**). Initial rates of reaction are reported as a function of caffeoylquinic acid (**Figure 5A**) and oxygen (**Figure 5B**) concentrations. Caffeoylquinic acid concentrations were close to the value of the Km_{φ}, which could be determined precisely, while the oxygen concentrations were not high enough to obtain a precise value of KmO_2 and KiO_2. Our results were in good agreement with those of Janovitz-Klapp (*6*) (**Table 2**) except for KiO_2.

Characterization of Procyanidins Fractions. Thiolysis (acid-catalyzed cleavage of the interflavanyl linkages of procyanidins in the presence of a nucleophile reagent such as toluene−α-thiol) followed by reverse-phase HPLC was used to calculate the average degree of polymerization of procyanidins and to characterize and quantitate the phenolic compounds (*16*, *20*).

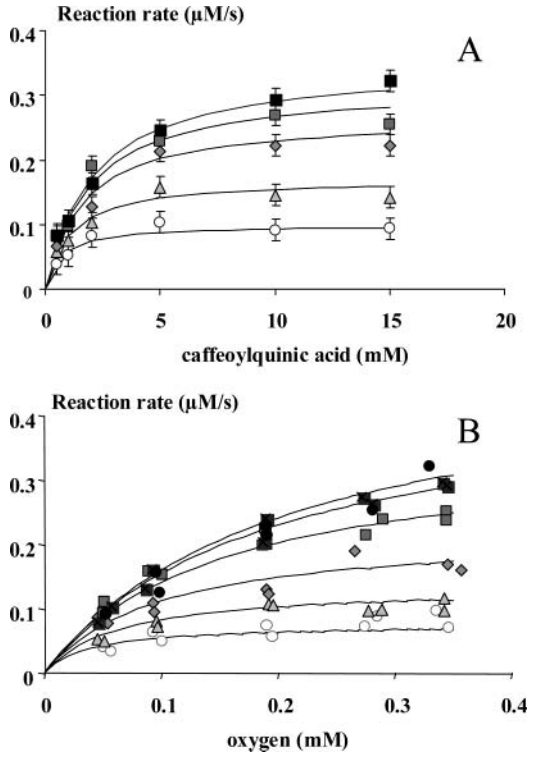

Figure 5. (A) Initial speed of the reaction as a function of the caffeoylquinic acid concentration (0.5, 1, 2, 5, 10, and 15 mM) with fixed oxygen concentration (0.05, 0.1, 0.2, 0.28, 0.35 mM). (B) Initial speed of the reaction as a function of the oxygen concentration (0.05 mM, 0.1 mM, 0.2 M, 0.28 mM, 0.35 mM) with fixed caffeoylquinic concentration (0.5, 1, 2, 5, 10, and 15 mM). The curves correspond to model curves with different oxygen concentration and with the kinetics parameters we have determined (0.05, 0.1, 0.2, 0.28, and 0.35 mM oxygen; 0.5, 1, 2, 5, 10, and 15 mM caffeoylquinic acid).

Table 3. Composition of the Identified Compounds (%) and Yields (wt %) of the Procyanidins Fractions Tested on Apple PPO[a]

fraction	CAT (%)	HCA (%)	PC (%)	$\overline{DP}$n	yields
Pro JR	11	0	89	4	87%
Pro JR Ox	2	0.3	97	3.8	44%
variation	–11				–50%
Pro KE	0	3	91	10.5	82%
Pro AV	1	7	73	80.5	95%

[a] CAT, monomer of catechin; HCA, hydroxycinnamic acid; PC, procyanidins; $\overline{DP}$n, average degree of polymerization. Yield: calculated from dry weight of procyanidins fraction.

The three apple varieties, Jeanne Renard, Kermerrien, and Avrolles, were chosen for the contrasting characteristics of their procyanidins (*17*) (**Table 3**). The extracts were essentially composed of flavan-3-ols, themselves mainly constituted of (−)-epicatechin (95%) (*20*), and $\overline{DP}$n ranged between 4 and 80. Thioacidolysis yields calculated according to weight were between 44% and 95%, which was consistent with previous results (*20*); procyanidins represented between 73% and 97% of identified phenolic compounds.

Characterization of Chemically Oxidized Fractions. The Pro JR Ox fraction was obtained by coupled oxidation with

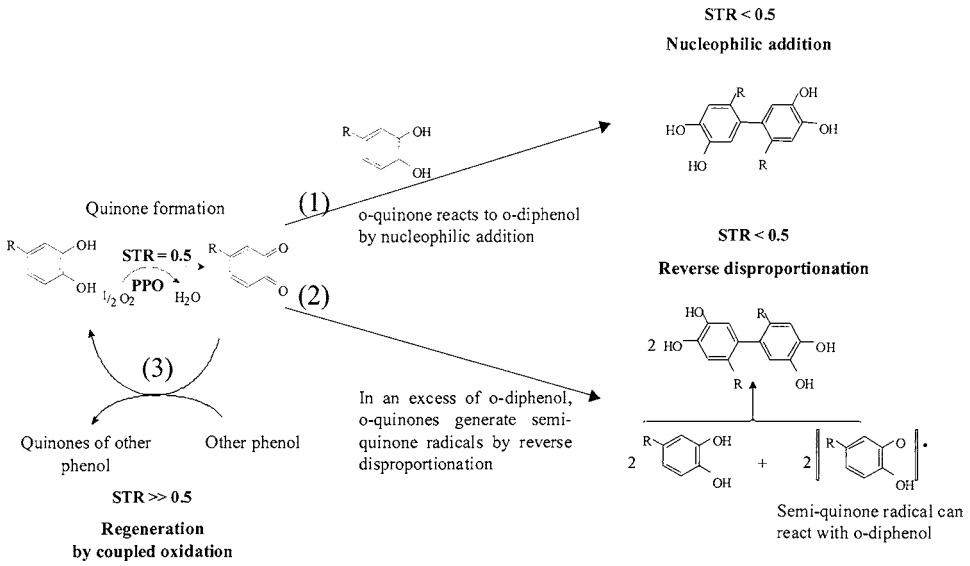

Figure 6. Possible pathways for further reaction of the *o*-quinone of caffeoylquinic acid and their consequences on the stoichiometric ratio (STR): (1) nucleophilic addition, (2) reverse disproportionation, (3) coupled oxidation.

caffeoylquinic acid *o*-quinone. This resulted in a 50% reduction of the concentration of measurable procyanidins (**Table 3**). The "loss" in procyanidins was probably due to formation of new bonds, resistant to thioacidolysis, with two possible mechanisms: formation of *o*-quinone of procyanidins by reaction with the *o*-quinone of caffeoylquinic acid and subsequent intermolecular addition with other molecules such as caffeoylquinic acid and procyanidins and conversion of the native B-type procyanidins to A-type (*23*, *24*).

CQ Ox and EC Ox fractions were obtained by oxidation of caffeoylquinic acid or (−)-epicatechin by periodate. This led to a 60% reduction of the concentration of caffeoylquinic acid and a 44% reduction of the concentration of (−)-epicatechin. These losses were interpreted as the result of the transformation into *o*-quinones, which themselves reacted with a hydroquinone to yield condensation products (*25*). According to Oszmianski and Lee (*26*), the oxidation products of caffeoylquinic acid might be essentially dimers. Oxidation compounds of (+)-catechin (*27*) were mainly dimers and trimers called dehydrodi- and tricatechins. Therefore, such oligomers based on (−)-epicatechin structure may be constitutive of the EC Ox fraction.

ECQ Ox fraction was obtained by coupled oxidation between the *o*-quinone of caffeoylquinic acid and (−)-epicatechin at a molar ratio of 1/1. This led to a 60% reduction of the concentrations of both caffeoylquinic acid and (−)-epicatechin. The products might be heterooligomers and homooligomers of caffeoylquinic acid and (−)-epicatechin as proposed by Oszmiansky and Lee (*26*).

Inhibition of PPO. *Stoichiometry of the Reaction.* PPOs catalyze conversion of *o*-diphenol into *o*-quinone with the theoretical stoichiometry of 0.5 mol of O_2 consumed for each mole of oxidized phenol (*3*, *4*). In the literature the ratios O_2/consumed phenol vary from 0.4 to 2 mol/mol (*25*, *28*−*30*) and seem to depend on the conditions of oxidation, the substrate used, and its concentration.

The *o*-quinones are very reactive species with an electrophilic character which may react in a different manner (**Figure 6**).

(1) The *o*-quinone may react with an *o*-diphenol to yield a condensation product (*26*). This mechanism, analogous to a Michael 1,4-nucleophilic addition, leads to the consumption of an additional molecule of *o*-diphenol per *o*-quinone formed resulting in a O_2/phenol ratio lower than 0.5.

Table 4. Variations of the Stoichiometric Ratio O_2/Caffeoylquinic Acid for the Inhibition of PPO by Procyanidins and Oxidized Compounds at Different Concentrations

	inhibitor (g/L)							
fraction	0	0.05	0.1	0.15	0.25	0.5	0.75	1
CQ Ox	0.36	0.27		0.26				
ECQ Ox	0.36	0.27		0.27				
EC Ox	0.36					0.55		0.58
Pro JR	0.36		0.57		0.64	0.86	1.11	1.07
Pro JR Ox	0.36		0.48		0.55	0.67	0.64	0.69
Pro KE	0.36						1.06	1.26
Pro AV	0.36		0.5			0.74	0.95	1.15

(2) In the presence of a large excess of *o*-diphenol, *o*-quinones generate semiquinone radicals by reverse disproportionation. According to this mechanism, one *o*-diphenol reacts with one *o*-quinone to produce two semiquinone radicals. Each semiquinone radical then reacts with an *o*-diphenol (*31*). Therefore, this mechanism leads to the consumption in total of three molecules of *o*-diphenol per *o*-quinone formed, resulting in a larger decrease of the O_2/phenol ratio.

(3) *o*-Quinones were shown to oxidize other phenols by coupled oxidation with reduction of *o*-quinone back to *o*-diphenol (*15*, *29*). With this mechanism, no caffeoylquinic acid is consumed and the stoichiometric ratio can become very high.

The ratios observed after the oxidation of caffeoylquinic acid by PPO with or without inhibitor compounds are given in **Table 4**.

With caffeoylquinic alone or mixed with its oxidation products CQ Ox or ECQ Ox, the stoichiometric ratio was 0.36 or 0.27, much lower than 0.5. Caffeoylquinic acid was thus consumed both directly by PPO and by reactions of its *o*-quinone. In the absence of substrates with redox potentials lower than caffeoylquinic acid *o*-quinone, the *o*-quinone may condense with *o*-diphenol by any of the first two mechanisms described above. Such condensation products have been described for caffeic acid (*21*) and identified for caffeoylquinic acid in our experimental conditions (*32*).

When procyanidins (Pro JR, Pro KE, and Pro AV) were present, the stoichiometric ratio was higher than 0.36 and increased as a function of the concentration of added procya-

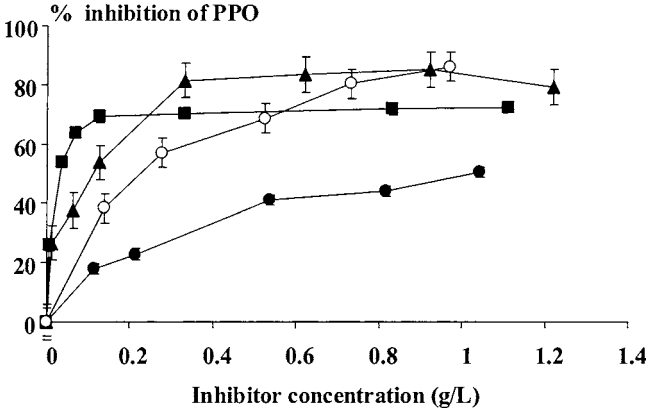

Figure 7. Inhibitory effect of native and oxidized procyanidins on apple PPO activity. Conditions were 20 μL of apple PPO, 2 mM caffeoylquinic acid as substrate in a malic acid buffer solution (pH 3.8, 2 mL, and 20 °C). PPO activity was assayed by polarography: (■) Pro AV, (▲) Pro KE, (●) Pro JR, (○) Pro JR Ox.

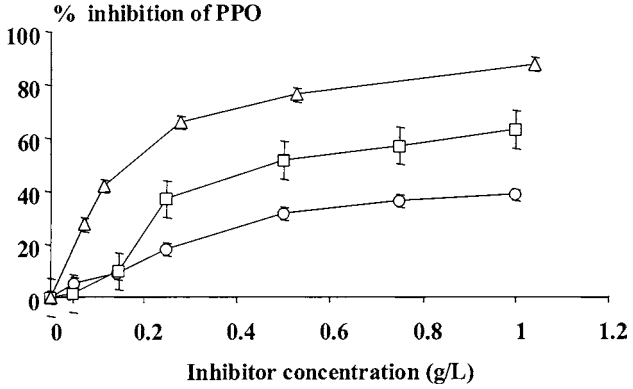

Figure 8. Inhibitory effect of oxidized phenolic compounds on apple PPO activity. Conditions were 20 μL of apple PPO, 2 mM caffeoylquinic acid as substrate in a malic acid buffer solution (pH 3.8, 2 mL, and 20 °C). PPO activity was assayed by polarography: (○) CQ Ox, (□) EQ Ox, and (△) EC Ox.

nidins (**Table 4**). This could be explained by the predominance of coupled oxidations. The more procyanidins are added, the higher the probability of coupled oxidation.

In the presence of the EC Ox fraction, corresponding to (−)-epicatechin oxidation products, the mean stoichiometric ratio was higher than 0.36 (**Table 4**), which was the stoichiometric ratio without any addition of oxidation products. This may be due to coupled oxidation between the *o*-quinone of caffeoylquinic acid and oxidized compounds of (−)-epicatechin, which might have a lower redox potential than the *o*-quinone of caffeoylquinic acid.

PPO Inhibition by Procyanidins and Their Oxidation Products. All the tested fractions inhibited PPO (**Figure 7**), and the percentage of inhibition increased with the concentration of procyanidins up to a plateau. The level of this plateau and the concentration at which it was reached depended upon the degree of polymerization of the procyanidins. For Pro AV, 50% of inhibition was reached for a concentration of 0.026 g/L, whereas for Pro KE and Pro JR, 50% inhibition was reached, respectively, for concentrations of 0.17 and 1 g/L. A nonspecific inhibition has usually been used to describe the inhibition of different enzymes by procyanidins and assumed to be related to a tanning effect (*9*, *33*). This tanning effect of polyphenols is a function of both the presence of functional groups able to form hydrogen bonds and hydrophobic interactions with proteins and their molecular weights (*33*). Helsper et al. (*11*) showed that the degree of polymerization and the number of phenolic hydroxyls may affect the inhibitory effect of procyanidins with respect to trypsin. The larger polyphenols have been reported to bind more strongly with proteins (*34*, *35*). The efficiency of polyphenol binding to proteins is due to the fact that polyphenols are polydentate ligands able to bind simultaneously at several sites of the protein surface (*34*, *35*). This could explain the differences between Pro AV, Pro KE, and Pro JR. Here the inhibitory effect of Pro AV of $\overline{DP}$n 82 is 7-times that of Pro KE of $\overline{DP}$n 10.5 and 40-times that of Pro JR of $\overline{DP}$n 4. The binding of the polyphenol to the protein may affect the catalytic activity of the enzyme by preventing the binding of the substrate to the active site or by forming an inactive enzyme−polyphenol−substrate complex (*36*). However, the existence of an inhibition plateau rules out binding at the active site, and an explanation of the inhibition and its plateau might be sought in terms of accessibility to the enzymatic protein and to its hydrophobic surface sites.

Proteins have only a few sites of binding for polyphenols, and the affinity of polyphenols for these sites increases with their $\overline{DP}$ (*37*, *38*). With procyanidins of very high MWs, such as Pro AV, we can suppose that the binding of a few molecules to each protein will induce a "cluttering" of the surrounding space, thus limiting access to the other sites, though procyanidins can continue to accumulate by stacking (*35*, *39*). Therefore, the maximum level of inhibition is quickly reached as the sites of high affinity are bound to procyanidin molecules for low concentrations, but these molecules inhibit further access. With polyphenols of low MWs, more binding sites still stay accessible so that it is possible to reach higher levels of inhibition with the requirement of higher concentration. In **Figure 7** the effects of Pro JR and Pro JR Ox are compared. The two graphs had the same appearance, but the level of inhibition of oxidized procyanidins was twice that of the native procyanidins, though in the case of Pro JR the plateau of inhibition was not reached at 1 g/L. The oxidized procyanidins keep their basic phenolic structure but may have higher molecular weights due to condensation reaction with caffeoylquinic acid *o*-quinone. Insofar as the Pro JR Ox fraction was obtained by coupled oxidation with caffeoylquinic acid *o*-quinone and subsequent intermolecular addition with other molecules such as caffeoylquinic acid and procyanidins can occur.

Oxidation Products of (−)-Epicatechin and Caffeoylquinic Acid. All three fractions tested did lead to inhibition of the PPO (**Figure 8**). The percentage of inhibition increased with the inhibitor concentration. However, the relation was not linear. The (−)-epicatechin oxidation products, EC Ox, showed stronger inhibition than caffeoylquinic acid oxidation products, CQ Ox. At 1 g/L, the level of inhibition was 87% for the (−)-epicatechin oxidation products and 40% for caffeoylquinic acid oxidation products. The inhibition power of ECQ Ox was intermediary between CQ Ox and EC Ox.

There is little work on the inhibition of PPO by its oxidation products, and the mechanism is not clear. Guyot et al. (*12*) showed that oxidation products of (+)-catechin inhibit the β-glucosidase of sweet almond with a noncompetitive mechanism in which polyphenol and substrate were assumed to bind simultaneously to the enzyme. The inhibitory effects found by Guyot et al. (*12*) depended not only on the number of *o*-diphenols function but also on the conformations of the molecules, which modified their ability to interact with proteins due to differences in the respective space orientations of hydroxyl functions and aromatic rings. Yellow oxidation

products from (+)-catechin were the strongest inhibitors of the β-glucosidases. Oxidized compounds from EC Ox were the most colored compounds and the most inhibitory with respect to the PPO.

The differences of inhibitor power observed between oxidation products from caffeoylquinic acid and those from (−)-epicatechin confirmed that the nature of the constitutive unit was an important factor of the inhibitory power.

CONCLUSION

The hypothesis of an inhibition of PPO by procyanidins, native and oxidized, and by oxidation products of caffeoylquinic acid and (−)-epicatechin was confirmed. The percentage of inhibition of PPO increased with the molecular weight of procyanidins. Oxidation of procyanidins doubled their effect for the inhibition of PPO. Oxidation products from (−)-epicatechin were more effective than those from caffeoylquinic acid.

The inhibition of apple PPO by procyanidins, the main polyphenols in apple fruits, can have a number of consequences on apple processing to juices and ciders. Among these and not the least is the different color evolution which can be seen between cultivars containing procyanidins of very different $\overline{DP}$n (*17*) with a quite similar initial PPO activity. This observation can explain the difficulties encountered during work on apple browning. Indeed, correlation between concentrations of individual or total phenolic compounds and browning is not sufficient to explain browning sensibilities of all varieties (*40*). Increased inhibitory effects of oxidation products mean that the system is self-limiting, i.e., the anaerobic conditions induced by polyphenol oxidation will be lifted after an interval which depends on the polyphenol composition of the must and the oxygen availability. This has important implications for the evolution of yeast populations during cider fermentation. Indeed, PPO oxidation does affect yeast growth in practical conditions during the first steps of the fermentation process, i.e., before any clarification.

ABBREVIATIONS USED

CQ Ox, oxidation product from caffeoylquinic acid; EC Ox, oxidation product from (−)-epicatechin; ECQ Ox, oxidation product from (−)-epicatechin and caffeoylquinic acid; $\overline{DP}$n, average degree of polymerization; B2, procyanidin B2; Pro AV, procyanidins from Avrolles cultivar; Pro KE, procyanidins from Kermerrien cultivar; Pro JR, procyanidins from Jeanne Renard cultivar; Pro JR Ox, oxidized procyanidins from Jeanne Renard cultivar; PPO, polyphenol oxidase; Var., variety; STR, stoichiometric ratio.

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