Studies on the Acetaldehyde-Induced Condensation of (−)-Epicatechin and Malvidin 3-*O*-Glucoside in a Model Solution System

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The reaction between (−)-epicatechin, malvidin 3-*O*-glucoside, and acetaldehyde was studied in a model solution system. Ethyl-linked flavanol oligomers and anthocyanin−flavanol derivatives were observed, showing that the two polyphenols competed in the condensation process. Among the anthocyanin−ethyl−flavanol adducts, dimeric compounds in which the flavanol was linked to the anthocyanin with CH_3−CH bridges were observed. In addition, trimeric and tetrameric products containing one anthocyanin and one, two, or three flavanols units were detected. A tetrameric product containing two anthocyanin and two flavanol units was also found as a doubly charged ion. No compound containing more than two malvidin 3-*O*-glucosides was detected, suggesting that only one anthocyanin A ring summit can be included in the polymerization process, which thus stops when both ends are occupied by an anthocyanin moiety. Thioacidolysis of the two isolated anthocyanin−ethyl−flavanol dimeric derivatives showed that anthocyanin−ethyl linkage was not sensitive to such reactants, whereas the flavanol−ethyl one was. In addition, flavanol−ethyl linkages involved in anthocyanin−ethyl−flavanol adducts were found to be less sensitive to those involved in flavan−ethyl dimers.

Keywords: *(−)-Epicatechin; malvidin 3-O-glucoside; acetaldehyde; condensation; thiolysis; LC/DAD; LC/MS*

INTRODUCTION

During the storage and aging of red wines, progressive changes of phenolic compounds initially extracted from grapes occur. There have been many studies on this complex phenomenon and the mechanisms involved in these transformations as well as the structures of the resulting compounds in model solution systems (Somers, 1971; Timberlake and Bridle, 1976; Haslam, 1980; Baranowski and Nagel, 1983; Ribereau-Gayon, 1983; Bishop and Nagel, 1984; Singleton and Trousdale, 1992; Liao et al., 1992; Bakker et al., 1993; Picinelli et al., 1994).

Among these transformations, the decrease of astringency occurring during wine aging has been considered as resulting from anthocyanin−flavanol condensation either involving acetaldehyde or not. This can be also explained, at least partly, by condensation of flavanols with acetaldehyde as reported in persimmon fruits (Tanaka et al., 1994). Recent works have thus shown the formation of flavanol ethyl-bridged compounds in model solution systems (Es-Safi et al., 1996; Fulcrand et al., 1996a,b; Saucier et al., 1997a,b; Cheynier et al., 1997). More recently, Saucier et al. (1997c) have shown the presence of such bridged compounds in red wine, proving thus the occurrence of this pathway condensation.

In addition to deastringency, change of wine color has been studied by many authors. The role of anthocyanins and flavanols in these transformations has been examined, and the additional presence of acetaldehyde is known to produce rapid change in color and intensity. Two major reaction pathways have been postulated to explain such phenomena. The first one is a direct reaction between anthocyanins and flavanols giving yellow-orange pigments (Jurd, 1967; Jurd and Somers, 1970; Somers, 1971; Liao et al., 1992; Santos-Buelga et al., 1995, 1996), and the second process is a condensation of the above phenolic compounds with acetaldehyde with formation of violet pigments (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; Fulcrand et al., 1996a,b; Francia-Aricha et al., 1997). Acetaldehyde is thus considered to cause an intense red color in wines and then take part in the aging process. When added to model solution systems containing anthocyanins and flavanols, various oligomeric bridged compounds were formed and the polymerization rate was found to be accelerated.

Thus, using malvidin 3-*O*-glucoside or malvidin 3,5-*O*-diglucoside with (+)-catechin or (−)-epicatechin and acetaldehyde, many authors have obtained ethyl-linked pigments in model solution systems. These products have been partially characterized using spectroscopic methods (Timberlake and Bridle, 1976; Baranowski and Nagel, 1983; Roggero et al., 1987; Liao et al., 1992; Bakker et al., 1993; Rivas-Gonzalo et al., 1995; Es-Safi et al., 1996). These experiments gave substantial evidence for the existence of anthocyanin−ethyl−flavanol polymers formed as a result of wine aging. However, most reported data concerned dimeric derivatives and

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did not give any exact information on the nature of the obtained polymerized compounds.

In this study, new information is offered that concerns the structures of the more polymerized pigments formed when (−)-epicatechin was incubated with malvidin 3-*O*-glucoside in the presence of acetaldehyde, and possible structures of them are suggested. In addition, a sensitive thiolysis and HPLC analytical method suitable for the obtained fractions identification, on the basis of the released free and thiol units, is proposed.

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). (−)-Epicatechin and mercaptoethanol were purchased from Sigma (St. Louis, MO). Malvidin 3-*O*-glucoside was isolated from red grape skin and purified in laboratory as described elsewhere (Fulcrand et al., 1996c). Acetaldehyde was obtained from Merck (Darmstadt, Germany).

(−)-Epicatechin−Malvidin 3-*O*-Glucoside−Acetaldehyde 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. An equimolar mixture of (−)-epicatechin and malvidin 3-*O*-glucoside (20 mM) was prepared in the above-described medium (0.5 mL, pH 2.2), and 60 μL of acetaldehyde was then added. The reaction was monitored by liquid chromatography coupled with a diode array detector (DAD) and with a mass spectrometry (MS) detector.

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. Thiolysis was conducted on solutions of pure dimeric compounds at a concentration of 1 mg/mL or by mixing 10 μL of collected solutions with 90 μL of thiolysis reactant. After sealing, reactions were carried out at 60 °C, and kinetics were monitored by HPLC.

HPLC/DAD analyses were performed by means of a Kontron Instruments system (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; temperature, 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 source. The mass spectrometer was operated in the positive mode ionization. Ion spray voltage was selected at +5 kV and orifice voltage at +60 V.

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 (3 μm packing, 125 × 2 mm i.d., Merck) column by using a two-step linear gradient at a flow rate of 200 μL/min. The elution was achieved with solvents A and B used in HPLC/DAD analyses and the conditions adapted as follows: 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. The flow was split so that 50 μL/min went to the electrospray source.

RESULTS AND DISCUSSION

The condensation between (−)-epicatechin and malvidin 3-*O*-glucoside was studied in the presence of ethanal at pH 2.2. The concentrations of the reagents 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 malvidin 3-*O*-glucoside was observed along with the appearance of oligomeric products in which the flavanol was linked either to another flavanol or to an anthocyanin unit by an ethyl bridge.

Figure 1A is a typical HPLC chromatogram, recorded at 280 nm, showing (−)-epicatechin, malvidin 3-*O*-glucoside, and the newly formed compounds that were eluted later than flavanol. Figure 1B shows the same chromatogram recorded at 520 nm, at which the presence of residual malvidin 3-*O*-glucoside and of new colored substances was observed. Two major compounds (MED1 and MED2) were detected corresponding to the two condensed pigments previously reported (Roggero et al., 1987; Bakker et al., 1993; Rivas-Gonzalo et al., 1995; Es-Safi et al., 1996). The UV−visible spectra of these products were similar to that of malvidin 3-*O*-glucoside, indicating that the flavylium chromophore was still present in both pigments. Moreover, the spectra of compounds MED1 and MED2 showed an additional shoulder around 450 nm, and the wavelengths of their maximum absorbances in the visible range (545 nm) were significantly higher than that of malvidin 3-*O*-glucoside (525 nm). This fact is probably due to some inter- or intramolecular copigmentation effect as described earlier in the case of the colored ethyl-bridged compounds obtained through interaction between (+)-catechin, acetaldehyde, and a synthetic flavylium pigment (Escribano-Bailon et al., 1996).

Upon LC/MS analysis, a molecular ion at *m*/*z* 809 was observed for both pigments. This is consistent with one flavylium moiety linked to one (−)-epicatechin moiety through an ethyl bridge, in agreement with the previously reported data and the postulated mechanism (Timberlake and Bridle, 1976; Bakker et al., 1993; Es-Safi et al., 1996).

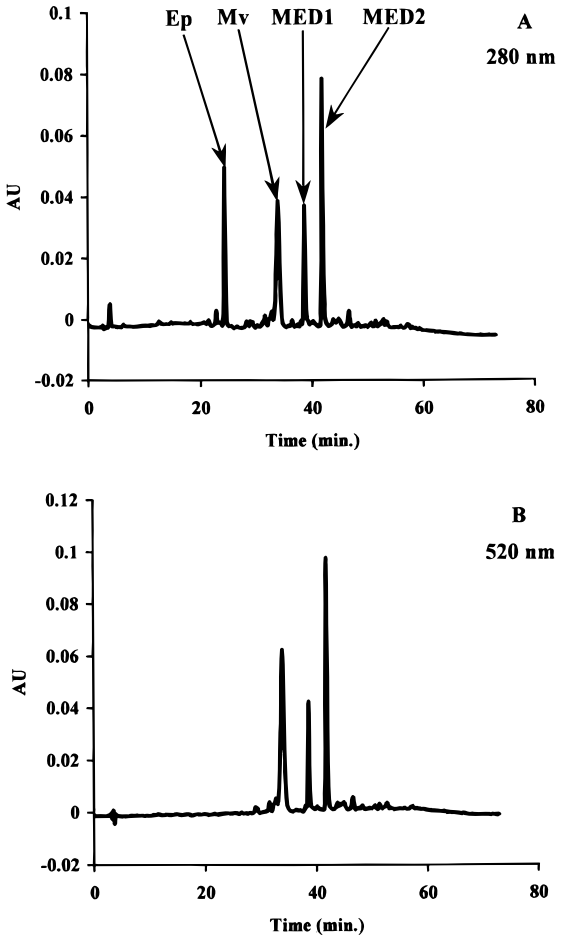

Figure 1. HPLC chromatograms recorded at 280 nm (A) and 520 nm (B) of a mixture of (−)-epicatechin, malvidin 3-*O*-glucoside, and acetaldehyde showing residual reagents and newly formed compounds [Mv, malvidin 3-*O*-glucoside; Ep, (−)-epicatechin].

To study their structures by thiolysis, isolation of the two dimers was achieved by HPLC at the semipreparative scale. The presence of two types of bonds linking the ethyl bridge, respectively, to the flavanol and to the anthocyanin moieties must be noted. The cleavage could concern the first and/or the second linkage. As shown in Figure 2, if the anthocyanin−ethyl bridge linkage was broken, free malvidin 3-*O*-glucoside would be obtained in addition to (−)-epicatechin−ethyl thiol derivatives, whereas free (−)-epicatechin and malvidin 3-*O*-glucoside ethyl thiol derivatives would be obtained in the other case. Finally, if both linkages were cleaved, a mixture of free (−)-epicatechin and free malvidin 3-*O*-glucoside in addition to their corresponding ethylthiol derivatives would be obtained.

Examination of the chromatograms obtained after complete thiolysis of the two dimers, conducted at 60 °C, shows the presence of free (−)-epicatechin and two thiol products named TEM1 and TEM2 as shown in Figure 3. The spectra of these compounds have absorption maxima in the visible region, indicating that the flavylium chromophore is still present in both of them (Figure 4). This means that the cleavage took place between (−)-epicatechin and the bridge, which was also confirmed by the absence of free malvidin 3-*O*-glucoside and the fact that the obtained thiol derivatives were different from those released from ethyl-linked (−)-epicatechin dimers (Es-Safi et al., 1999). This suggested that the linkage between (−)-epicatechin and the bridge

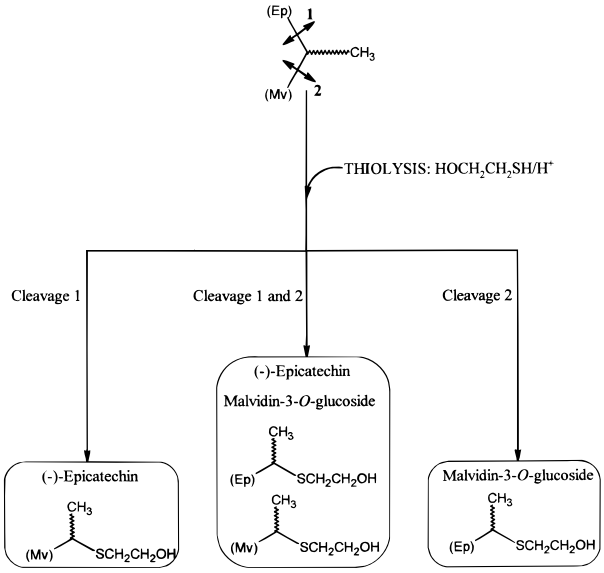

Figure 2. Scheme of anthocyanin−ethyl−flavanol adduct thiolysis [Mv, malvidin 3-*O*-glucoside moiety; Ep, (−)-epicatechin moiety].

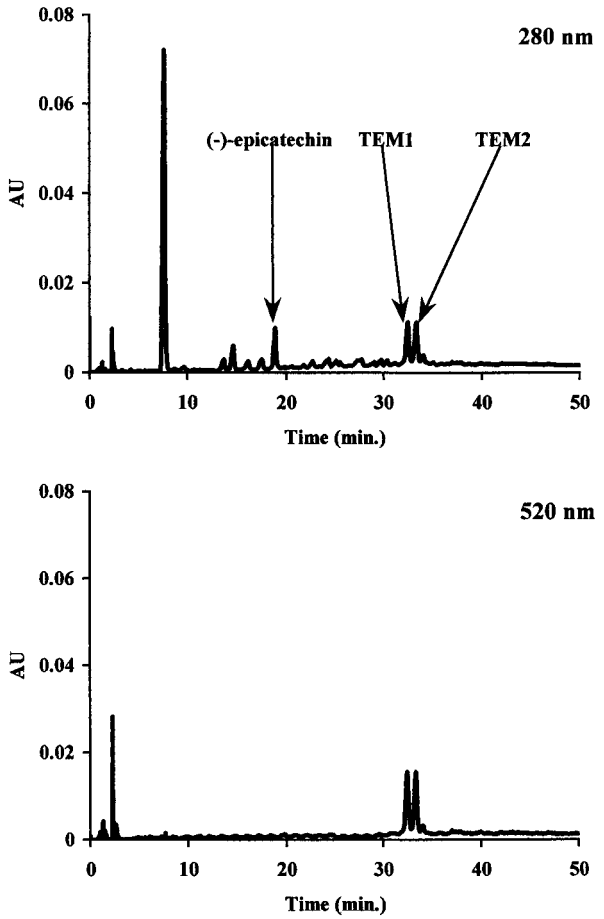

Figure 3. HPLC chromatograms recorded at 280 nm (top) and 520 nm (bottom) showing free (−)-epicatechin and the two colored thiol derivatives (TEM1 and TEM2) released after complete MED1 adduct thiolysis at 60 °C.

is more sensitive to thiolysis than that between the ethyl group and malvidin 3-*O*-glucoside.

The fact that the reaction requires heat contribution indicated that anthocyanin−ethyl−flavanol adducts are more stable than their flavanol equivalents. This also shows that flavanol−ethyl linkages involved in antho-

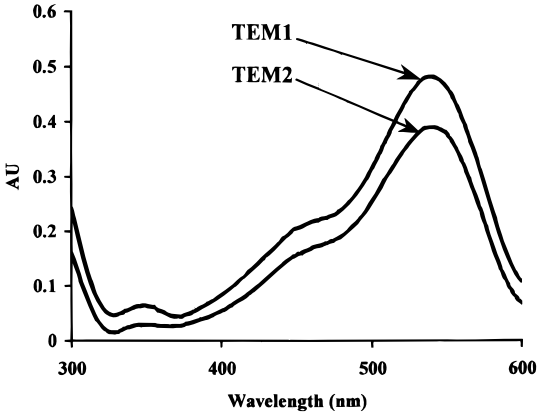

Figure 4. Visible spectra of the colored compounds TEM1 and TEM2.

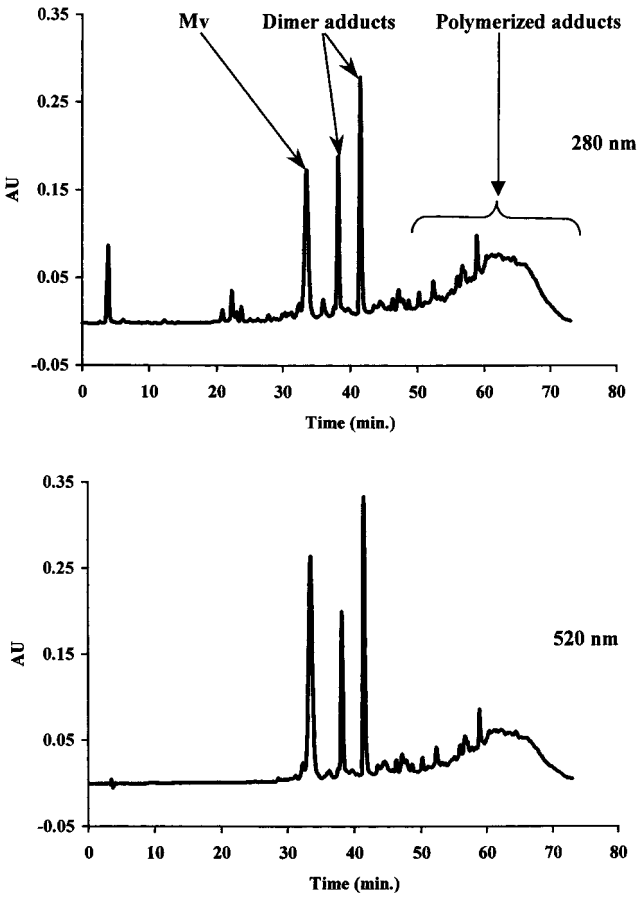

Figure 5. HPLC chromatograms measured at 280 nm (top) and 520 nm (bottom) of a mixture of (−)-epicatechin, malvidin 3-*O*-glucoside, and acetaldehyde after 24 h of reaction at ambient temperature (Mv, malvidin 3-*O*-glucoside).

cyanin−ethyl−flavanol oligomers are more stable than those involved in flavanol−ethyl ones, which were cleaved at ambient temperature. The obtention of the same two thiol derivatives from the two dimers showed that the ethyl group was fixed to the same summit of the malvidin 3-*O*-glucoside A ring. The two thiol derivatives corresponded to *R* and *S* isomers caused by the presence of an asymmetric carbon atom as observed in the case of (−)-epicatechin-ethyl thiol derivatives (Es-Safi et al., 1998).

It has been suggested that dimers MED1 and MED2 are transient and that they later evolve to form substances with greater degrees of condensation which

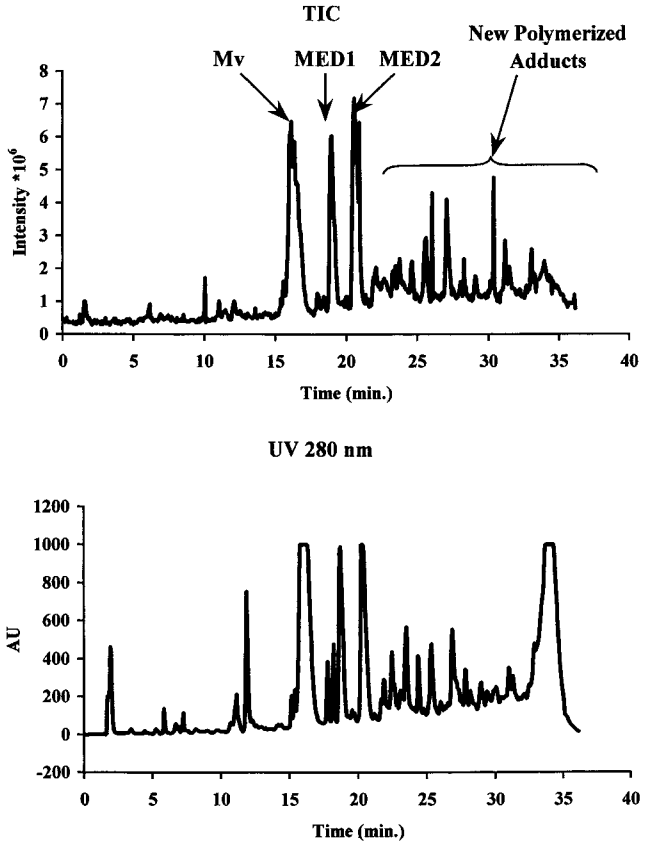

Figure 6. HPLC chromatograms measured with an electrospray mass detector (top) and with a UV detector at 280 nm (bottom) of a mixture of (−)-epicatechin, malvidin 3-*O*-glucoside, and acetaldehyde after 19 h of reaction at ambient temperature (Mv, malvidin 3-*O*-glucoside).

finally precipitate (Bakker et al., 1993; Garcia-Viguera et al., 1994). This fact was confirmed in the case of (+)-catechin−ethyl adducts conducted by ourselves by LC/ESI-MS analysis where octamers were detected (Fulcrand et al., 1996a; Es-Safi et al., 1996). In the case of anthocyanin−ethyl−flavanol condensation products, most reported results concerned dimer derivatives, and up to now, no exact information on the structure of the more polymerized adducts has been reported.

To investigate the structures of such compounds, the changes in solution containing malvidin 3-*O*-glucoside, (−)-epicatechin, and acetaldehyde were monitored by HPLC/DAD and HPLC/MS during 24 h. The results showed the formation of oligomeric pigments in which both anthocyanin and flavanol were incorporated. The reaction evolved later to more polymerized compounds observed as a bump on the chromatograms recorded at 280 and 520 nm (Figure 5), indicating that the basic flavylium structure is present in these polymerized adducts.

Figure 6 shows a chromatogram recorded after 19 h of reaction obtained either by UV−visible or by MS detection. In addition to (−)-epicatechin, malvidin 3-*O*-glucoside, and the two bridged dimers MED1 and MED2, other peaks with longer retention times appeared, corresponding probably to polymeric pigments and involving more than one (−)-epicatechin and/or one malvidin 3-*O*-glucoside moiety. Like compounds MED1 and MED2, these compounds exhibited UV−visible spectra similar to that of malvidin 3-*O*-glucoside but with maxima in the visible region shifted bathochromically.

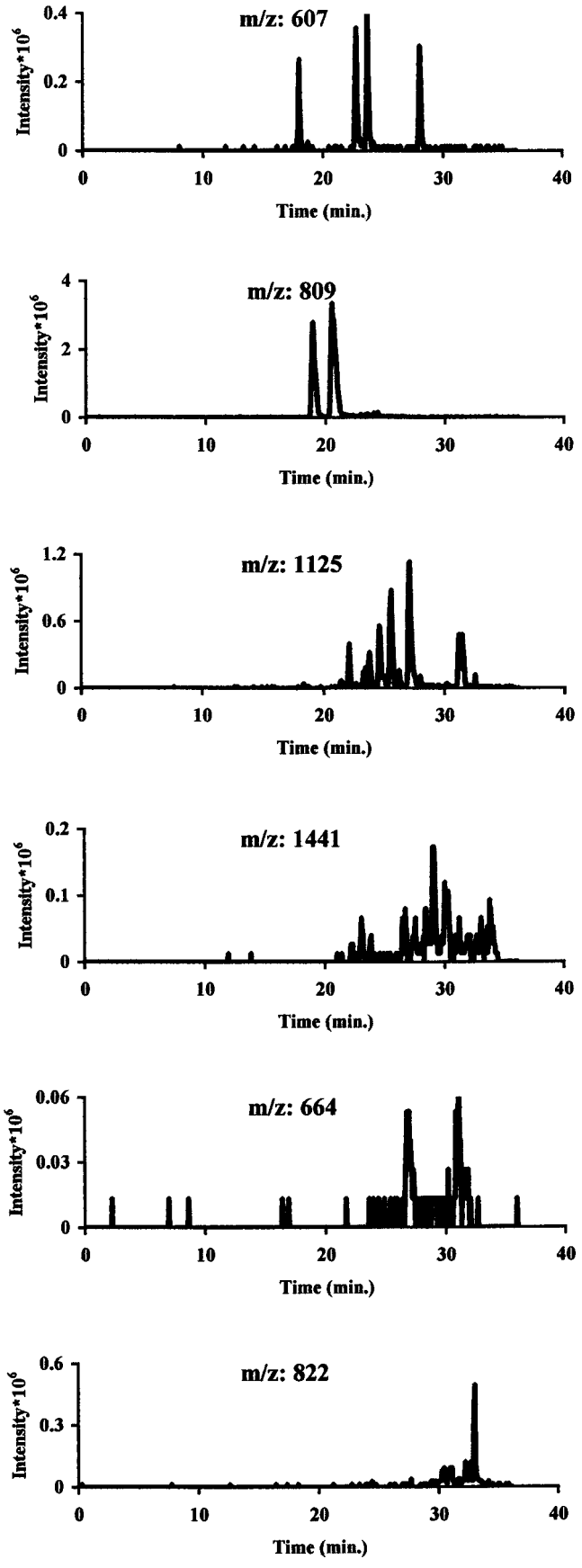

Figure 7. LC/MS analysis of the solution incubated during 19 h, performed with an ion-spray source. The chromatograms show traces of ion currents extracted (XIC) from the total ion current (TIC), each one corresponding to an *m/z* value of flavanol−ethyl or anthocyanin−ethyl−flavanol adducts.

LC/ESI-MS analysis of the solution, conducted in the positive-ion mode, showed the presence of both (−)-epicatechin−ethyl adducts detected as $[M + H]^+$ ions and malvidin 3-*O*-glucoside−ethyl−(−)-epicatechin oligomers detected as flavylium cations M^+, indicating that the anthocyanin competed with the flavanol in the condensation process. Figure 7 represents traces of ion currents extracted (XIC) from the total ion current chromatogram (TIC), each one corresponding to the *m/z* value of a given type of adduct. The MS spectra corresponding to these *m/z* values are shown in Figure 8.

Among the detected compounds, dimeric ethyl-linked derivatives consisting of (−)-epicatechin (*m/z* 607) and (−)-epicatechin and malvidin 3-*O*-glucoside (*m/z* 809) were observed. In addition, trimeric ethyl-linked pigments consisting of two (−)-epicatechin moieties and one malvidin 3-*O*-glucoside units (*m/z* 1125), two anthocyanin units, and one flavanol unit (*m/z* 664) were observed. The latter was detected as a doubly charged M^{2+} ion. Tetrameric species containing, respectively, one onthocyanin linked via an ethyl bridge to three (−)-epicatechin ethyl-linked moieties (*m/z* 1441) and two anthocyanins linked to two (−)-epicatechin units (*m/z* 822), were detected as a monocharged flavylium cation M^+ and a doubly charged ion M^{2+}. Figure 9 shows simplified representations of these compound structures.

It must be noted that no compound containing more than two malvidin 3-*O*-glucoside units was detected, suggesting that only one A ring summit can be involved in the polymerization process, whereas the two summits 6 and 8 were involved in the case of (−)-epicatechin, confirming the results obtained above by thiolysis analysis. This fact showed that the anthocyanin must be considered as a chain ending in the reaction, and as a consequence, the polymerization process stops when both ends are occupied by a malvidin moiety.

It must also be noted that no monomer intermediate adduct or dimeric compound consisting only of malvidin 3-*O*-glucoside units was detected. This showed that the anthocyanin moiety was not attacked by the first carbocation formed by protonation of acetaldehyde. On the contrary, both (−)-epicatechin−ethanol intermediate and (−)-epicatechin−ethyl dimer were detected, showing thus that the first carbocation attacks the flavanol, which after further loss of water molecule attacks either another flavanol or an anthocyanin, giving, respectively, a flavanol−ethyl dimer or an anthocyanin−ethyl−flavanol adduct.

CONCLUSION

On the basis of this investigation, it appears that acetaldehyde plays a major role in the condensation of flavanol, in the presence of anthocyanins, giving various bridged oligomers and polymers that finally precipitate. Such reaction may explain the decrease of astringency and the change of color observed during aging of grape-derived foods. The competetive action of (−)-epicatechin and malvidin 3-*O*-glucoside in the condensation process was demonstrated by the obtention of both flavanol−ethyl and anthocyanin−ethyl−flavanol adducts. More polymerized derivatives, in which more than one flavanol and up to two anthocyanin moieties were involved, were detected, suggesting that the polymerization process stops when the obtained product chain was ended by two malvidin 3-*O*-glucoside units.

Application of thiolysis analytical methods promotes isomer degradation and thus enables the differentiation

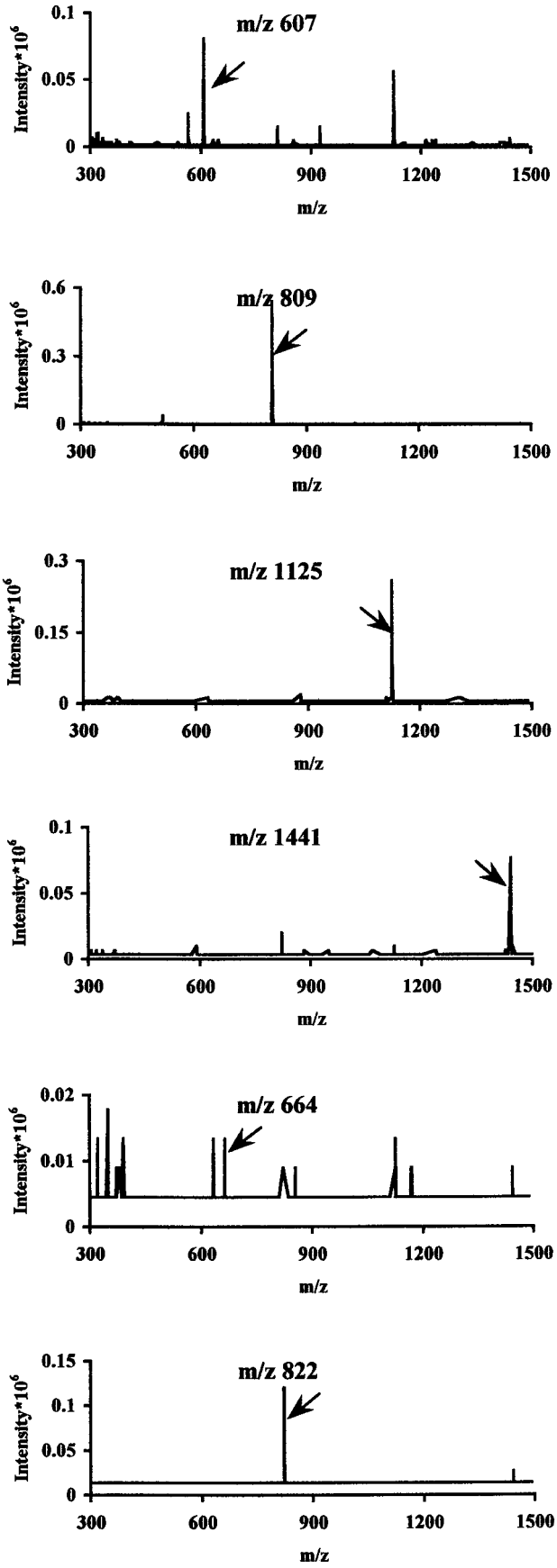

Figure 8. Mass spectra of the detected flavanol−ethyl and anthocyanin−ethyl−flavanol adducts.

of these compounds. The use of mercaptoethanol as a thiolyzing agent allows a faster reaction and gives precious information on the structures of these compounds. This thiol-promoted degradation reaction may be used in the case of products available in small amounts and eventually applied for the depolymerization of more condensed anthocyanin−ethyl−tannin complexes.

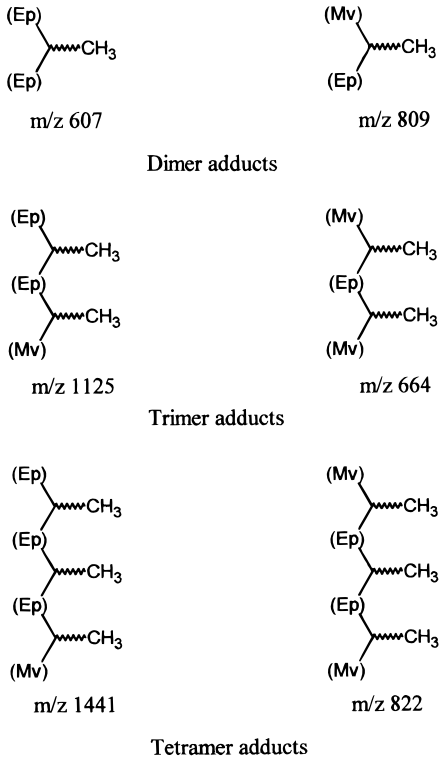

Figure 9. Simplified structures of the detected flavanol−ethyl and anthocyanin−ethyl−flavanol adducts [Mv, malvidin 3-*O*-glucoside moiety; Ep, (−)-epicatechin moiety].

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