Influence of pH on the Enzymatic Oxidation of (+)-Catechin in Model Systems

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PPO-catalyzed oxidation of (+)-catechin was performed in aqueous buffers in the pH range 3–7, using the same level of PPO activity at all pH values. Concentration of (+)-catechin and major oxidation products was monitored by HPLC. The nature and amounts of products formed were highly pH dependent. The solutions oxidized at pH below 4 contained mostly colorless products (λ_{max} 280 nm), whereas yellow compounds (λ_{max} 385–415 nm), eluting later than the colorless ones, predominated at higher pH values. Seven of them were purified by semipreparative HPLC. FAB-MS and acid hydrolysis data indicated they were dimers differing from natural procyanidins.

Keywords: *(+)-Catechin; oxidation products; polyphenol oxidase; pH; HPLC*

INTRODUCTION

Browning resulting from oxidation and coupling of phenols is a ubiquitous phenomenon and a major cause of quality degradation during handling, storage, and processing of fruits (Mathew and Parpia, 1971; Lee, 1992). The fundamental step is the enzymatic oxidation of phenolic compounds to *o*-quinones, catalyzed by polyphenol oxidase. *o*-Quinones can be monitored by HPLC either directly (Rouet-Mayer *et al.*, 1990; Cheynier and Moutounet, 1992; Richard-Forget *et al.*, 1992) or after derivatization with benzenesulfinic acid (Cheynier *et al.*, 1989a). They can undergo various types of reactions, namely formation of adducts with nucleophilic substrates (e.g. thiols, amines, sulfones, phenols, and water) and oxidation of lower redox potential molecules, including ascorbic acid, sulfur dioxide, and other phenols, which are then oxidized to the corresponding *o*-quinones. The fate and stability of *o*-quinones vary widely, depending both on the phenolic precursor and on environmental factors. In particular, the *o*-quinones of (+)-catechin and (−)-epicatechin were shown to be much less stable than those of chlorogenic acid (Rouet-Mayer *et al.*, 1990; Richard-Forget *et al.*, 1992) or caffeoyltartaric acid (Cheynier *et al.*, 1989b), and their instability increased at higher pH values (Rouet-Mayer *et al.*, 1990).

Hydroxycinnamic acids and flavan-3-ols seemed equally involved in the apple browning susceptibility (Amiot *et al.*, 1992). As well, grape must oxidative browning was shown to depend largely on the hydroxycinnamic acid level (Cheynier *et al.*, 1990) but was greatly enhanced by addition of flavan-3-ols (Rigaud *et al.*, 1991), whereas browning susceptibility of wines appeared mostly related to their flavan-3-ol content (Cheynier *et al.*, 1989c). Oxidized (epi)catechin solutions were highly colored compared to those derived from phenolic acids (Lea, 1984; Lee and Jaworski, 1988; Oszmianski and Lee, 1990; Rouet-Mayer *et al.*, 1990). The products formed by enzymatic oxidation of (+)-catechin could be separated by HPLC (Goodenough *et al.*, 1983; Oszmianski and Lee, 1990; Rouet-Mayer *et al.*, 1990).

According to Goodenough *et al.* (1983), polyphenol oxidase oxidation of (+)-catechin yielded colored material (λ_{max} 390 nm) which may be analogous to the dehydrodicatechin A identified in solutions oxidized using peroxidase (Weinges *et al.*, 1969) and smaller amounts of a colorless product, presumably 8-hydroxycatechin, also formed by peroxidase-catalyzed oxidation of (+)-catechin (Weinges and Ebert, 1968). The formation of other yellow pigments (Oszmianski and Lee, 1990; Rouet-Mayer *et al.*, 1990), showing maximum absorbance at 380 and 420 nm, and of procyanidin B3 (Oszmianski and Lee, 1990) was also reported. On the other hand, peroxidase oxidation of (+)-catechin afforded four colorless isomers of procyanidins of the B group (dehydrodicatechins B) and a dehydrotricatechin (Weinges, 1971), besides the above-mentioned dehydrodicatechin A (Weinges *et al.*, 1969). As well, a complex mixture of colorless C–C- and O–C-linked dimers was obtained by treatment of (+)-catechin with $K_3[Fe(CN)_6]$ at pH 9 (Young *et al.*, 1987), along with dehydrodicatechin A, whereas alkaline air oxidation of (+)-catechin led to generation of 2′-and 6′-hydroxycatechinic acids (Jensen and Pedersen, 1983).

These differences may be due to the type of reaction, which is reported to be one-electron oxidation, yielding a semiquinone radical, in the case of peroxidase or $K_3[Fe(CN)_6]$ oxidation (McDonald and Hamilton, 1973), and two-electron oxidation yielding the *o*-quinone in the case of polyphenol oxidase (Kalyanamaran *et al.*, 1984). They may also be related to incubation conditions such as pH and ionic strength which can influence the coupling reactions between the chemical species present in solution. According to Bresler *et al.* (1979), polyphenol oxidase-catalyzed oxidation at pH 7 also involved radical mechanisms. However, the effect of pH cannot be ruled out, as the unusually high pH used should increase the proportion of phenolate anions, which are more easily oxidized to the semiquinone free radical than the protonated phenol. Besides, pH variations modified the degradation rate of various *o*-quinones and the nature of the oxidation products formed from 4-methylcatechol or chlorogenic acid both in enzymati-

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cally (Richard-Forget *et al.*, 1992) and in chemically (Fulcrand *et al.*, 1994) oxidized model systems.

The purpose of our work was to study the products of (+)-catechin oxidation under the conditions prevailing in plant-derived food products (polyphenol oxidase-catalyzed oxidation, pH in the range 3–7) and in particular to evaluate the effect of pH changes on the composition of the oxidized solutions.

MATERIALS AND METHODS

Preparation of Crude Grape Polyphenol Oxidase. Frozen Grenache blanc grapes (750 g) were homogenized in 350 mL of 0.1 M acetate buffer (pH 4.0) containing 10 g/L ascorbic acid by using a Waring Blendor, filtered on cheesecloth, and centrifuged at 1750*g* for 5 min. The deposit was dispersed in 80 mL of the same buffer and centrifuged again (1200*g*; 5 min). The deposit was dispersed in 50 mL of acetate buffer (0.05 M, pH 4.0) containing 1% Triton X-100. The suspension was sonicated for 30 min and centrifuged at 5000*g* for 10 min. The supernatant containing soluble PPO was mixed with acetone until the acetone/buffer ratio was 80:20 (v/v) and placed at −18 °C for 3 h to flocculate proteins. After centrifugation (5000*g*) and elimination of the supernatant, the deposit was lyophilized and constituted soluble PPO extract, which was stored at −18 °C until used.

Assay of Enzymatic Activity. Enzymatic activity was assayed polarographically at each pH by means of an OXI 196 (WTW, Weilheim) oxymeter equipped with a Clark-type electrode, using (+)-catechin (5 mM) as substrate. Activity was expressed as nanomoles of oxygen consumed per second (nanokatals) under the assay conditions. Oxygen solubility in each buffer was calculated (Schumpe *et al.*, 1982), and its variations were taken into account in the determination of oxygen consumption.

Oxidation Procedure. (+)-Catechin and benzene-1,2,4-tricarboxylic acid, used as an internal standard, were purchased from Sigma (St. Louis, MO). Enzymatic incubations were carried out in 0.1 M citrate/phosphate buffers (pH 3, 4, 5, 6, 7), at 30 °C, with air agitation on a magnetic stirrer. The enzyme extract was suspended in the same buffer solutions and sonicated for 3 min immediately before use to break the protein aggregates and homogenize the suspension. All model solutions contained initially 4 mM catechin and 0.2 mM benzene-1,2,4-tricarboxylic acid. The reaction was started by addition of the enzyme suspension. Concentration was calculated to provide 42 nkat of activity in the total incubation medium (10 mL) at each given pH.

Sampling. One-milliliter aliquots were withdrawn after 5 min, 1 h, and 24 h of incubation, immediately filtered through 0.45 μm membrane filters (Sartorius, Goettingen, Germany) protected with glass microfiber prefilters (Whatmann, Maidstone, England), and injected (injection volume of 20 μL) onto the HPLC system. Filters and prefilters were washed by sonication for 3 min in 1 mL of acetone. After filtration, removal of acetone, and resolubilization in precisely 1 mL of methanol, the sample was also injected (20 μL) onto the HPLC system. All incubations were performed in triplicate so that each data point is the mean of three measurements.

HPLC Analyses. The HPLC apparatus was a Kontron Instruments (Milano, Italy) system including a 460 autosampler, a 325 pump system, a 430 double-wavelenghth UV–visible detector set at 280 and 400 nm, and a 450-MT2 data system. The column was a reversed-phase Lichrospher 100-RP18 (5 μm packing) (250 × 4 mm i.d.) protected with a guard column of the same material (Merck, Darmstadt, Germany). The UV–visible spectra were recorded from 240 to 500 nm, and the purity of each peak was checked by means of a Millipore-Waters (Millipore Corp., Milford, MA) photodiode array detector connected on-line to the HPLC system described above.

Elution conditions were as follows: flow rate, 1 mL min^{-1}; oven temperature, 30 °C; solvent A, 2.5% acetic acid in water; solvent B, acetonitrile/solvent A (80/20 v/v); linear gradients from 5 to 20% B in 20 min and from 20 to 32% B in 12 min, followed by washing and reconditioning of the column.

Calibrations were performed for (+)-catechin by injections of known dilutions. Quantitations of (+)-catechin and oxidation products were based on peak areas at 280 nm. Areas of the peaks presenting identical retention times and UV–visible spectra in the aqueous solution and corresponding acetone extract were added for each sample to take into account eventual tanning of oxidation products on proteins of the PPO extract. Eventual concentration of the solution during incubation was taken into account by reporting each peak area to that of the internal standard.

Purification of the major reaction products was achieved by oxidizing 12 mM (+)-catechin with 1 g/L enzyme extract at pH 4 for 10 min, filtering the resulting solution (2 mL volume), and injecting it (1.75 mL injection volume) onto the HPLC system equipped with a Spherisorb ODS-2 (5 μm packing) (250 × 7 mm i.d.) column (Knauer, Bad Hamburg, Germany). The elution conditions were modified as follows: flow rate, 2 mL min^{-1}; linear gradients from 10 to 15% B in 10 min and from 15 to 35% B in 20 min, followed by washing and reconditioning of the column. The major reaction products were collected, concentrated by rotary evaporation under vacuum, frozen, and lyophilized.

Characterization of the Isolated Products. *Thin Layer Chromatography.* The fractions corresponding to the seven major peaks were analyzed by TLC on silica gel plates (Kieselgel 60 F_{254}, Merck). Elution conditions were as follows: acetone/toluene/formic acid 4:3:1; detection under UV light at 254 nm; and pulverization of a solution containing 5 g/L of vanillin in 12 N HCl. A procyanidin extract from cacao was used as a reference (Rigaud *et al.*, 1993).

Acidic Hydrolysis. An amount of 0.3 mg of each fraction was dissolved in 100 μL of methanol, mixed with 100 μL of a $Fe(NH_4)(SO_4)_2$ solution at 20 g/L in 2 N HCl and 2.5 mL of 1-butanol/HCl 95:5 (v/v), and placed at 95 °C for 45 min.

Mass Spectrometry Analysis. FAB-MS analysis was performed on each fraction collected with a ZAB-HF (Fisons, England) mass spectrophotometer.

Table 1. pH Effect on PPO Extract Activity Measured by Polarography

	pH				
	3	4	5	6	7
PPO activity (nkat/mg)	26	32	34	30	42

RESULTS AND DISCUSSION

Polyphenol oxidase activity in the crude enzyme extract varied from 26 nkat/mg at pH 3 to 42 nkat/mg at pH 7 (Table 1). Note that activity remained high at pH 7 as reported for mitochondrial polyphenol oxidases (Brown, 1967), probably owing to the mode of enzyme preparation used.

The amount of enzyme extract was thus calculated for each pH so as to oxidize all solutions using the same level of polyphenol oxidase activity.

The degradation of (+)-catechin and formation of oxidation products after 5 min, 1 h, and 24 h of incubation at pH 3, 4, 5, 6, and 7 were monitored by HPLC. Quantitation, based on peak areas at 280 nm, was corrected by means of internal standard (i.e. benzene-1,2,4-tricarboxylic acid). It was checked that the latter was stable at all pH values throughout the incubation period and did not affect oxidase activity.

Figure 1 shows typical chromatographic profiles at 280 and 400 nm of catechin solution oxidized for 1 h at pH 3 and 6.

After 1 h, the remaining (+)-catechin was the major component in all solutions, meaning that enzymatic activity was not sufficient to oxidize the available substrate. It could also result from an inhibitory effect

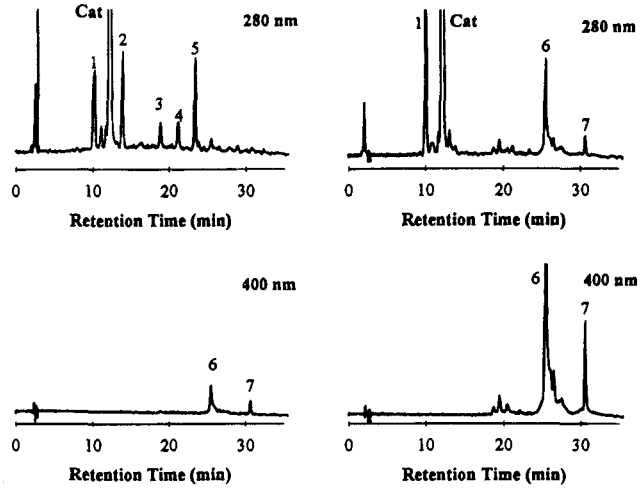

Figure 1. Chromatograms at 280 and 400 nm of enzymatic (+)-catechin oxidation mixture at pH 3 (left) and 6 (right). Cat, (+)-catechin; peaks 1–7, oxidation products.

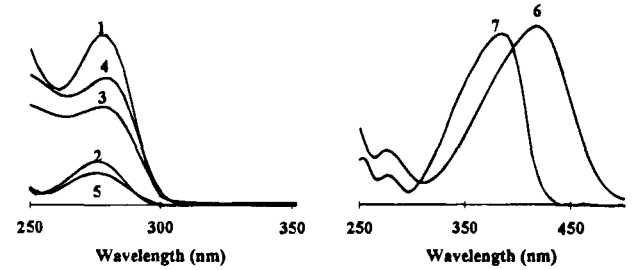

Figure 2. UV–visible spectra registered on diode array detector and corresponding to peaks 1–7.

of (+)-catechin oxidation products on PPO activity. However, use of larger amounts of enzyme extract decreased the level of secondary oxidation products in the solution, suggesting that they were tanned by proteins in the enzyme extract or oxidized either by the enzyme or by the enzymatically generated catechin quinones.

Most of the products formed eluted later than catechin and should therefore be less polar and/or larger molecules, as observed earlier by Oszmianski and Lee (1990). With the exception of product under peak 1, which eluted earlier than catechin and was present at both pH values, the products formed at pH 3 eluted earlier than those formed at pH 6. Besides, the solution oxidized at pH 3 exhibited essentially colorless products and only traces of material absorbing at 400 nm, whereas that oxidized at pH 6 contained mostly yellow compounds. Seven major peaks corresponding to five colorless products, denoted **1–5**, and two yellow pigments, denoted **6** and **7**, characterized by their retention time and UV–visible spectra, will be discussed further. The UV–visible spectra of these oxidation products, recorded between 240 and 500 nm, are shown in Figure 2. Diode array analysis of each of the seven peaks suggested that they corresponded to pure coumponds. Although we are aware of possible coelutions, product(s) eluted under peak X will be referred to as "product X".

The spectra of products **1–5** were similar to those of (epi)catechin and procyanidins, with a maximum absorbance near 280 nm, suggesting that the original flavan-3-ol structure was retained. Products **6** and **7** showed absorbance maxima, respectively, at 280 and 412 nm and at 256, 280 and 385 nm, indicating more complex structures.

The relative amounts of residual catechin and of the seven products mentioned above in the solutions oxidized for 5 min, 1 h, and 24 h at each pH are presented in Figure 3. Catechin *o*-quinone was detected only after 5 min at pH 3 and, in much lower amount, at pH 4 and is therefore not represented. This confirms that it is fairly unstable, as reported earlier (Cheynier *et al.*, 1989; Richard-Forget *et al.*, 1992), especially as the pH increases (Rouet-Mayer *et al.*, 1990).

In the first 5 min, the catechin degradation rate increased with pH, up to pH 6, and decreased slightly at higher pH, although the effect of pH on polyphenol oxidase activity was compensated by adjusting the amount of enzymatic extract. The amount of residual catechin was almost stable after 1 h of incubation. It equaled approximately half of the initial value at all pH values, with the exception of solutions incubated at pH 6, in which catechin losses were much larger.

Comparison of the solutions oxidized at different pH values allows two groups of products to be distinguished. The first one, consisting of colorless products **2–5**, was formed in larger amounts at pH 3. Accumulation of these four compounds decreased as the pH increased, to reach approximately zero at pH 6. The second group contained colorless product **1** and the two yellow pigments, **6** and **7**, which were formed in larger amounts as the pH increased, with a maximum at pH 6. The concentration of most products was maximum after 1 h at all pH values, with the exception of compounds **1** and **3** which were present in larger amounts after 5 min. This suggests that the latter were intermediate compounds, probably formed directly from the quinone, and fairly unstable. Compounds **4** and **5** were formed in smaller amounts but reached their maximum level earlier at pH 4 and 5 than at pH 3, presumably meaning that they were degraded more rapidly as the pH was raised.

The composition differences observed at the various pH values may be due to competition between two mechanisms leading to different products, one prevailing at pH <4 and the other at pH >4. Another possible explanation is that colorless products are intermediate products on the reaction pathways leading to yellow compounds.

The seven compounds were then purified from solutions oxidized at pH 4 for 10 min. These incubation conditions were chosen so as to obtain the seven major peaks in the same solution. The five colorless compounds were then analyzed by TLC following the procedure described by Lea (1978) to separate procyanidin oligomers according to their molecular weight. Under these conditions, the R_f values of peaks **1–5** equaled 0.63, 0.71, 0.54, 0.74, and 0.68, respectively, and were close to those of catechin monomer and dimeric procyanidins from a cacao extract, suggesting a procyanidin dimer structure or modified monomer structure. In particular, compound **1** coeluted with procyanidin dimer B3 [catechin-(4α-8)-catechin] both upon HPLC and upon TLC. FAB-MS spectra confirmed that products **1–5** were isomers of procyanidin dimers of the B type, giving $[M + H]^+$ and $[M + Na]^+$ ion peaks at *m/z* 579 and 601, respectively. However, all of them were resistant to acidic hydrolysis, meaning that they were not procyanidins of the B type but presumably dehydrodicatechins of the B type linked by one interflavan bond, either C–C or C–O–C, as reported earlier (Weinges, 1971; Young *et al.*, 1987). As well, FAB-MS spectra of yellow compounds **6** and **7** gave $[M + H]^+$ and $[M + Na]^+$ ion peaks at *m/z* 577 and 599, respectively,

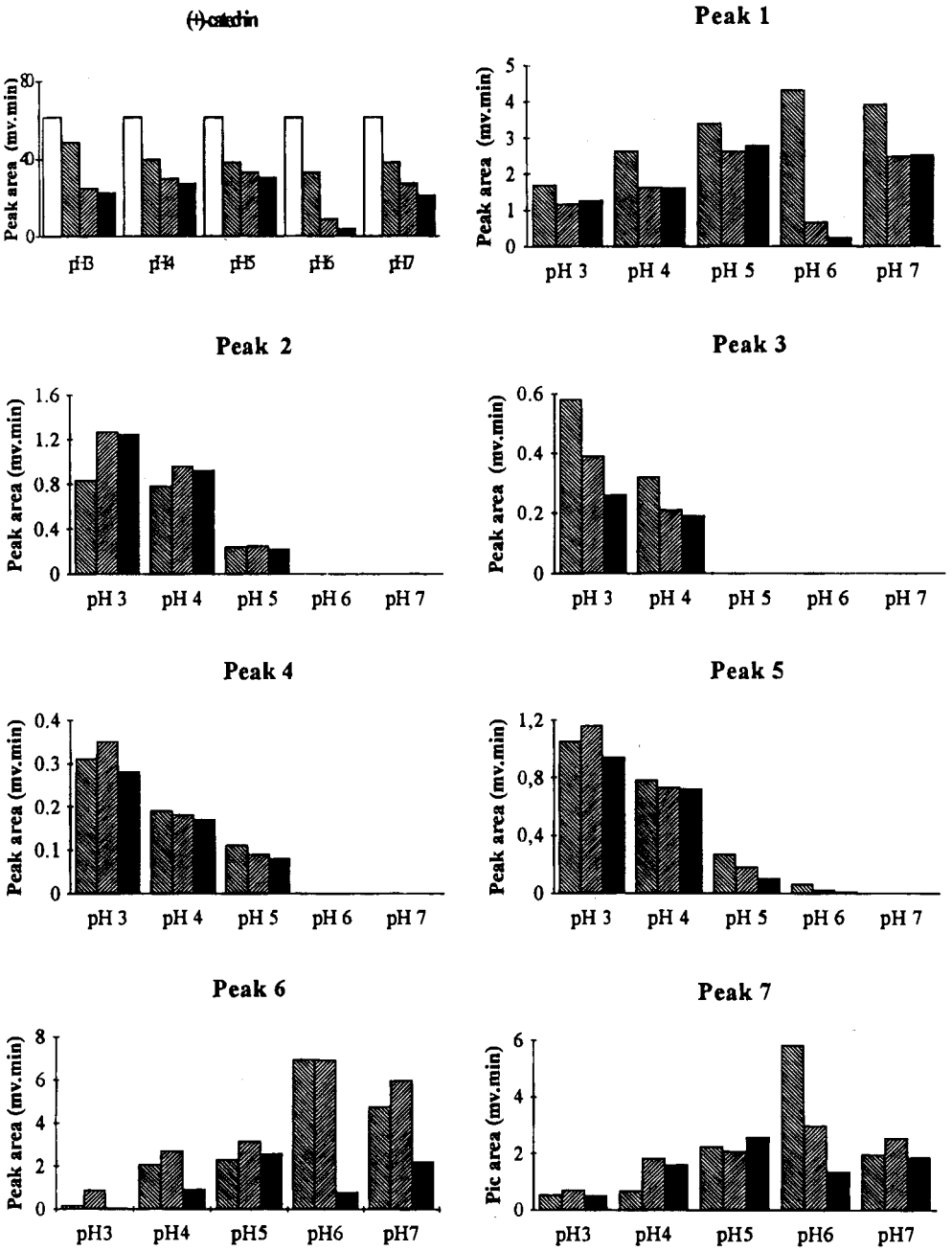

Figure 3. Influence of incubation pH on relative concentrations (based on peak area at 280 nm) of (+)-catechin and oxidation products. Incubation time: (open bars) 0 min; (backslashed bars) 5 min; (slashed bars) 1 h; (solid bars) 24 h.

indicating dimeric structures linked by two interflavan bonds, which may be similar to dehydrodicatechin A (Weinges *et al.*, 1969). NMR studies are under way to confirm the above hypotheses and achieve structural characterization of the oxidation products. However, the present study demonstrates that the products formed by oxidation of catechin during fruit processing differ from procyanidin dimers originating from biosynthetic pathways.

Oxidation of (+)-catechin, catalyzed by grape PPO, gives complex mixtures. *o*-Quinones, initially formed, are involved in several coupling reactions leading to formation of various dimers whose nature and relative abundance depend on incubation pH. The color of oxidized solutions seems rather to result from products formed by coupling than from (+)-catechin quinones, which are very unstable. Low pH values give almost colorless solutions, whereas higher pH values yield yellow solutions containing higher concentrations of yellow pigments.

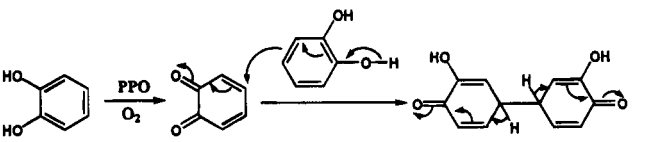

Figure 4. Michael-type nucleophilic addition (McDonald and Hamilton, 1973).

At present, available information on the structure of the different products formed is insufficient to formulate their formation mechanisms. However, some hypotheses may be proposed to discuss the influence of pH on coupling reactions. Thus, *o*-quinones formed at pH 3 may react differently with residual (+)-catechin than those formed at pH 6. Two different ways may be expected:

(1) Nucleophilic Michael 1−4 addition of a phenolic ring onto an *o*-quinone (Figure 4) was often mentioned as the mechanism responsible for coupling of phenols

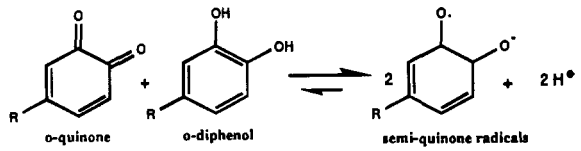

Figure 5. Reaction of reverse disproportionation (Nilges *et al.*, 1984).

in the presence of polyphenol oxidase. (McDonald and Hamilton, 1973).

(2) Coupling of semi-quinone radicals, formed by reverse disproportionation (Figure 5), could be another alternative since incubation conditions (large excess of polyphenol in reduced form) may facilitate reverse disproportionation (Peter, 1989).

Michael 1−4 addition could be favored by high pH, which increases the nucleophilic character of (+)-catechin, whereas low pH could favor radical mechanisms by increasing the reactivity of semi-quinones radicals (Nilges *et al.*, 1984).

Complete identification of the major products obtained by enzymatic oxidation of (+)-catechin is under way and should provide more information relative to the mechanisms responsible for their formation.

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