Competition between (+)-Catechin and (−)-Epicatechin in Acetaldehyde-Induced Polymerization of Flavanols

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The reactions of (+)-catechin and (−)-epicatechin in the presence of acetaldehyde were studied in model solution systems. When incubated separately with acetaldehyde and at pH values varying from 2.2 to 4.0, reactions were faster with (−)-epicatechin than with (+)-catechin. In mixtures containing both (+)-catechin and (−)-epicatechin with acetaldehyde, new compounds besides the homogeneous bridged derivatives were detected. These compounds were concluded to be hetero-oligomers consisting of (+)-catechin and (−)-epicatechin linked with an ethyl bridge. In this case, the reaction of (−)-epicatechin was faster than that of (+)-catechin. This was also observed in solutions containing the two flavanols and the (+)-catechin−ethanol intermediate. Under these conditions, the homogeneous (+)-catechin bridged dimers and heterogeneous dimers were obtained by action of the intermediate on (+)-catechin and (−)-epicatechin, respectively. In addition, the homogeneous (−)-epicatechin ethyl-bridged dimers were also detected, showing that ethyl linkages underwent depolymerization and recombination reactions.

Keywords: *(+)-Catechin; (−)-epicatechin; acetaldehyde; condensation; thiolysis; LC/DAD; LC/MS; competitive action*

INTRODUCTION

Flavanols are polyphenolic compounds found in many plants, fruits, and beverages such as fruit juices, beer, and wine. They have attracted much attention in relation to their potential physiological activities, and their role has become an important issue in the relationship between health and human diet. The presence of these natural compounds in wine has created considerable interest with respect to the potential health effects associated with moderate consumption.

Polyphenols are also compounds of high reactivity which react with other phenolic or nonphenolic compounds, giving rise to various new compounds so that the resulting mixtures become progressively more complex. These transformations affect sensorial properties such as color, taste, and colloidal stability during storage and aging of polyphenolic rich foods (Ribereau-Gayon et al., 1983; Liao et al., 1992; Singleton et al., 1992; Fulcrand et al., 1996a; Saucier et al., 1996, 1997a).

The color changes produced during storage of red wine have attracted attention for many years. This has been attributed to the progressive formation of condensed pigments resulting from the interaction between free anthocyanins initially extracted from grapes and other phenolic compounds, particularly flavanols. Various mechanisms have been suggested to explain the formation of these pigments. Processes including copigmentation (Brouillard et al., 1990; Mistry et al., 1991; Dangles et al., 1992; Figueiredo et al., 1996), direct condensation between flavanols and anthocyanins (Jurd, 1967; Jurd and Somers, 1970; Somers, 1971; Liao et al., 1992; Santos-Buelga et al., 1995, 1996), and reactions between them mediated by acetaldehyde (Timberlake and Bridle, 1976; Baranowski and Nagel, 1983; Roggero et al., 1987; Bakker et al., 1993; Rivas-Gonzalo et al., 1995; Dallas et al., 1996a,b; Es-Safi et al., 1996; Escribano-Bailon et al., 1996; Francia-Aricha et al., 1997; Fulcrand et al., 1996a,b) have been proposed.

In addition, changes in taste and astringency also occur during conservation of red wine. The deastringency phenomenon has been attributed first as being a consequence of anthocyanin−flavanol-derived pigment formation either by direct condensation (Timberlake and Bridle, 1976; Haslam, 1980; Baranowski and Nagel, 1983; Liao et al., 1992) or through acetaldehyde-mediated reactions (Timberlake and Bridle, 1976; Baranowski and Nagel, 1983; Bakker et al., 1993). However, a studies conducted on (+)-catechin autopolymerization induced by acetaldehyde (Fulcrand et al., 1996a; Saucier et al., 1997a) have proven that this reaction could also occur and be responsible for flavanol decrease. Occurrence of such reaction in grape-derived foods has been recently proven (Saucier et al., 1997b; Cheynier et al., 1997) as (+)-catechin ethyl-bridged dimers and trimers have been detected in red wine samples. This may explain the partial deastringency observed during aging of red wine as observed in the case of persimmon fruits (Tanaka et al., 1994).

The aim of the present work was to study and to compare the condensation kinetics of flavanols with acetaldehyde and to establish the effect of pH on these reactions. The purpose of this work was also to develop a sensitive thioacidolysis and HPLC analytical method suitable for identification of the obtained fractions, on the basis of the released free and thiol units and of thiolysis kinetics. The major base units of flavanols

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occurring in wines, that is, (+)-catechin and (−)-epicatechin, were used in this study.

MATERIALS AND METHODS

Reagents. Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA) prior to use. Acetonitrile was purchased from BDH (Poole, U.K.). Methanol, formic acid, and acetic acid were obtained from Prolabo (Fontenay S/Bois, France). (+)-Catechin, (−)-epicatechin and mercaptoethanol were purchased from Sigma (St. Louis, MO). Acetaldehyde was obtained from Merck (Darmstadt, Germany).

Reactions. An acidic solution was prepared with 17 μL of acetic acid and 50 μL of ethanol in 373 μL of water, giving a pH value of 2.2. Six milligrams of (+)-catechin or (−)-epicatechin and 60 μL of acetaldehyde were then added. The reactions were monitored by liquid chromatography coupled with a diode array detector (DAD) and with a mass spectrometry (MS) detector. When (+)-catechin and (−)-epicatechin were incubated together, 3 mg of each was used and the reaction was conducted as described above. The various pH values were obtained by addition of 1 M sodium hydroxide to the medium described above and were adjusted using a 93313 Bioblock pHmeter.

Analytical HPLC/DAD Analyses. HPLC/DAD analyses were performed by means of a Waters system including two M510 pumps, a U6K manual injector, an automated gradient controller, and a 990 diode array detector. UV−visible spectra were recorded from 250 to 600 nm, and peak areas were measured at 280 nm. 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. Elution conditions were as follows: 1 mL/min flow rate; temperature, 30 °C; solvent A, water/formic acid (98:2, v/v); solvent B, acetonitrile/water/formic acid (80:18:2, v/v); elution from 5 to 30% B in 40 min, from 30 to 50% B in 20 min, and from 50 to 80% B in 10 min, followed by washing and re-equilibrating of the column.

Semipreparative HPLC Purification. Semipreparative HPLC separations were performed by means of a Gilson system including a 305 master and a 306 slave pump, an 806 manometric module, an 811 dynamic mixer, a 7161 Rheodyne valve injector, and an 875 UV−visible Jasco detector set at 280 nm. The column was a reversed-phase Microsorb C18 (3 μm packing, 250 × 50 mm i.d.). Elution conditions were as follows: 15 mL/min flow rate; solvent A, water/acetic acid (99:1, v/v); solvent B, methanol/solvent A (80:20, v/v); elution from 5 to 30% B in 3 min, isocratic 30% B in 2 min, from 30 to 50% B in 5 min, and from 50 to 80% B in 5 min, followed by washing and re-equilibrating of the column.

Thiolysis Reactions and HPLC Analyses. The thiolysis reagent was prepared by mixing 1 mL of HCl, 7.5 mL of mercaptoethanol ($HSCH_2CH_2OH$), and 29 mL of water. The thiolysis reaction was conducted on solutions of pure dimeric compounds at concentration of 1 mg/mL. After sealing, reactions were carried out at ambient temperature and kinetics were monitored by HPLC.

HPLC/DAD analyses were performed by means of a Kontron Instrument systems (Milano, Italy) including a 460 autosampler, a 325 pump system, a 430 detector, and an MSTI 450 data system. UV−visible spectra were recorded from 250 to 600 nm, and peak areas were measured at 280 nm. 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. Elution conditions were as follows: 1 mL/min flow rate; temperaturr, 30 °C; solvent A, water/formic acid (98:2, v/v); solvent B, acetonitrile/water/formic acid (80:18:2, v/v); elution begins with isocratic 5% B in 11 min, from 5 to 40% B in 44 min, isocratic 40% B in 5 min, followed by washing and re-equilibrating of the column.

MS Apparatus and LC/MS Analyses. MS measurements were performed on a Sciex API I Plus simple quadruple mass spectrometer with mass range of 2400 amu, equipped with an ion spray ion. The mass spectrometer was operated in the negative-ion mode. Ion spray voltage was selected at −4 kV and orifice voltage at −60 V.

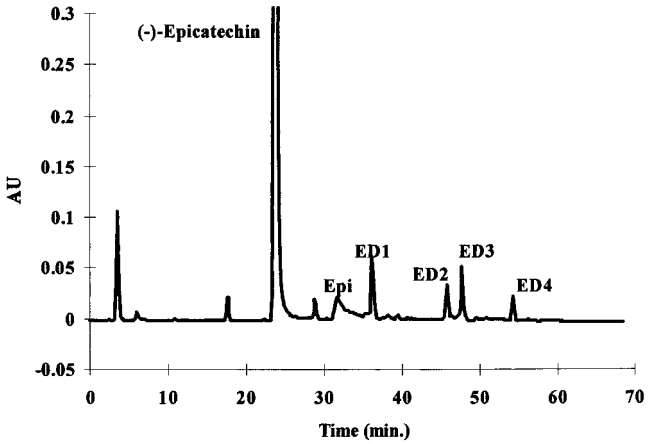

Figure 1. HPLC chromatogram recorded at 280 nm of a mixture of (−)-epicatechin and acetaldehyde showing residual flavanol and the newly formed compounds.

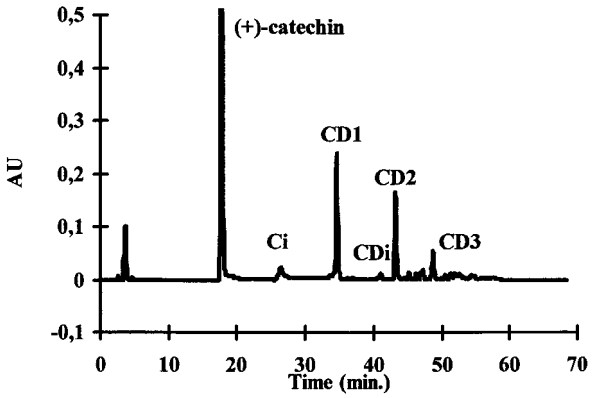

Figure 2. HPLC chromatogram recorded at 280 nm of a mixture of (+)-catechin and acetaldehyde showing residual flavanol and the newly formed compounds.

HPLC separations were carried out on a narrow-bore reversed-phase column with an ABI 140 B solvent delivery system (Applied Biosystems, Weiterstadt, Germany). The column was connected with the ES interface via a fused-silica capillary (length = 100 cm, 100 μm i.d.). The reaction mixture was injected with a rotary valve (Rheodyne model 8125) fitted with a 20 μL sample loop. The separation was achieved on a Superspher 100-RP18 column (3 μm packing, 125 × 2 mm i.d., Merck), using a two-step linear gradient at a flow rate of 200 μL/min. The elution was done with solvents A and B used in HPLC/DAD analyses and the conditions adapted as follows: linear gradients from 5 to 30% B in 20 min and from 30 to 50% B in 10 min, followed by washing and reconditioning of the column. The absorbance at 280 nm was monitored by an ABI 785A programmable absorbance detector.

RESULTS AND DISCUSSION

To investigate reactions between flavanols and acetaldehyde, (−)-epicatechin and (+)-catechin were individually incubated with ethanal at various pH values. The concentrations of the reactants and of the newly formed compounds were monitored by HPLC with diode array detection, and their molecular weights were determined by electrospray mass spectrometry. A general decrease in the concentrations of (−)-epicatechin and (+)-catechin and appearance of numerous products were observed at all pH values. Figures 1 and 2 represent typical HPLC chromatograms showing, respectively, residual (−)-epicatechin or (+)-catechin and

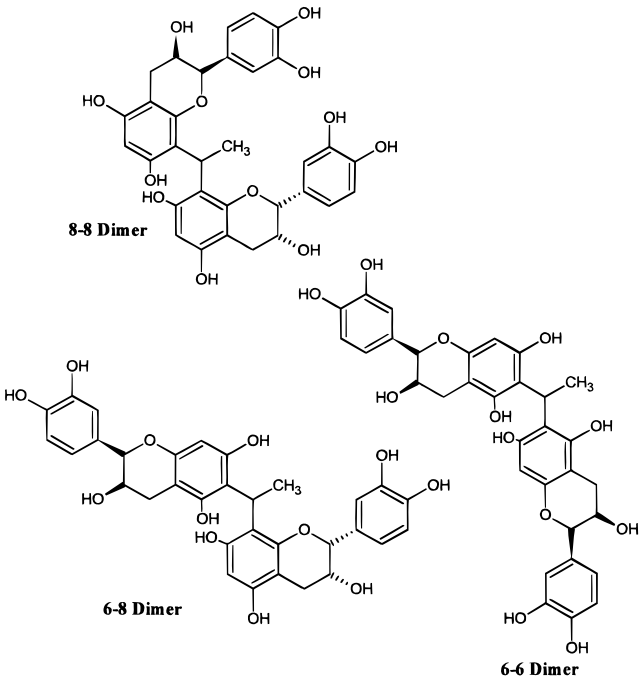

Figure 3. General structure of (−)-epicatechin−ethyl dimers.

the newly formed compounds that were eluted later than their precursors, thus indicating therefore that they were less polar and/or larger molecules, as observed in enzymatic oxidation of (+)-catechin (Guyot et al., 1995).

The UV−visible spectra of the formed compounds recorded between 250 and 500 nm were similar to that of (+)-catechin with a maximum absorbance near 280 nm, suggesting that the original flavanol structure was retained.

LC/MS analysis, conducted in the negative-ion mode, allowed molecular weight determination of the major peaks formed through these reactions. The results obtained indicated that these products are oligomeric compounds consisting of flavanol units [(−)-epicatechin or (+)-catechin] bridged by ethyl groups formed according to the mechanism postulated by Timberlake and Bridle (1976) and recently confirmed by Fulcrand et al. (1996a).

Thus, fractions ED1, ED2, ED3, and ED4, formed when (−)-epicatechin was incubated with acetaldehyde, gave a signal at *m*/*z* 605, which corresponds to a molecular weight of 606 and thus to a structure in which two (−)-epicatechin units are linked by an ethyl bridge as shown in Figure 3. Recent studies on condensation of (+)-catechin in the presence of acetaldehyde have shown the presence of similar derivatives (Es-Safi et al., 1996; Fulcrand et al., 1996a; Saucier et al., 1997c). According to the evidence recently contributed (Es-Safi et al., 1996), flavanols can be linked through C6 or C8, yielding four products with C6−C6, C8−C8, and C6−C8 (*R* and *S*) bonds, taking into account the presence of an asymmetric carbon for the C6−C8 isomers (Figure 3). The formation of such bridged products was also obtained in the case of (+)-catechin, (−)-epicatechin, procyanidin B2, and malvidin 3-*O*-glucoside (Es-Safi et al., 1996; Francia-Aricha et al., 1997; Fulcrand et al., 1996a,b).

In addition to these dimers, various oligomeric bridged compounds up to the tetramer level were also detected. Among them, intermediate acetaldehyde adducts of

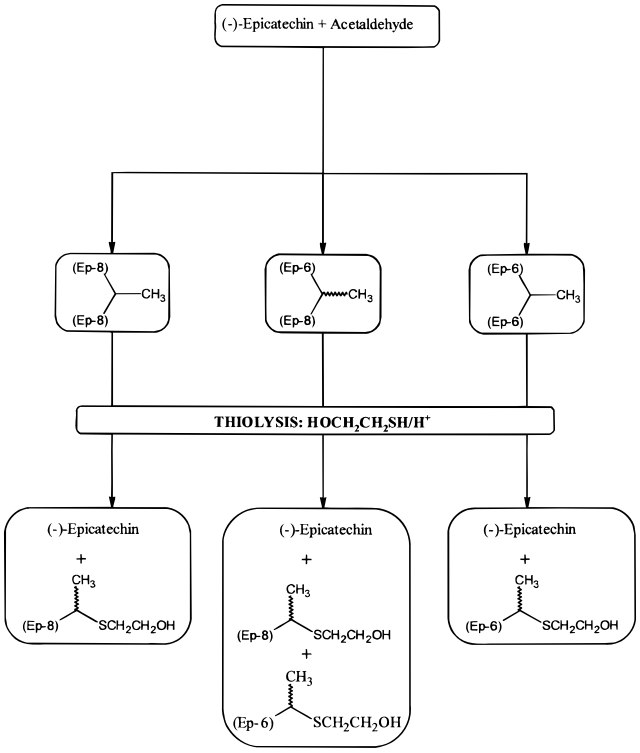

Figure 4. Scheme of (−)-epicatechin−ethyl fraction thiolysis.

monomer (Epi; *m*/*z* 333), dimer (*m*/*z* 921), and trimer derivatives (*m*/*z* 649) were observed.

In the case of (+)-catechin, fractions CD1, CD2, and CD3 gave a mass signal at *m*/*z* 605, which corresponds to a molecular weight of 606 and thus to a structure in which two (+)-catechin units are linked by an ethyl bridge similar to those shown in Figure 3. A recent study conducted on the condensation of (+)-catechin in the presence of acetaldehyde has shown the presence of four dimer derivatives (Saucier et al., 1997a). Fraction CD2 probably consists of two products that coelute, as previously observed by Es-Safi et al. (1996), Fulcrand et al. (1996a,b), and Saucier et al. (1997c).

In addition to these compounds, intermediate adducts of monomer (Ci; *m*/*z* 333), dimer (Cdi; *m*/*z* 649), and at least four trimer derivatives (*m*/*z* 921) were also observed (Es-Safi et al., 1996; Fulcrand et al., 1996a,b).

To elucidate the obtained dimeric structures, the four fractions obtained in the case of (−)-epicatechin (ED1, ED2, ED3, and ED4) and those formed in the case of (+)-catechin (CD1, CD2, and CD3) were collected by HPLC at the semipreparative scale. All of them proved to be very unstable once isolated, rendering identification difficult to achieve by spectral methods (Es-Safi et al., 1996; Fulcrand et al., 1996b). Nevertheless, degradation by thiolysis (Tanaka et al., 1994) provides essential information on the structures of these compounds. This consists of mixing the obtained flavanol−ethyl derivatives with thiol reactant in acidic medium. A free flavanol is thus released in addition to flavanol ethyl−thiol derivatives according to the considered thiolyzed isomer. Thus, 6- or 8-flavanol−ethyl thioether derivatives are released in the case of 6−6 or 8−8 dimer, respectively, whereas the two thioethers are obtained starting from a 6−8 dimer (Figure 4). This method has been used by Tanaka et al. (1994) to elucidate tannin−acetaldehyde condensed products isolated from persimmon fruits.

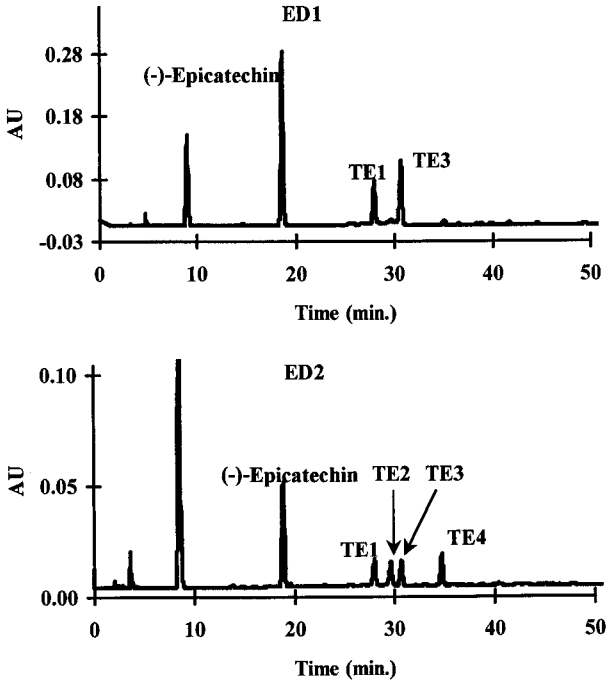

Figure 5. HPLC chromatograms recorded at 280 nm of ED1 and ED2 fraction thiolysis.

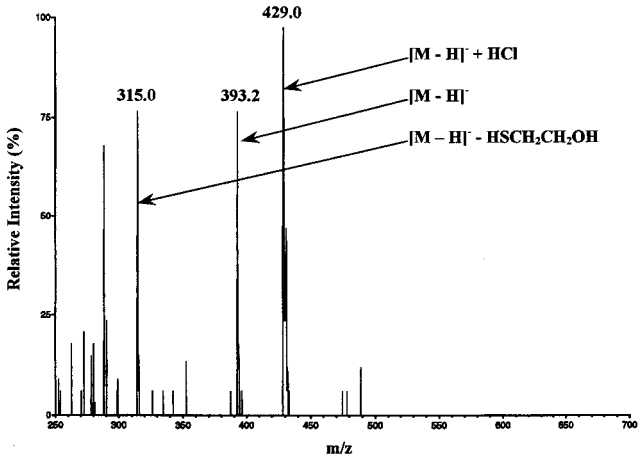

Figure 6. Mass spectrum of a (−)-epicatechin−ethyl thiol derivative (TE1).

Examination of the chromatograms obtained after complete thiolysis of fractions ED1, ED2, ED3, and ED4 showed that, in addition to free (−)-epicatechin, two differents pairs of thiol derivatives were obtained in the case of fractions ED1 and ED3, whereas the four thiol derivatives named TEE1, TEE2, TEE3, and TEE4 were obtained in the case of fractions ED2 and ED3 (Figure 5). These compounds exhibit spectra similar to that of (−)-epicatechin with a maximum absorbance near 280 nm. Identical molecular weights of 429 ($[M + Cl]^-$) were determined by LC/MS analysis conducted in the negative-ion mode as shown in Figure 6, indicating that an additional hydroxyethylsulfanyl group is attached to the ethyl group. This confirmed that the obtained flavanol−ethyl compounds are sensitive to thiol reagents and that thiolysis can be used to study their isomerization (Tanaka et al., 1994). The four thiol derivatives are evidently 6- and 8-position isomers because the presence of an asymmetric carbon gives two derivatives for each position, due to *R* and *S* configurations.

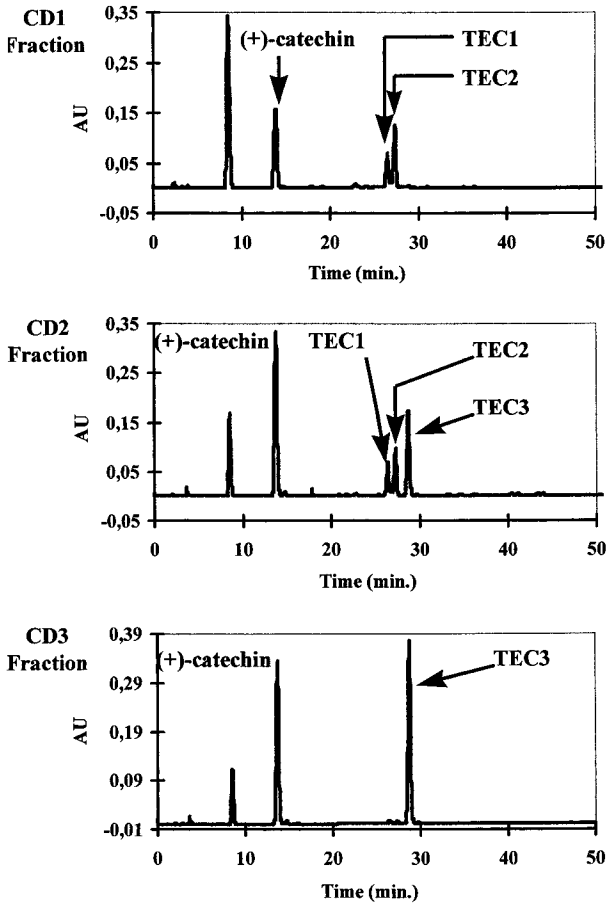

Figure 7. HPLC chromatograms recorded at 280 nm of CD1, CD2, and ED3 fraction thiolysis.

Fractions ED1 and ED4, which gave two differents pairs of thioethers, corresponded to the 8−8 and 6−6 isomers, whereas fractions ED2 and ED3, which gave all thioethers, corresponded to 6−8 isomers. Kinetics studies of ED1 and ED4 thiolysis showed that ED1 was degraded more rapidly than ED4 and should correspond therefore to an 8−8 isomer (Tanaka et al., 1994), whereas ED4 corresponds to the 6−6 dimer.

The results obtained in the case of the three (+)-catechin−ethyl fractions (CD1, CD2, and CD3) are shown in Figure 7. Examination of the three chromatograms obtained after complete thiolysis showed that, in addition to free (+)-catechin, one, two, or three thiol derivatives named TEC1, TEC2, and TEC3 are obtained in the case of CD3, CD1, and CD2, respectively. The three thiol derivatives are 6- and 8-position isomers with one position giving two derivatives due to *R* and *S* configurations.

Fractions CD1 and CD3, which gave respectively one and two different thioethers, probably correspond to 8−8 and 6−6 isomers, whereas compound CD2, which gave all thioethers, corresponds to the mixture of both 6−8 dimers. Kinetic studies of CD1 and CD3 thiolysis showed that CD1 was degraded more rapidly than CD3 and should correspond therefore to an 8−8 isomer as observed in the case of (−)-epigallocatechin 3-gallate (Tanaka et al., 1994). CD3 corresponds finally to a 6−6 isomer.

To evaluate the effect of pH changes on the composition of the incubated solutions, the individual condensations of (−)-epicatechin and (+)-catechin with acetaldehyde were monitored by HPLC in the pH range from

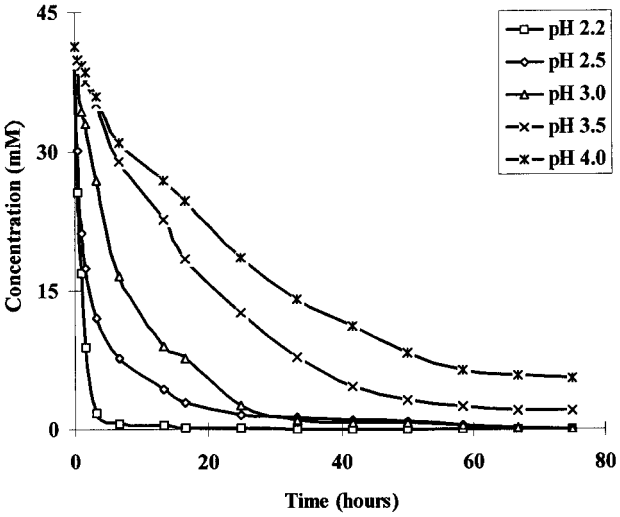

Figure 8. Changes in (−)-epicatechin concentration with time according to pH.

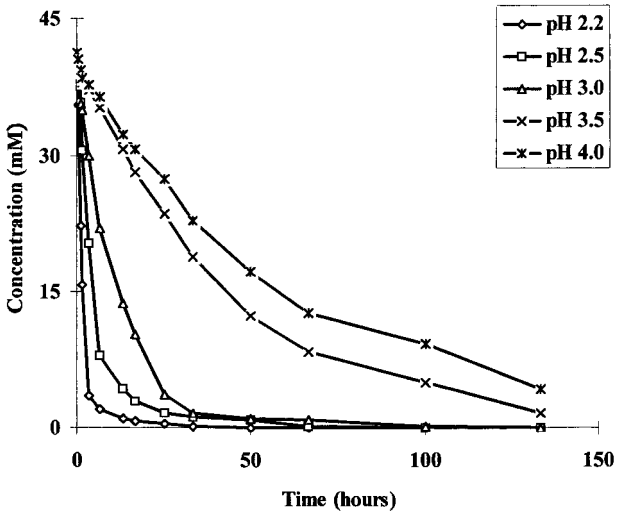

Figure 9. Changes in (+)-epicatechin concentration with time according to pH.

2.2 to 4.0. Quantification of all products was achieved on the basis of peak areas at 280 nm, using (−)-epicatechin and (+)-catechin as standards. The concentrations of residual (−)-epicatechin and (+)-catechin at various pH values are presented in Figures 8 and 9.

(−)-Epicatechin and (+)-catechin concentrations showed a gradual decrease at all pH values, but the reaction rate increased with the decrease of pH, owing to the higher availability of acetaldehyde carbocation at lower pH values. This phenomenon was also observed in the case of condensation of malvidin 3-*O*-glucoside with (+)-catechin in the presence of acetaldehyde (Garcia-Viguera et al., 1994) and is in agreement with the mechanism initially postulated by Timberlake and Bridle (1976) and recently confirmed by us (Es-Safi et al., 1996; Fulcrand et al., 1996a,b).

The variations in relative amounts of the obtained dimer fractions during incubation of the reaction media at various pH values are presented in Figures 10 and 11. Fractions accumulated gradually at the beginning of the reaction and then decreased, probably as more polymerized products were formed.

The comparison of the results obtained above, when the two flavanols were individually incubated with acetaldehyde, showed that reactions were faster with

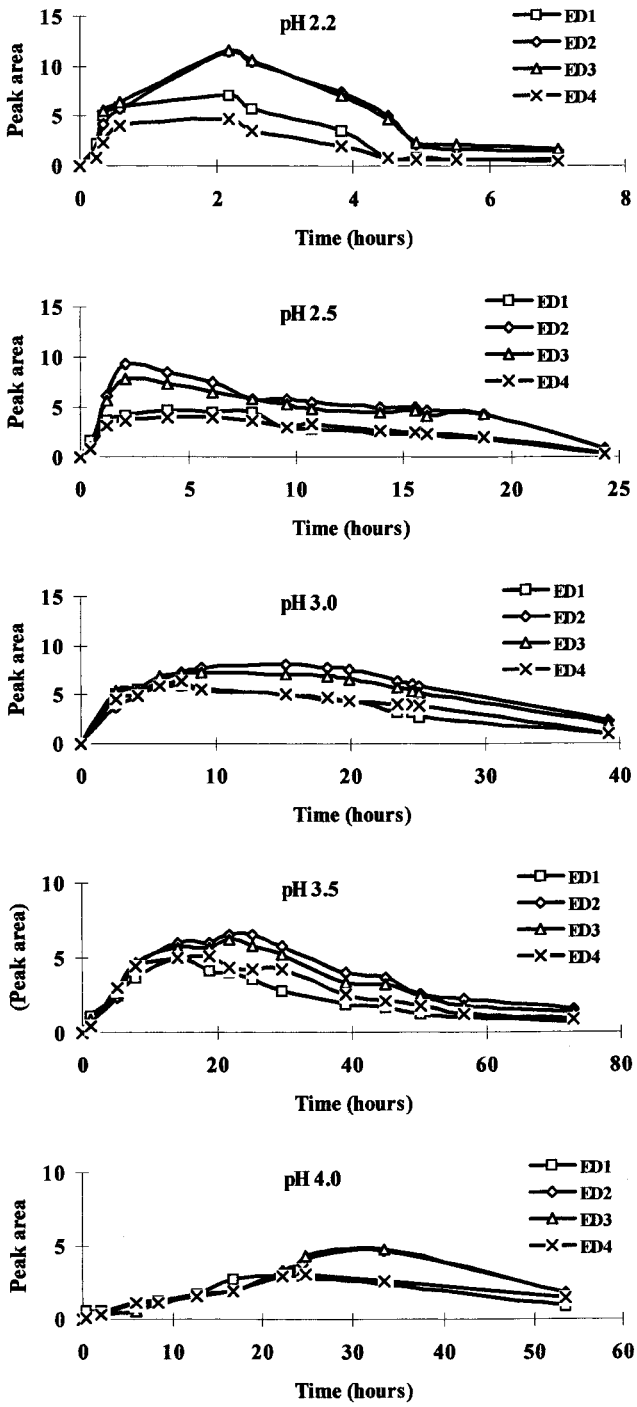

Figure 10. Changes in relative amounts of fractions ED1, ED2, ED3, and ED4 according to time and pH.

(−)-epicatechin than with (+)-catechin at all pH values (Figure 12). The difference was more important as the pH increased.

To compare the reactivity of the two studied flavanols, solutions containing (+)-catechin, (−)-epicatechin, and acetaldehyde were prepared at pH 2.2 and incubated at room temperature. Changes taking place were monitored by LC/DAD and LC/MS analysis.

Quantification of the two flavanols during the course of the reaction gave the results shown in Figure 13. The rate of loss of (−)-epicatechin was higher than that of (+)-catechin, especially at the beginning of the reaction. This showed that (−)-epicatechin is more easily incor-

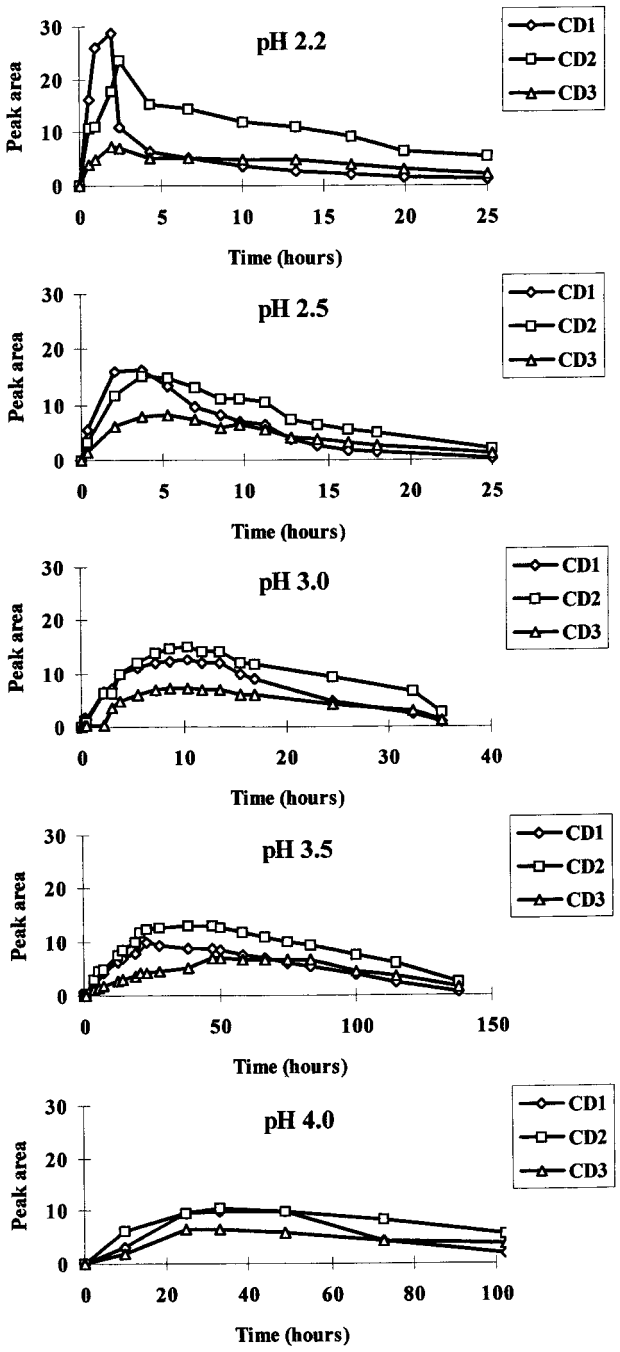

Figure 11. Changes in relative amounts of fractions CD1, CD2, and CD3 according to time and pH.

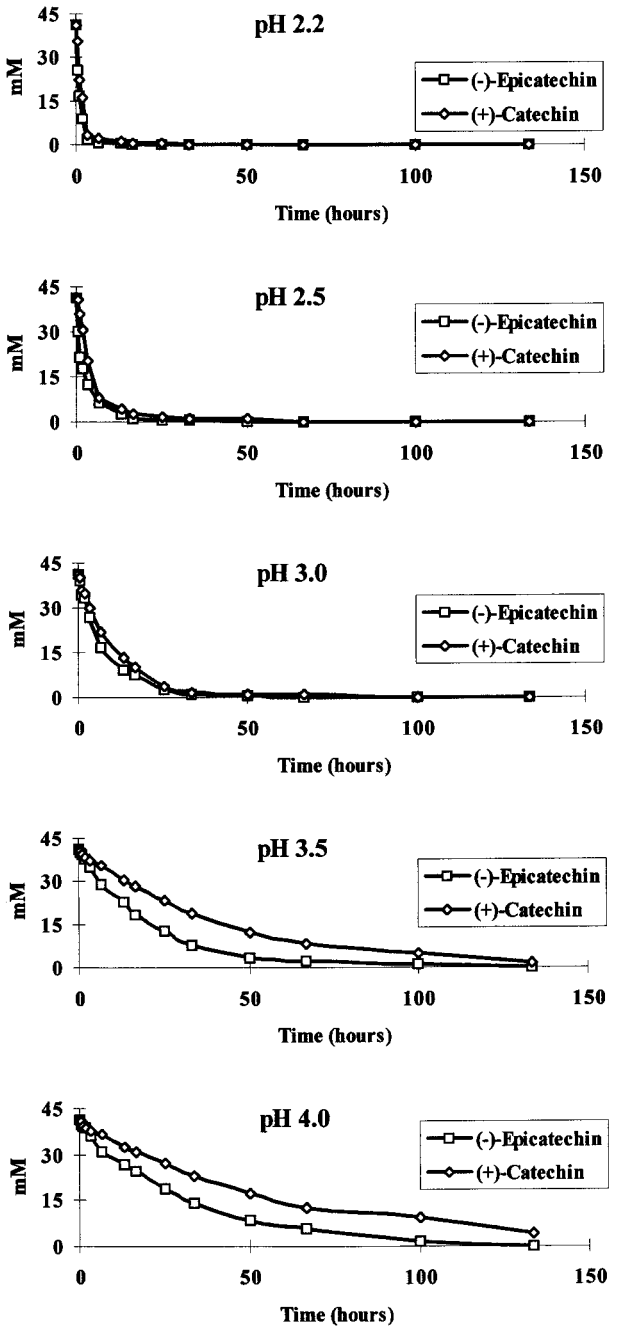

Figure 12. Changes in concentrations of (−)-epicatechin and (+)-catechin when individually incubated with acetaldehyde according to time and pH.

porated in the formed bridged oligomers than (+)-catechin.

LC/MS study conducted in the negative ion mode showed the presence of new dimers (*m*/*z* 605) besides the condensation products obtained when each flavanol was incubated individually with acetaldehyde at the same pH. These compounds were concluded to be heterodimers consisting of (+)-catechin and (−)-epicatechin units linked with an ethyl bridge. The chromatographic profiles of solutions containing both flavanols with acetaldehyde and incubated at pH 2.2 were very complex due to the presence of many new peaks, which caused peak overlapping.

After comparison of the reactivity of (−)-epicatechin and (+)-catechin separately and together on acetaldehyde, it seemed interesting to study the interaction of the two flavanols with the intermediate formed by addition of protonated acetaldehyde onto (+)-catechin. Thus, after preparation and isolation of the (+)-catechin−ethanol intermediate by semipreparative scale HPLC, it was incubated with both flavanols at pH 2.2 and at room temperature. The mixture composition was monitored by HPLC as indicated above. The formation of the homogeneous (+)-catechin bridged dimers and of the heterogeneous dimers was first observed. These products are obviously formed by action of the intermediate on (+)-catechin and (−)-epicatechin, respectively. It was, however, interesting to note the appearance of the (−)-epicatechin homogeneous bridged dimers. These compounds could not be formed directly by interaction of the reactants introduced at the beginning of the reaction. This indicates that the first heteroge-

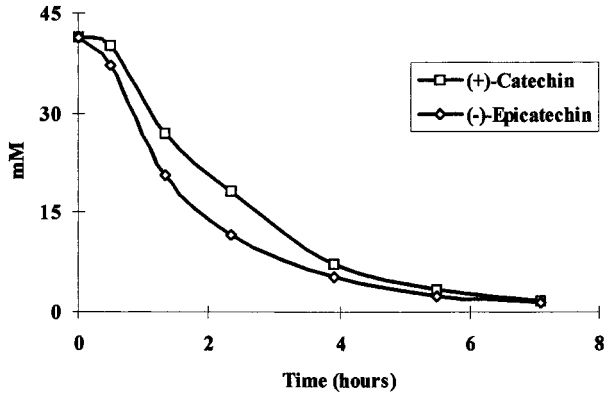

Figure 13. Changes in concentrations of (−)-epicatechin and (+)-catechin when incubated together with acetaldehyde.

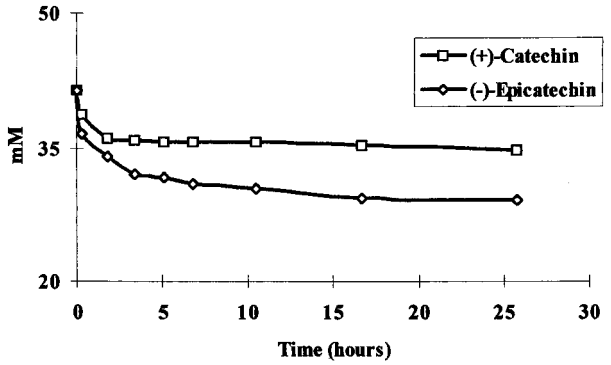

Figure 14. Changes in concentrations of (−)-epicatechin and (+)-catechin when incubated together with the (+)-catechin−ethanol intermediate.

neous dimers formed underwent decomposition, releasing a mixture of (−)-epicatechin and (+)-catechin intermediates and free (−)-epicatechin and (+)-catechin units, which by further condensation gave a mixture of compounds among which were homogeneous (−)-epicatechin bridged compounds. This shows the complex evolution of such mixtures and the interest of working on model solutions.

The quantification of (+)-catechin and (−)-epicatechin relative amounts is represented in Figure 14, which shows that, in this case, the loss of (−)-epicatechin was also higher than that of (+)-catechin, meaning that it reacts more readily with the intermediate. The reaction stopped when the intermediate was depleted.

CONCLUSION

The results obtained in this paper showed that the acetaldehyde autopolymerizations of (−)-epicatechin and (+)-catechin are greatly affected by the conditions used to induce the reaction and that the two studied flavanols showed different reactivities in such a reaction.

When incubated individually with acetaldehyde, the reaction was faster with (−)-epicatechin than with (+)-catechin at all studied pH values. Larger amounts of bridged compounds and also faster condensation rates were observed at lower pH values. These phenomena were related to the availability of the acetaldehyde cation.

When (−)-epicatechin and (+)-catechin were incubated together with acetaldehyde or with the (+)-catechin intermediate, heterodimeric ethyl-bridged compounds were obtained in addition to the homo-oligomers. The results showed that the first formed products underwent decomposition and recombination, giving various bridged oligomers. In both cases, the rate of loss of (−)-epicatechin was higher than that of (+)-catechin.

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