Characterization of Pigments from Different High Speed Countercurrent Chromatography Wine Fractions

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A red wine, made from Cabernet Sauvignon (60%) and Tannat (40%) cultivars, was fractionated by high speed countercurrent chromatography (HSCCC). The biphasic solvent system consisting of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (2/2/1/5, acidified with 0.1% trifluoroacetic acid) was chosen for its demonstrated efficiency in separating anthocyanins. The different native and derived anthocyanins were identified on the basis of their UV–visible spectra, their elution time on reversed-phase high-performance liquid chromatography (HPLC), and their mass spectra, before and after thiolysis. The HSCCC method allowed the separation of different families of anthocyanin-derived pigments that were eluted in different fractions according to their structures. The hydrosoluble fraction was almost devoid of native anthocyanins. Further characterization (glucose quantification, UV–visible absorbance measurements) indicated that it contained flavanol and anthocyanin copolymers in which parts of the anthocyanin units were in colorless forms. Pigments in the hydrosoluble fraction showed increased resistance to sulfite bleaching and to the nucleophilic attack of water.

KEYWORDS: Wine; HSCCC; anthocyanins; flavanols; adducts; LC/DAD/ESI-MS; derived pigments

INTRODUCTION

Red wine is a very complex medium, which evolves during its storage and aging. With time, the color of young red wine changes from red-bluish toward the reddish-brown color of matured wines and the astringency decreases. Color changes are due to gradual conversion of the anthocyanin pigments extracted from red grapes into various derivatives through different reaction mechanisms.

The development of techniques to fractionate and isolate anthocyanins (native and derived) is a prerequisite to identify them and allows a better understanding of their properties. As an alternative to the classical gel chromatographic techniques, countercurrent chromatography (CCC) has been recently examined and has proven to be efficient in the preparative fractionation of anthocyanins (*1*, *2*).

CCC can be roughly described as a chromatographic separation process in which a liquid phase is retained in a coil by centrifugal force, while a second immiscible liquid phase continuously passes through it. Because it is a support-free liquid–liquid chromatographic technique, the retention of the solutes is determined exclusively by their partition coefficients, and, equally important, the problem of the irreversible adsorption of the solutes onto the stationary phase is excluded (*3*, *4*).

CCC has been successfully used to separate native anthocyanins from some derived anthocyanins in model solutions (*5*) and in wine (*6*). This is an analytical improvement since in young wines, the high concentrations of native anthocyanins usually hamper the analysis of derived wine pigments, which are only present in trace amounts. As a preparative method, CCC easily removes the excess of native anthocyanins, thus allowing a closer look at the newly formed wine pigments. The aim of this study was to characterize the anthocyanin profile of a wine by means of high-performance liquid chromatography (HPLC)/diode array detection (DAD)/mass spectrometry (MS) and thiolysis after fractionation using high speed (HS) CCC.

MATERIALS AND METHODS

The analyzed wine was a blended red wine (4 months of age), made from Cabernet Sauvignon (60%) and Tannat (40%) cultivars. Twenty-one liters of wine was loaded onto a 60 cm × 18 cm (i.d.) column filled with vinyldivinylbenzene. The resin was washed with 12.5 L of water to eliminate proteins, residual sugars, organic acids, and ions. Polyphenolic compounds were eluted with 21 L of methanol and then concentrated with a rotary evaporator under vacuum to 2 L. This extract was then further concentrated with a dryer (model B 190, Büchi, Flawil, Switzerland), yielding 112.53 g of a crude polyphenolic powder (*7*). This extract was fractionated with a CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research Corp., Baltimore, MD) equipped with three coils connected in series (sample loop, 20 mL; total volume, 850 mL; speed, 800 rpm). The solvent system consisted of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (2/2/1/5, acidified

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with 0.1% trifluoroacetice acid) with the lighter (organic) phase acting as the stationary phase and the aqueous phase as the mobile phase. The elution mode was head to tail (*1*), with a 3.5 mL/min flow rate. One gram of the wine powder was dissolved in 20 mL of a mixture of the upper (organic) and lower (aqueous) phases (50:50; v/v) before injection. Five different fractions and the organic stationary phase were collected and freeze-dried.

Analysis of Fractions by HPLC/DAD/MS. Chromatographic separation was achieved using a Waters 2690 system equipped with an autosampler system, a Waters 996 photodiode array detector, and Millennium 32 chromatography manager software. The column was a Lichrospher 100-RP18 (5 μm packing, 250 mm × 2 mm i.d.) protected with a guard column of the same material (Merck, Darmstadt, Germany). The elution conditions were as follows: 0.250 mL/min flow rate; oven temperature, 30 °C; solvent A, water/formic acid (95:5, v/v); solvent B, acetonitrile/water/formic acid (80:15:5, v/v/v); elution started isocratically with 2% B for 7 min, continued with linear gradient from 2 to 20% B in 15 min, from 20 to 30% B in 8 min, from 30 to 40% B in 10 min, from 40 to 50% B in 5 min, and from 50 to 80% B in 5 min, followed by washing and reequilibration of the column. UV−vis spectra were recorded from 220 to 600 nm, and peak areas were measured at 280 and 520 nm. MS analysis and fragmentation experiments were performed on a ThermoFinnigan LCQ Advantage (San Jose, California) MS equipped with an electrospray ionization source and an ion trap mass analyzer, which were controlled by the LCQ navigator software. The mass spectrometer was operated in the positive ion mode in the range of *m/z* 150−2000 and under the following conditions: source voltage, 4.5 kV; capillary voltage, 23.5 V; capillary temperature, 250 °C; collision energy for fragmentation, 25% for MS[2], 30% for MS[3], and 35% for MS[4].

Thiolysis Conditions. A few milligrams of each fraction were dissolved in 1 mL of methanol, and 100 μL of this solution was added to an equal volume of thiolytic reagent (toluene-α-thiol 5% in methanol containing 0.2 M HCl). After it was sealed, the mixture was shaken and heated at 90 °C for 2 min. The solutions were then analyzed by LC/DAD/MS as described above. Quantification of each terminal and extension unit was based on peak areas at 280 nm, and calibration was undertaken with external standards purified in the laboratory (*8*).

Quantification of Glucose by Gas Chromatography (GC). The glucose content of fraction 1 was determined by GC analysis of the alditol acetates derivatives (*9*), obtained after hydrolysis with 2 M trifluoroacetic acid. Separation was achieved with a HP-5890 engine (Hewlett-Packard, Avondale, PA) on a fused-silica DB-225 (210 °C) capillary column (30 m × 0.32 i.d., 0.25 μm film) with H$_2$ as the carrier gas; injection volume, 2 μL; temperature of the injector and the detector, 250 °C. The identification and quantification of each peak were based on injection of alditol acetate standards prepared in our laboratory.

Spectrophotometric Measurements. Twelve milligrams of fraction 1 was dissolved in 550 μL of methanol, 250 μL of this solution was diluted in 25 mL of 0.1 M HCl to give solution A, and 250 μL of the methanolic solution was diluted in 25 mL of 0.1 M Na$_2$PO$_4$·12H$_2$O (solution B). The ionic strength was fixed by 1 M NaCl in both solutions A and B. The solutions A and B were mixed in variable proportions in order to obtain a series of pH values ranging from 1 to 7 with a step size of 0.5 pH unit.

Absorbance measurements were performed with a SAFAS UV mc² spectrophotometer, with a 10 mm cell width. Absorbance measurements were performed 12 h later to ensure that the hydration equilibrium was reached. Absorbance measurements were done from 250 to 700 nm.

Sulfite Bleaching. Sulfite bleaching experiments were performed by adding 15 μL of two aqueous Na$_2$S$_2$O$_5$ solutions (0.2 and 200 g/L) to 1 mL of fraction 1 dissolved in a HCl solution adjusted by the addition of a 0.01 N NaOH solution at two different pH values: 2.5 and 3.5. Similar experiments were performed on the wine polyphenolic extract and on commercial Mv3glc. Before and after sulfite bleaching, the absorbance measurements were done from 250 to 700 nm, and the absorbance value at 520 nm was taken from the spectra.

RESULTS

The anthocyanin composition of the wine sample was established by HPLC/DAD/MS analysis. The HPLC profile at

Table 1. Mass Spectra and UV–Vis Data of the Anthocyanins Identified in Wine

	wine	*m/z*	λ_{max}
1	epi-Mv3glc	781	529
2	epi-epi-Mv3glc	1069	530
3	Dp 3glc	465	525
4	Cy3glc	449	516
5	Pt3glc	479	526
6	carboxyl-pyrano-Pt3glc	547	520
7	Mv3,5glc	655	525
8	Pn3glc	463	516
9	Mv3glc	493	530
10	carboxyl-pyrano-Mv3glc	561	511
11	Dp3glc-ac	507	530
12	carboxyl-pyrano-Mv3glc-ac	603	511
13	(epi)cat-ethyl-Mv3glc	809	550
14	Pt3glc-ac	521	530
15	carboxyl-pyrano-Mv3glc-cou	707	530
16	Pn3glc-ac and Dp3glc-cou	505,611	530
17	Mv3glc-Ac	535	535
18	Cy3glc-cou	595	521
19	Pt3glc-cou	625	530
20	Pn3glc-cou	609	521
21	Mv3glc-cou	639	535

520 nm of the wine sample before fractionation is shown in **Figure 1** (peak identification given in **Table 1**). The chromatogram shows the presence of both native anthocyanins (largely predominant) originating from grape- and anthocyanin-derived pigments. Peaks 3, 4, 5, 8, and 9 correspond to the 3-O-glucosides of delphinidin (Dp3glc), cyanidin (Cy3glc), petunidin (Pt3glc), peonidin (Pn3glc), and malvidin (Mv3glc), respectively. The corresponding 3-O-acetylglucosides are eluted in peaks 11 (Dp3glc-ac), 14 (Pt3glc-ac), 16 (Pn3glc-ac), and 17 (Mv3glc-ac) and the 3-O-*p*-coumaroylglucosides in peaks 16 (Dp3glc-cou coeluted with Pn3glc-ac), 18 (Cy3glc-cou), 19 (Pt3glc-cou), 20 (Pn3glc-cou), and 21 (Mv3glc-cou). In addition to the native grape pigments, the chromatogram shows the presence of direct reaction products between flavanols and anthocyanins (peaks 1 and 2) that have earlier been detected in wine (*10*, *11*) and obtained by hemisynthesis (*12*). Peak 1 was tentatively identified to be a dimer (epicatechin-Mv3glc) (**Figure 2A**) since it had the same retention time, mass signal (*m/z* 781), and λ_{max} (535 nm) as epicatechin-Mv3glc (epicatechin being the upper unit and Mv3glc the lower unit) obtained in a model solution containing Mv3glc and procyanidin dimer B2 3′-O-gallate (*12*). Peak 2 might correspond to a trimer [(epi)cat-(epi)-cat-Mv3glc] with a mass signal at *m/z* 1069, in the positive ion mode. Another family of anthocyanin-derived compounds, namely, carboxyl-pyranoanthocyanins (**Figure 2B**), resulting from the reaction of anthocyanins with pyruvic acid (*13*) was detected on the basis of their λ_{max} at 510 nm, which is the distinctive λ_{max} of the pyranoanthocyanin family and of their mass spectra. Thus, peaks 6, 10, 12, and 15, showing mass signals at *m/z* 547, *m/z* 561, *m/z* 603, and *m/z* 707, respectively, were identified to carboxyl-pyrano-Pt3glc, carboxyl-pyrano-Mv3glc also called vitisin A (*14*), carboxyl-pyrano-Mv3glcAc, and carboxyl-pyrano-Mv3glc-cou. Another peak (13) had the distinctive UV−vis spectrum of acetaldehyde-mediated condensation product between anthocyanins and flavanols (**Figure 2C**) and a mass signal at *m/z* 809 and was thus identified as (epi)cat-ethyl-Mv3glc. This kind of compound has already been shown to occur in wine (*15*−*17*). Their formation mechanism has been studied (*18*), and their structures have been elucidated by NMR (*19*). A small hump can also be observed in the chromatogram (between 20 and 45 min); this hump is due to the elution of unresolved polydisperse-derived pigments.
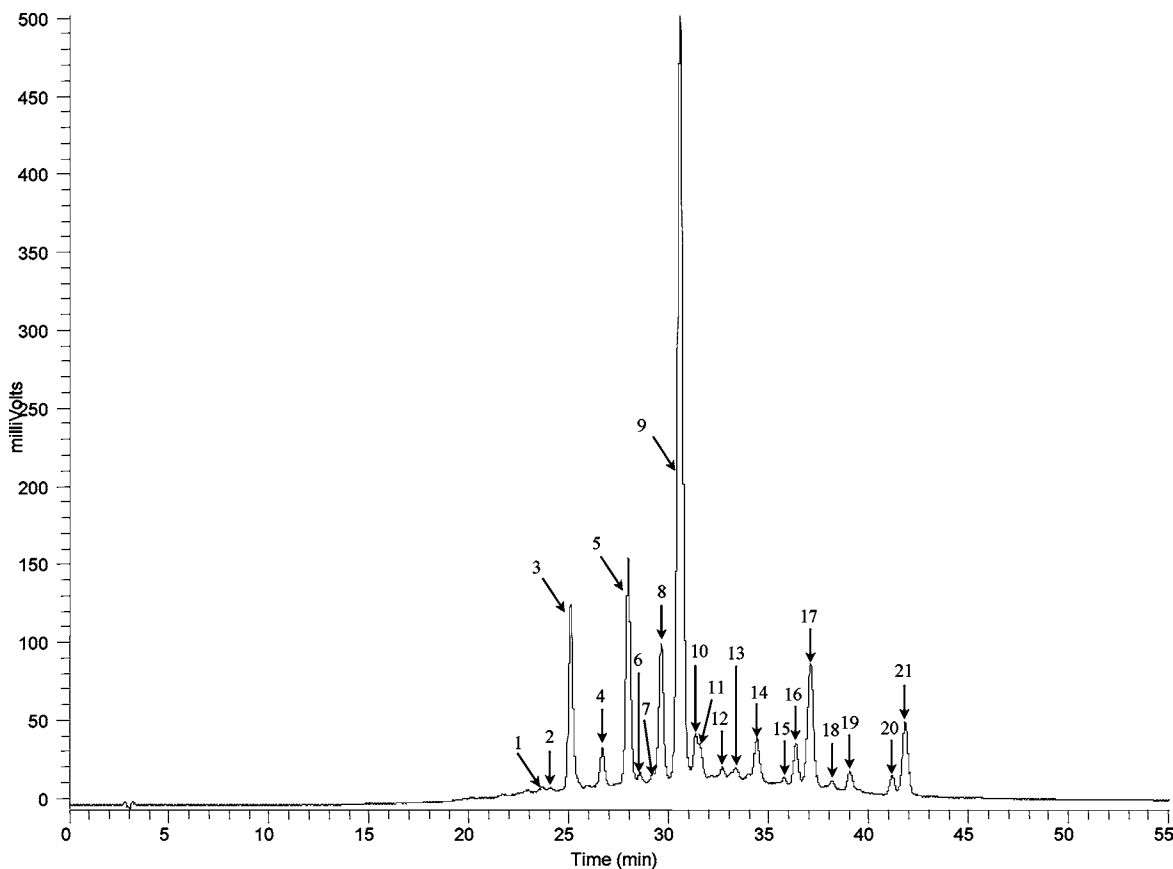
Figure 1. HPLC profile at 520 nm of the wine polyphenolic extract.

A) (epi)cat-Mv3glc

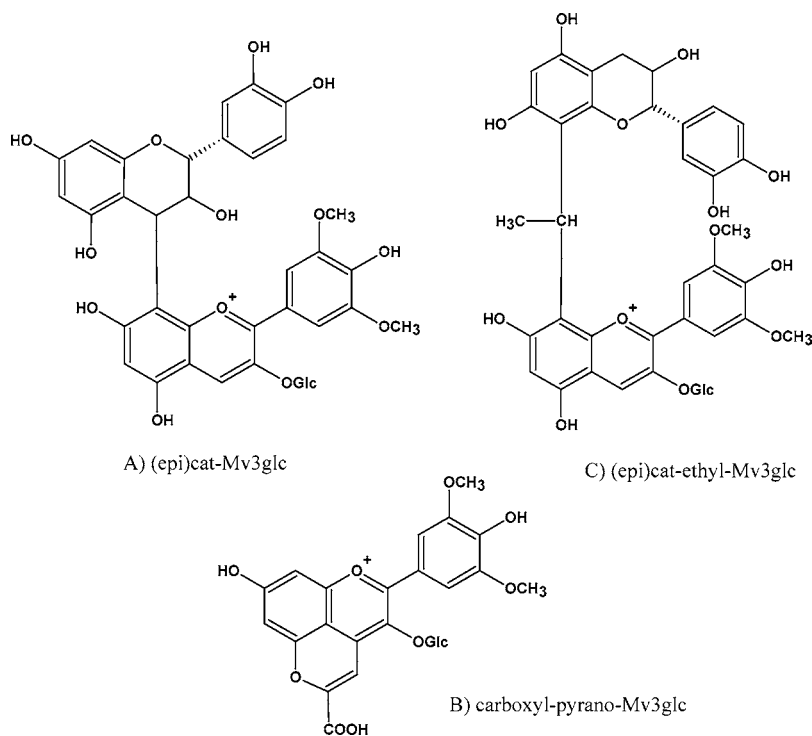C) (epi)cat-ethyl-Mv3glc

B) carboxyl-pyrano-Mv3glc

Figure 2. Structures of anthocyanin-derived adducts.

HSCCC separation profile of the wine sample recorded at 520 nm is shown in **Figure 3**. Five different fractions noted 1−5 and the organic stationary phase were collected and freeze-dried. The HSCCC separation lasted over 9 h. The delineation of the five fractions collected was based on the apparent rough separation exhibited by the HSCCC profile, and because baseline separation was not achieved according to this profile, considerable overlap in the contents of adjacent fractions might be anticipated.

The general separation profile of the native anthocyanins was similar to that obtained by multilayer coil (ML) CCC fractionation of a grape skin extract (*20*). A list of compounds identified
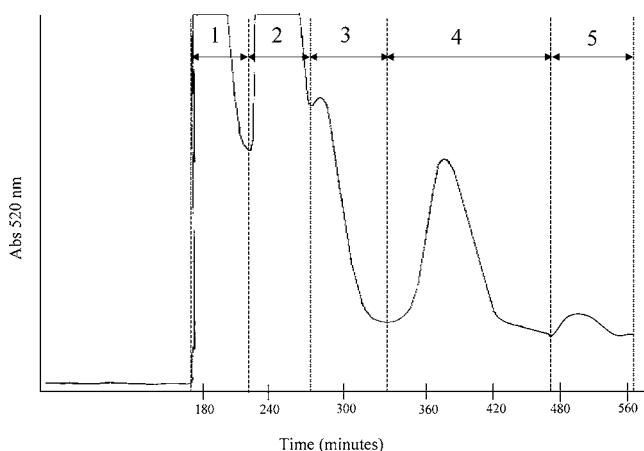
Figure 3. HSCCC profile at 520 nm of the wine.

Table 2. Molecular Ions and λ_{max} in the Visible Region of the Anthocyanins and Derived Products Detected in the Different HSCCC Fractions

		m/z	λ_{max}
	fraction 1		
22	carboxyl-pyrano-Dp3glc	533	512
6	carboxyl-pyrano-Pt3glc	547	520
7	Mv 3,5glc; Dp3glc-Mv3glc	655, 957	525
8	Pn3glc, carboxyl-pyrano-Pt3glc-ac	463, 589, 1523	521
9	Mv3glc, carboxyl-pyrano-Pn3glc, Pt3glc-Mv3glc	493, 531, 971	521
10	carboxyl-pyrano-Mv3glc	561	511
12	carboxyl-pyrano-Mv3glc-ac, Mv3glc-Mv3glc	603, 985	511
23	Mv3glc-ethyl-Mv3glc	1029	525
	fraction 2		
1	epi-Mv3glc	781	529
3	Dp3glc	465	525
2	(epi)cat-(epi)cat-Mv3glc	1069	536
4	Cy3glc	449	516
5	Pt3glc	479	526
8	Pn3glc	463	516
9	Mv3glc	493	530
	fraction 3		
3	Dp3glc	465	525
4	Cy3glc and cat-Mv3glc	449, 781	520
5	Pt3glc	479	525
8	Pn3glc	463	516
9	Mv3glc	493	530
13	Mv3glc-ethyl-(epi)cat	809	540
17	Mv3glc-ac	535	535
	fraction 4		
3	Dp 3glc and unknown	465, 1359	535
5	Pt 3glc	479, 783, 799, 631	535
24	Dp3glc-ethyl-(epi)cat	781, 657,769	550
8	Pn3glc	463, 823, 553	529
9	Pt3glc-ethyl-cat and Mv3glc	493, 795	530
11	Dp3glc-ac	507, 621, 783, 799	530
25	Pn3glc-ethyl-(epi)cat	779	540
26	unknown	535, 693, 581	526
13	Mv3glc-ethyl-epi(cat)	809	550
14	Pt3glc-ac	521, 317	530
15	carboxyl-pyrano-Mv3glc-cou- and unknown	707, 1117	515
17	Mv3glc-ac	535, 331	535
	fraction 5		
27	Cy3glc-ac	491, 287	521
28	carboxyl-pyrano- Pn3glc-cou	677, 1087	506
16	Pn3glc-ac	505, 817	521
17	Mv3glc-ac	535, 791, 837	535
	stationary phase		
3	Dp3glc	465	525
4	Cy3glc	449	516
5	Pt3glc	479	530
8	Pn3glc	463	516
9	Mv3glc	493	530
16	Pn3glc-ac and Dp3glc-cou	505, 611	530
17	Mv3glc-ac	535	535
18	Cy3glc-cou	595	521
19	Pt3glc-cou	625	530
29	Cat-ethyl-Mv3glc-cou	955	550
20	Pn3glc-cou	609	521
21	Mv3glc-cou	639	535
30	Mv3glc-vinylphenol	609	501

in each fraction, along with their λ_{max} and mass signals, is given in **Table 2**. Anthocyanins are separated according to the degree of substitution of their B ring and to the nature of the anthocyanidin substituent (glucoside, acetylglucoside, or coumaroylglucoside). Thus, there was a rough separation between Dp3glc, Pt3glc, and Mv3glc, recovered mostly in fraction 2, and Cy3glc and Pn3glc, eluted in fraction 3. Similarly, their 3-acetylglucosides were recovered in fractions 4 and 5, respectively, whereas all 3-*p*-coumaroylglucosides were recovered in the stationary phase, due to their higher hydrophobicity.

Fraction 1 consisted mostly of a colored hump as shown in **Figure 4**. A similar fraction was earlier obtained after HSCCC separation of wine and referred to as "polymeric pigments" (*6*). Another similar hydrophilic fraction was obtained from a grape skin extract by MLCCC using the reverse mode (tail to head) with the organic phase as the mobile phase (*20*) and shown to contain oligomeric anthocyanins (*21*). Water soluble pigments are very interesting from the enological point of view in relation with wine age. Indeed, it has been observed that pigments remaining in the aqueous phase after extraction with isoamyl alcohol were responsible for 50% of the color intensity of a one year old wine and for most of the color of older wines (*22*). Water soluble pigments obtained by CCC were also responsible for almost all of the color of a 10 year old merlot wine (*1*).

Trace amounts of grape anthocyanins, i.e., Mv3glc (peak 9), Pn3glc (peak 8), and Mv3,5glc (peak 7), were detected in fraction 1. The last compound is expected to be rather polar, due to the presence of two glucose units, and was earlier observed to elute with the water soluble compounds using the same solvent system (*1*).

Small peaks of derived pigments are also present in fraction 1. The major series consists of carboxyl-pyranoanthocyanins. These include carboxyl-pyrano-Mv3glc (vitisin A) (peak 10) that has already been observed to elute with a similar fraction obtained using the same solvent system (*6*) and the other carboxyl-pyranoanthocyanins detected by analysis of the wine extract plus minor related compounds: carboxyl-pyrano-Dp3glc (peak 22), carboxyl-pyrano-Pt3glc-ac (coeluted with Pn3glc in peak 8), and carboxyl-pyrano-Pn3glc (coeluted with Mv3glc in peak 9).

Peak 23 (**Figure 4**) with a mass signal at *m/z* 1029 and a λ_{max} at 525 nm was identified as Mv3glc-ethyl-Mv3glc, with one of the anthocyanins in its hydrated form. This compound has been characterized by NMR (*23*) and corresponds to the product of the acetaldehyde-mediated dimerization of anthocyanins.

Colorless anthocyanin-flavanol adducts linked by an A type bond (C4−C8) and (C2−O−C7) (**Figure 5A**) were detected in the extracted ion chromatograms (XIC) at *m/z* 783 (dimer) and at *m/z* 1071 (trimer). These colorless adducts, notably anthocyanin-O-(epi)cat, have been detected in wine (*24*), and the structure of a Mv3glc-O-epicatechin dimer has been characterized by NMR (*25*).
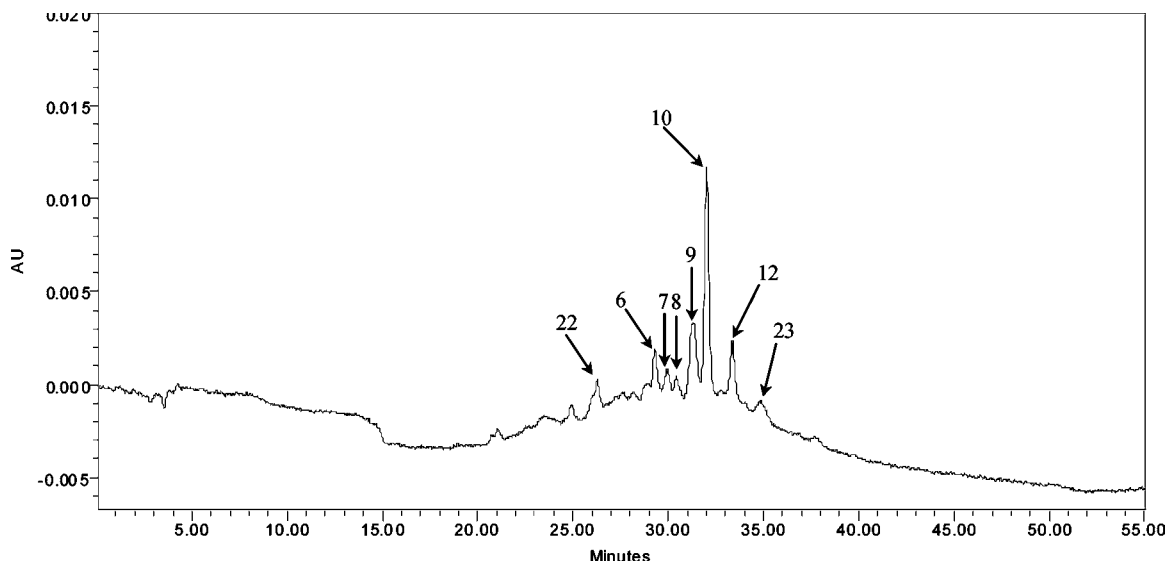
Figure 4. HPLC profile at 520 nm of fraction 1.

B) *m/z* 985

C) *m/z* 985

A) R= H *m/z* 783

R= (epi)catechin *m/z* 1071
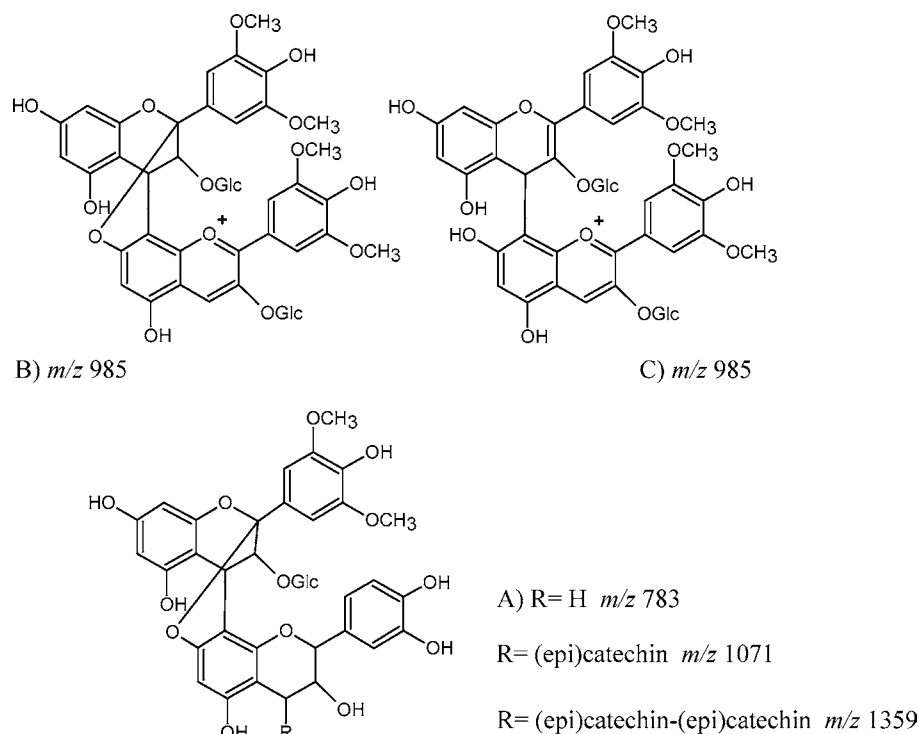
R= (epi)catechin-(epi)catechin *m/z* 1359

Figure 5. Possible structures of anthocyanin-derived adducts.

Finally, a signal detected at *m/z* 985 (coeluted with carboxyl-pyranoMv3-glc-ac in peak 12) was interpreted as a Mv3glc dimer. Such dimers have been recently detected in the aqueous stationary phase obtained after MLCCC separation of a grape skin extract (*21*). Two structures have been proposed. In the first one, the anthocyanin in the upper position is in the flavan-3-glucoside form and connected through a A type bond to the lower anthocyanin unit in the flavylium form (**Figure 5B**). The second structure consists of an anthocyanin upper unit in the flavene form linked to the terminal unit in the flavylium form through a B type bond (**Figure 5C**). Three other dimers of the same family, which correspond, respectively, to Pt3glc-Mv3glc, Dp3glc-Mv3glc, and Pn3glc-Mv3glc, were detected in the XIC at *m/z* 971, *m/z* 957, and *m/z* 955.

Fraction 1 was also analyzed by HPLC/DAD/MS after thiolysis (**Figure 6**). This method was initially developed for the analysis of proanthocyanidins (*8, 26, 27*) but can also provide

structural information on tannin-anthocyanin derivatives (*12, 17, 24*). Its relies upon acid-catalyzed depolymerization in the presence of toluene-α-thiol, used as a nucleophilic agent to trap the carbocations released by cleavage of the interflavanic bonds. Analysis of the resulting solution permits one to distinguish between the terminal units (substituted at C-6 or C-8 in the original structure) that are released as such and the upper and intermediate units (substituted at the C-4 position in the original structure) that yield the corresponding benzylthioether derivatives.

Analysis of the fraction 1 after thiolysis (**Figure 6**) showed the presence of the pyranoanthocyanin family (described above) confirming its resistance to thiolysis. The group of pigments consisting of two anthocyanin units linked by an A type bond with the Mv3glc upper unit in the flavan form or linked by a B type bond with the Mv3glc upper unit in the flavene form, was also detected after thiolysis. The resistance toward thiolysis of
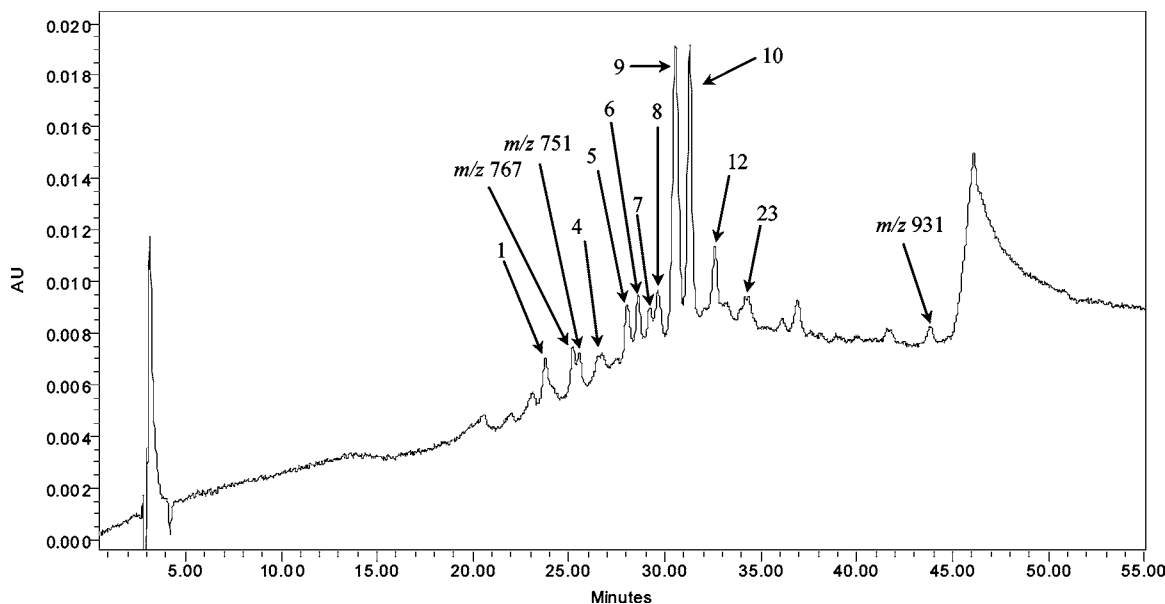
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Figure 6. HPLC profile at 520 nm of fraction 1 after thiolysis.

this group may be due to the presence of an A type bond (*25*) as demonstrated for A type proanthocyanidins (*28*). It may also be explained by the fact that the presence of one flavylium anthocyanin in the structure of a dimer confers resistance to thiolysis regardless of the type of linkage and of its position.

Peak 1, detected in the first fraction after thiolysis, had the same retention time, mass signal (m/z 781), and λ_{max} (535 nm) as epicatechin-Mv3glc. This ion was present in the first fraction before thiolysis but in lower quantity as it was only detected in the XIC and no UV−vis peak was observed in the HPLC profile at 520 nm (**Figure 4**). Similar compounds with mass signals at m/z 767 and m/z 751 corresponding, respectively, to (epi)cat-Pt3glc and (epi)cat-Pn3glc, were detected after thiolysis. The increase of this group of compounds after thiolysis reflects the presence of larger (epi)cat$_n$-anthocyanin adducts (n >2) in fraction 1 before thiolysis since they release the flavanol-anthocyanin dimer as terminal unit. These dimers have proved to be resistant to thiolysis (*10*). Such larger tannin-anthocyanin adducts were earlier reported to be recovered in the aqueous phase after liquid−liquid extraction (*24*). However, we cannot exclude the possibility of UV−vis peaks of this family of compounds (flavanol-anthocyanin) being visible before thiolysis because they might have been hidden under the colored hump.

XIC detection of a signal at m/z 905 corresponding to the benzylthioether of anthocyanin-O-(epi)cat reflects the presence of larger colorless anthocyanin-O-(epi)cat$_n$ adducts (n > 2) in the first fraction before thiolysis as they form the colorless anthocyanin-O-flavanol benzylthioether derivatives from their upper dimeric unit.

The compound with a mass signal at m/z 1029, which was identified as Mv3glc-ethyl-Mv3glc, with one of the anthocyanins in its hydrated form, was still present after thiolysis.

The mean degree of polymerization (mDP) calculated for fraction 1 on the basis of flavanol units released by thiolysis was 10.7. It should, however, be emphasized that this result does not reflect the chain length as anthocyanins incorporated in the oligomeric and polymeric structures are not taken into account but indicates that most of the flavanol units in the molecular species present were in the upper position.

Fraction 2 consisted mainly of three of the five native anthocyanins that are trisubstituted in the B ring (see **Table 2** and **Figure 7A**): Dp3glc, Pt3glc, and Mv3glc. Small amounts

of Pn3glc and Cy3glc were also observed. Peak 1 tentatively identified as epicatechin-Mv3glc was detected. Another compound present in this wine fraction (peak 2), with a mass signal at m/z 1069 and a λ_{max} at 536 nm, can be interpreted as the flavylium form of (epi)catechin-(epi)catechin-Mv3glc.

Fraction 2 was also analyzed by HPLC/DAD/MS after thiolysis. The entire family of the (epi)catechin-anthocyanin adducts was detected and, most importantly, respecting the same elution order as found for their anthocyanin precursors. Thus, the first compound to be eluted corresponds to (epi)cat-Dp3glc, with a mass signal at m/z 753. The second is (epi)cat-Cy3glc, with a mass signal at m/z 737. The third is (epi)cat-Pt3glc, with a mass signal at m/z 767. The following one is (epi)cat-Pn3glc, with a mass signal at m/z 751. The last one of the series is epicat-Mv3glc already present in this fraction before thiolysis. As explained above, these compounds arise from acid-catalyzed cleavage of (epi)cat$_n$-anthocyanin adducts (n > 2).

Fraction 3 consisted primarily of Pn3glc, Cy3glc (disubstituted in the B ring), along with some Mv3glc (see **Table 2** and **Figure 7B**). One compound with a mass signal at m/z 781 was coeluted with Cy3glc, so that its λ_{max} could not be determined. This compound had the same retention time, mass signal, and fragmentation pattern as those of cat-Mv3glc dimer obtained by hemisynthesis (*10*) and was thus tentatively identified to cat-Mv3glc. Another peak (13) had a mass signal at m/z 809 and the distinctive UV−vis spectrum of acetaldehyde-mediated condensation product between anthocyanins and flavanols (λ_{max} 550 nm) and was thus tentatively identified as (epi)cat-ethyl-Mv3glc.

Fraction 3 was analyzed by HPLC/DAD/MS after thiolysis. The ion at m/z 809 was still present after thiolysis because the occurrence of a flavylium moiety within these structures increases their resistance to acid-catalyzed cleavage (*18*). A compound with a mass signal at m/z 931 and a UV−vis spectrum characteristic of ethyl-bridged anthocyanin derivatives was interpreted as the benzylthioether derivative of (epi)cat-ethyl-Mv3glc. Its presence after thiolysis reflects the presence of larger (epi)cat$_n$-ethyl-anthocyanin adducts (n > 2).

Fraction 4, (see **Table 2** and **Figure 7C**) is mainly constituted of the three acetylated anthocyanins that are trisubstituted in the B ring (Mv3glc-ac, Dp3glc-ac, and Pt3glc-ac). Three

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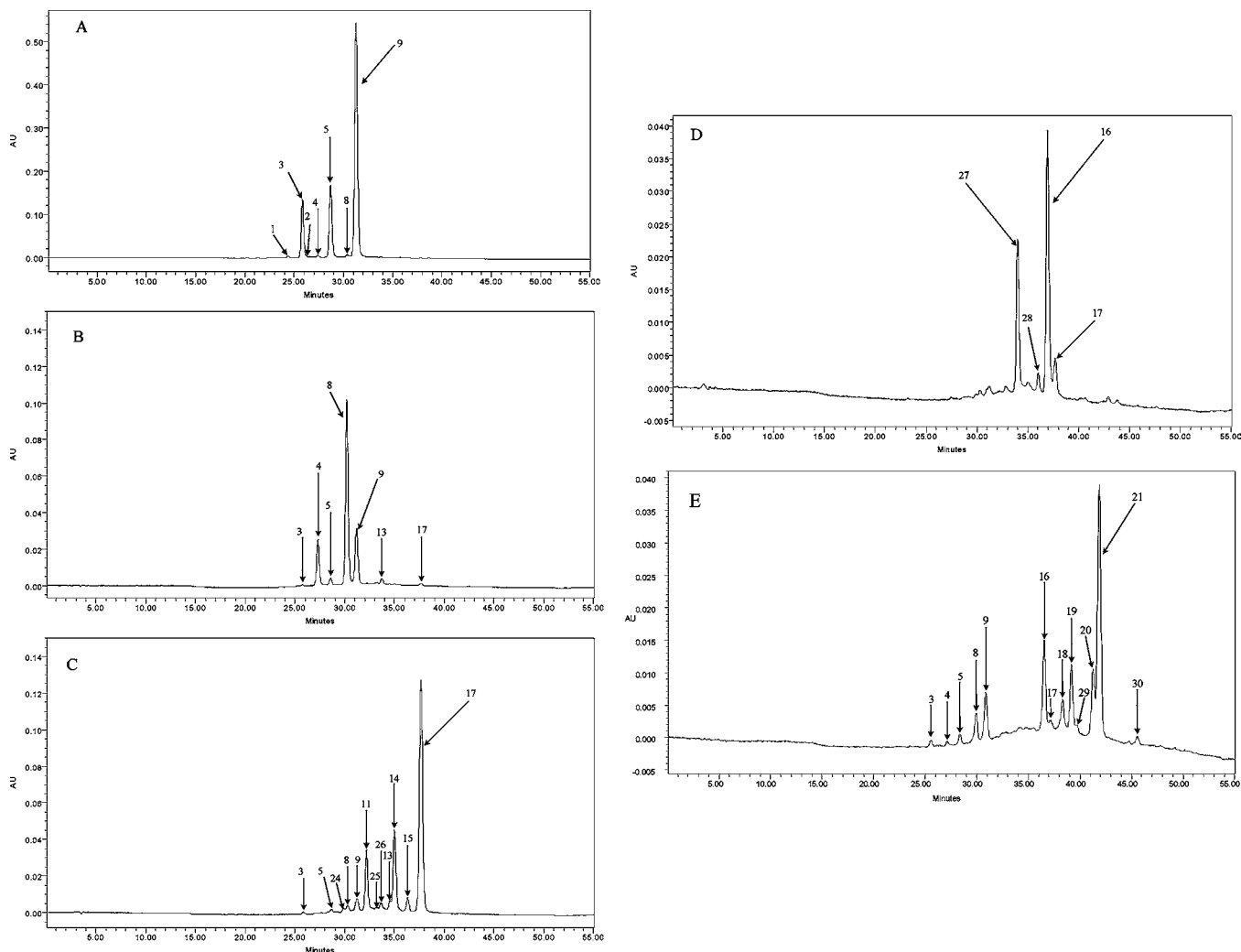
Figure 7. HPLC profile at 520 nm of fractions 2–5 and the stationary phase.

compounds of the same family, presenting UV–vis spectra characteristic of the ethyl-bridged family, were detected at m/z 781, m/z 779, and m/z 809 and interpreted as (epi)cat-ethyl-Dp3glc (24), (epi)cat-ethyl-Pn3glc (25), and (epi)cat-ethyl-Mv3glc (13), respectively. One compound with a mass signal at m/z 795 was attributed to (epi)cat-ethyl-Pt3glc. This compound had the same retention time as Mv3glc (9), so that its UV–visible spectrum was not available. Another peak (15) with a mass signal at m/z 707, presenting a λ_{max} of 506 nm characteristic of pyranoanthocyanins, was interpreted as carboxyl-pyrano-Mv3glc-cou. The lower polarity of carboxyl-pyrano-Mv3glc-cou as compared to other carboxyl-pyranoan-thocyanins eluted in fraction 1 is explained by the presence of the coumaric acid substituent also encountered in the native anthocyanins. Colorless anthocyanin-flavanol dimers linked by an A type bond (m/z 783) and the corresponding trimers (m/z 1071) and tetramer (m/z 1359) (**Figure 5A**) were detected in the XIC. One unknown compound (peak 26) was detected with a mass signal at 535 and with a fragment at m/z 331. This is the same fragmentation pattern as that of Mv3glc-ac, but the elution time under our chromatographic conditions was different from that of Mv3glc-ac. This unknown compound was earlier detected by Vidal and collaborators (*20*) who postulated that it might be an isoform with either a different OH group of the glucose esterified by acetic acid or a different sugar stereoisomer (*20*).

Fraction 4 was rich in derivatives of the ethyl-bridged group, as we detected four dimers made up with four of the five native anthocyanins. Ions at m/z 905 detected after thiolysis of fraction 4 were attributed to the colorless anthocyanin-O-flavanol benzylthioether derivative, reflecting the presence of larger colorless anthocyanin-O-(epi)cat$_n$ adducts ($n > 2$). Formation of this product confirms the resistance of the A type linkage to thiolysis. It may arise from the ions at m/z 1071 and m/z 1359, which correspond to an A type anthocyanin-flavanol dimer linked by a B bond to a terminal flavanol (m/z 1071) and to a flavanol dimer (m/z 1359), respectively, which disappeared after thiolysis.

Major pigments in fraction 5 (see **Table 2** and **Figure 7D**) consisted of the two acetylated anthocyanins that are disubstituted in the B ring (Pn3glc-ac, Cy3glc-ac). Fraction 5 also contained another pigment from the pyranoanthocyanin family, with a mass signal at m/z 677, which was attributed to carboxyl-pyrano-Pn3glc-cou. The main constituents of fraction 5 did not absorb in the visible range (data not shown) and were oligomeric procyanidins, namely, dimers (m/z 579) and trimers (m/z 867).

Fraction 5 was analyzed by HPLC/DAD/MS after thiolysis. All pigments (native and derived) were still present after thiolysis. Thiolysis allowed us to calculate mDP of fraction 5, which was estimated to be 3.44; this result is coherent with the presence of oligomeric procyanidins before thiolysis.

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The stationary phase (see **Table 2** and **Figure 7E**) contained the five coumaroylated anthocyanins along with trace amounts of the five anthocyanins glucosides. A compound (29) detected with a mass signal at m/z 955 and a λ_{max} at 550 was attributed to (epi)cat-ethyl-Mv3glc-cou; this compound was already detected in wine (*29*). All of these ions were still present after thiolysis.

Further experiments were performed on fraction 1 in order to investigate its composition and determine its color properties. Quantification of glucose after hydrolysis showed that fraction 1 contained 0.61 mmol/g of glucose, which thus represented 10% of its weight. The analysis performed without hydrolysis indicated that residual glucose accounted for 0.07 mmol/g (1% weight) of fraction 1. Assuming that the glucose can only be provided by anthocyanins, we can deduce that the first fraction contains 0.54 mmol/g of anthocyanin units, which corresponds to approximately 27% weight (270 mg/g), based on the molecular weight of Mv3glc.

The concentration of anthocyanins in the flavylium ion form was calculated from the absorbance at 520 nm. Prior calibration was done with a catechin solution and a Mv3glc solution to determine their absorption coefficients at 280 nm ($\epsilon_{cat} = 2994$ and $\epsilon_{Mv3glc} = 14886$) and at 520 nm for Mv3glc ($\epsilon_{Mv3glc} = 21018$), under the same conditions as used for fraction 1 (0.1 M HCl solution at pH 1, 1 M NaCl). The concentration of individual pigments detected by HPLC analysis of fraction 1 was also evaluated from their peak areas at 520 nm, using the response coefficient of Mv3glc.

The concentration of pigments under the flavylium form in fraction 1 was estimated to be 100 mg/g, which means that only 40% of the anthocyanins contribute, at pH 1, to the color and the remaining 60% are under colorless forms. In fact, anthocyanin structures detected in fraction 1 include genuine anthocyanins, pyranoanthocyanins, (epi)cat$_n$-anthocyanin adducts, ethyl-linked anthocyanin oligomers, anthocyanin oligomers, and A type anthocyanin-flavanol adducts (detected only after thiolysis). Among these compounds, the first three groups are known to be in the flavylium form at pH 1 (*30–32*) whereas the other three actually contain colorless anthocyanin units, in agreement with these data. The hydration pK calculated for Mv3glc-ethyl-Mv3glc is 1.81 (*33*) so that a small proportion of anthocyanin moieties are in the hemiketal form, even at pH 1. In the other two structures, the anthocyanin upper units are present either as flavene- or as flavan- 3-O-glucoside, which are both colorless.

The concentration of flavylium ions determined from the peaks in the HPLC profile represented only 11 mg equivalent Mv3glc per g of fraction 1, indicating that most of the pigment units were actually incorporated in unresolved larger molecular weight compounds. Detection of larger amounts of (epi)cat-anthocyanins and of anthocyanin-O-(epi)cat benzylthioether, after thiolysis of fraction 1, signals the presence of (epi)cat$_n$-anthocyanin and anthocyanin-O-(epi)cat$_n$ adducts, respectively, and is in agreement with this hypothesis.

The amount of flavanol units estimated by HPLC after thiolysis was 0.4 mmol/g (11.5% weight). However, parts of the flavanol units included in the polymeric structures are not released by thiolysis. This has been demonstrated in particular for flavanol units linked to anthocyanins encountered in (epi)-cat$_n$-anthocyanin adducts ($n > 2$) and anthocyanin-O-(epi)cat$_n$ adducts ($n > 2$), which are both present in fraction 1.

The concentration of flavan units (including colorless anthocyanins and flavanols) was estimated from the absorbance at 280 nm (A_{280}) using the following equations:

$$A_{280flavan} = A_{280} - A_{280flavylium}$$

$$A_{280flavylium} = (\epsilon_{280flavylium}/\epsilon_{520flavylium}) \times A_{520flavylium}$$

The concentration of flavan units estimated using the ϵ_{280} measured for catechin was 2.1 mmol of flavan units per gram of fraction 1. This calculation is based on the assumption that all flavan units have approximately the same molar extinction coefficient at 280 nm.

The molar amounts of the various types of units thus calculated (**Table 3**) indicate a molar ratio of three flavan-3-ol units to one anthocyanin. On the basis of the available data, flavanol units released by thiolysis were present mostly in upper position as encountered in (epi)cat$_n$-anthocyanin adduct. Nevertheless, they represented only about 25% of total flavanol units, the remaining units being involved in structures resistant to acid-catalyzed cleavage such as (epi)cat-anthocyanin adducts and A type anthocyanin-flavanol adducts. Oxidation products of flavanols were also shown to be resistant to thiolysis (*34*).

Spectrophotometric Measurements. UV−vis spectra of equilibrated solutions (12 h) of fraction 1 were recorded in buffers with pH values ranging from pH 1 to pH 7. The water soluble fraction reached maximum color expression at pH 1 and lost approximately 50% of the absorbance at 520 nm upon an increase in pH to pH 3.0 as shown in **Figure 8**. Two distinctive processes can be observed on this set of spectra, the first one prevailing in the pH range 1−3, and the second one appearing from pH 4 to pH 7. A continuous decrease of the visible band between pH 1 and pH 3.5 actually reflects the hydration of flavylium nuclei yielding the colorless hemiketal forms. Above pH 4, a new visible band appears in the 550−700 nm region, corresponding to the formation of quinoidal bases (neutral and anionic) by proton transfers. In contrast with native anthocyanins, the persistence of these species in equilibrated solutions indicates the high resistance of the corresponding flavylium nuclei toward hydration. In other words, this reflects the presence of derived pigments, which are particularly resistant to hydration. Among derived anthocyanins identified up to now, pyranoanthocyanins and ethyl-linked pigments were previously shown to be resistant to hydration although the origin of that resistance is probably different. Roughly, two kinds of pigments can be discriminated based on the set of spectra recorded in the pH range 1−7. The first type undergoes hydration similarly to native anthocyanins and includes, in particular, the (epi)cat$_n$-anthocyanin pigments, for which color properties have already been investigated (*32*). The other class of pigments is characterized by its high resistance toward hydration, which is actually a feature of pyranoanthocyanins and ethyl-bridged anthocyanin dimers detected in fraction 1.

An increase of the absorbance between 370 and 450 nm was observed at the higher pH values (6−7); this increase might be due to the oxidation of the flavanol units present in fraction 1 during the equilibration time (12 h).

Table 3. Molar Amounts of Units Present in Fraction 1

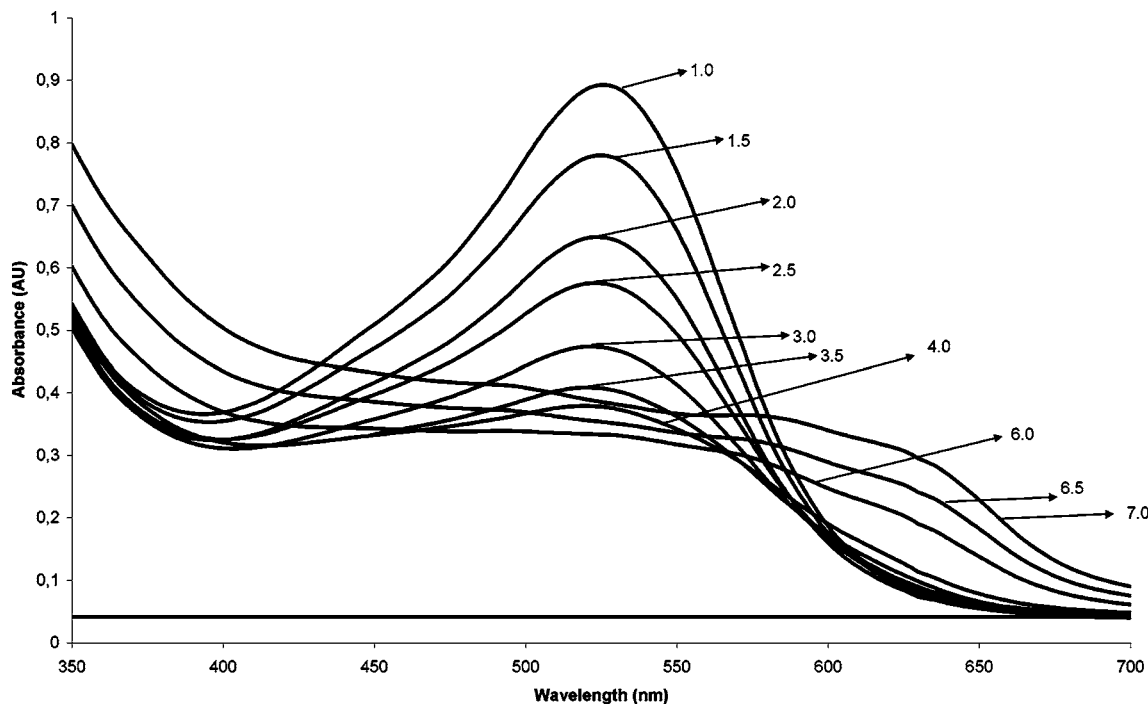|		mmol/g in fraction 1
1	total flavan (flavan-3-ol and flavan-3-O-glucoside) (Abs$_{280}$)	2.1
2	colored flavylium anthocyanin (Abs$_{520}$)	0.2
3	total anthocyanin (based on glucose quantification)	0.54
4	tannins (after thiolysis)	0.4
5	colorless anthocyanins (3-2)	0.34
6	total flavan-3-ol (1–5)	1.76

Figure 8. UV–vis spectra of fraction 1 at various pH values.

Sulfite Bleaching. The effect of SO_2 on fraction 1 was studied at pH 2.5 and 3.5, the latter being within the wine pH range. Sulfite bleaching is caused by the addition of hydrogen sulfite to the C ring of the flavylium cation, generating a colorless hydrogen sulfite adduct (*35, 36*). This process is reversible in acidic medium.

The more diluted SO_2 solution was used to mimic enological concentration, and the more concentrated one was used to be sure of SO_2 saturation and bleaching completion of the C4 unsubstituted pigments. As can be seen in **Figure 9**, Mv3glc solution lost around 20% of its color at enological concentration of SO_2 and almost 100% with the high SO_2 concentration. In fraction 1 at pH 2.5, 50% of the color remained after SO_2 bleaching (concentrated solution) although the flavylium concentration was estimated to be half of the concentration of the Mv3glc solution. Therefore, it can be concluded that this fraction is constituted by 50% of sulfite bleaching resistant pigments, some of them being pyranoanthocyanins as demonstrated by Sarni et al. (*31*) and ethyl-linked flavanol-anthocyanins dimers as described by Escribano-Bailon et al. (*37*). At pH 3.5, Mv3glc bleaching experiment was not carried out since the anthocyanin solution would already have been largely discolored by the prevalence of colorless hemiketal forms (approximate 89%) at this pH. For fraction 1, the absorbance at 520 nm is lower (35%) than the absorbance value at pH 2.5, indicating that a part of the pigments underwent hydration. Sixty percent of the color resisted sulfite bleaching, but the absolute value of absorbance is, however, lower than that at pH 2.5, showing that the SO_2 pigment adduct is formed more easily and is also more stable (pH effect on the sulfite addition equilibrium) with the higher pH. Besides, the higher percentage of sulfite bleaching resistance reflects that at pH 3.5 there is actually less colored flavylium forms, presumably because a larger proportion of anthocyanin units is in the hydrated form (**Figure 8**). Additionally, fraction 1 showed an increased resistance to sulfite bleaching in comparison to the crude wine polyphenolic extract.

In conclusion, HSCCC has already proved to be a powerful tool for large-scale isolation of anthocyanins (*2*). It is also an excellent way of prefractionation, instead of classical gel
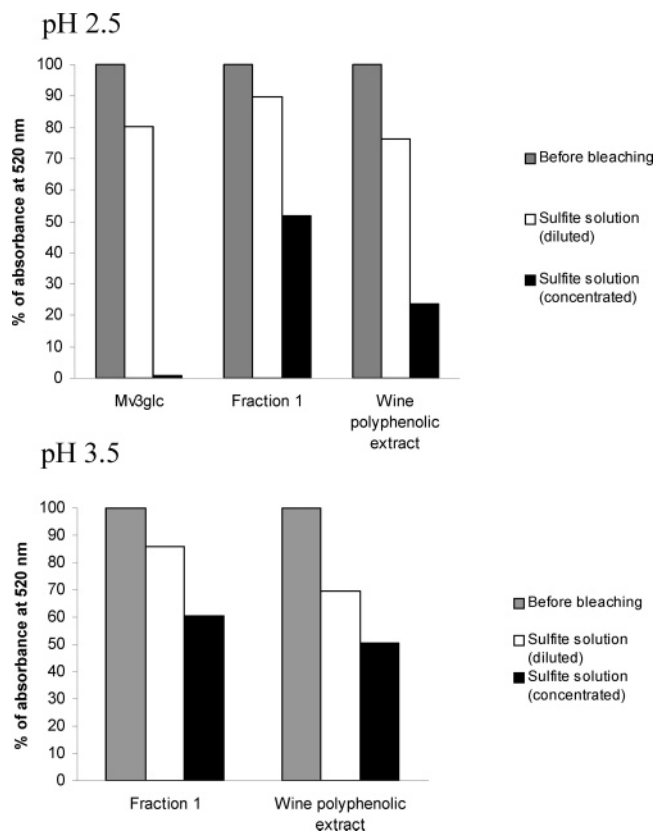

Figure 9. Absorbance values at 520 nm for fraction 1, Mv3glc, and the wine polyphenolic extract before and after sulfite bleaching.

chromatographic techniques or preparative HPLC, which are time consuming.

The different anthocyanin-derived pigments were separated in different families according to their structures. The pyranoanthocyanins and anthocyanin dimers were concentrated in fraction number 1; the ethyl-bridged flavanol anthocyanin dimers were concentrated in fraction 4. The direct flavanol-anthocyanin dimers were detected in fractions 2 and 3. Moreover, acylation

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of the glucose by *p*-coumaric acid decreased the polarity of the compounds and delayed the elution order of all corresponding anthocyanins (native and derivative).

HSCCC enables the recovery of the water soluble pigments in a single fraction (fraction 1), which is still too complex to be fully characterized. Nevertheless, thiolysis provided information on its composition, demonstrating the presence of anthocyanin-O-(epi)cat$_n$, (epi)cat$_n$-ethyl-anthocyanin, and (epi)cat$_n$-anthocyanin adducts ($n > 2$). All of these results suggest the polydisperse oligomeric nature of fraction 1. Glucose quantification and absorbance measurements also allowed a partial description of the composition of fraction 1, which was estimated to have a molar ratio of three flavan-3-ol units to one anthocyanin.

ACKNOWLEDGMENT

LITERATURE CITED

(1) Degenhardt, A.; Hofmann, S.; Knapp, H.; Winterhalter, P. Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. *J. Agric. Food Chem.* **2000**, *48*, 5812−5818.

(2) Schwarz, M.; Hillebrand, S.; Habben, S.; Degenhardt, A.; Winterhalter, P. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochem. Eng. J.* **2003**, *14*, 179−189.

(3) Ito, Y. Recent advances in counter-current chromatography. *J. Chromatogr. A* **1991**, *538*, 3−25.

(4) Conway, W. D. Counter-current chromatography. *J. Chromatogr. A* **1991**, *538*, 27−35.

(5) Salas, E.; Poncet-Legrand, C.; Fulcrand, H.; Meudec, E.; Kölher, N.; Winterhalter, P.; Cheynier, V. *Pigments in Food More Than Colours*; Quimper, France; Vol. Pigments in Food, pp 390−392.

(6) Schwarz, M.; Quast, P.; Baer, D. v.; Winterhalter, P. Vitisin A content in Chilean wines from *Vitis vinifera* cv. Cabernet Sauvignon and contribution to the color of aged red wines. *J. Agric. Food Chem.* **2003**, *51*, 6261−6267.

(7) Atanasova, V. Réactions des composés phénoliques induites dans les vins rouges par la technique de micro-oxygénation. Caractérisation de nouveaux produits de condensation des anthocyanes avec l'acétaldéhyde. Thesis, ENSAM, 2003.

(8) Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

(9) Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. A method for the analysis of sugars in plant cell-wall polysaccharides by gas−liquid chromatography. *Carbohydr. Res.* **1967**, *5*, 340−345.

(10) Salas, E.; Atanasova, V.; Poncet-Legrand, C.; Meudec, E.; Mazauric, J. P.; Cheynier, V. Demonstration of the occurrence of flavanol-anthocyanin adducts in wine and in model solutions. *Anal. Chim. Acta* **2004**, 325−332.

(11) Vivar-Quintana, A.; Santos-Buelga, C.; Rivas-Gonzalo, J. Anthocyanin-derived pigments and colour of red wines. *Anal. Chim. Acta* **2002**, *458*, 147−155.

(12) Salas, E.; Fulcrand, H.; Meudec, E.; Cheynier, V. Reactions of anthocyanins and tannins in model solutions. *J. Agric. Food Chem.* **2003**, *51*, 7951−7961.

(13) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments yielded by reactions between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401−1407.

(14) Bakker, J.; Bridle, P.; Honda, T.; Kuwano, H.; Saito, N.; Terahara, N.; Timberlake, C. F. Identification of an anthocyanin occurring in some red wines. *Phytochemistry* **1997**, *44*, 1375−1382.

(15) Timberlake, C. F.; Bridle, P. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. *Am. J. Enol. Vitic.* **1976**, *27*, 97−105.

(16) Cheynier, V.; Fulcrand, H.; Sarni, P.; Moutounet, M. Application des techniques analytiques à l'étude des composés phénoliques et de leurs réactions au cours de la vinification. *Analusis* **1997**, *25*, M14−M21.

(17) Atanasova, V.; Fulcrand, H.; Cheynier, V.; Moutounet, M. Effect of oxygenation on polyphenol changes occurring in the course of wine making. *Anal. Chim. Acta* **2002**, *458*, 15−27.

(18) Es-Safi, N.; Fulcrand, H.; Cheynier, V.; Moutounet, M. Studies on the acetaldehyde-induced condensation of (−)-epicatechin and malvidin 3-O-glucoside in a model solution system. *J. Agric. Food Chem.* **1999**, *47*, 2096−2102.

(19) Lee, D. F.; Swinny, E. E.; Jones, G. P. NMR identification of ethyl-linked anthocyanin-flavanol pigments formed in model wine ferments. *Tetrahedron Lett.* **2004**, *45*, 1671−1674.

(20) Vidal, S.; Hayasaka, Y.; Meudec, E.; Cheynier, V.; Skouroumounis, G. Fractionation of grape anthocyanin classes using multilayer coil countercurrent chromatography with step gradient elution. *J. Agric. Food Chem.* **2004**, *52*, 713−719.

(21) Vidal, S.; Meudec, E.; Cheynier, V.; Skouroumounis, G.; Hayasaka, Y. Mass spectrometric evidence for the existence of oligomeric anthocyanins in grape skins. *J. Agric. Food Chem.* **2004**, *52*, 7144−7151.

(22) Somers, T. C. The polymeric nature of wine pigments. *Phytochemistry* **1971**, *10*, 2175−2186.

(23) Atanasova, V.; Fulcrand, H.; Le Guerneve, C.; Cheynier, V.; Moutounet, M. Structure of a new dimeric acetaldehyde malvidin 3-glucoside condensation product. *Tetrahedron Lett.* **2002**, *43*, 6151−6153.

(24) Remy, S.; Fulcrand, H.; Labarbe, B.; Cheynier, V.; Moutounet, M. First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. *J. Sci. Food Agric.* **2000**, *80*, 745−751.

(25) Remy-Tanneau, S.; Guerneve, C. L.; Meudec, E.; Cheynier, V. Characterization of a colorless anthocyanin-flavan-3-ol dimer containing both carbon−carbon and ether interflavanoid linkages by NMR and mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3592−3597.

(26) Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, D. J. N. Plant proanthocyanidins. Part I. Introduction: The isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1387−1399.

(27) Rigaud, J.; Perez-Ilzarbe, X.; RicardodaSilva, J. M.; Cheynier, V. Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr.* **1991**, *540*, 401−405.

(28) Le Roux, E.; Doco, T.; Sarni-Manchado, P.; Lozano, Y.; Cheynier, V. Characterization of A-type proanthocyanidins from pericarp of litchi (*Litchi sinensis* Sonn). *Phytochemistry* **1998**, *48*, 1251−1258.

(29) Mateus, N.; de Pascual-Teresa, S.; Rivas-Gonzalo, J.; Santos-Buelga, C.; De Freitas, V. Structural diversity of anthocyanin-derived pigments in port wines. *Food Chem.* **2002**, *76*, 335−342.

(30) Brouillard, R.; Delaporte, B. Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton transfer, hydration, and tautomeric reactions of malvidin 3-glucoside. *J. Am. Chem. Soc.* **1977**, *99*, 8461−8468.

(31) Sarni-Manchado, P.; Fulcrand, H.; Souquet, J.-M.; Cheynier, V.; Moutounet, M. Stability and color of unreported wine anthocyanin-derived pigments. *J. Food Sci.* **1996**, *61*, 938−941.

(32) Salas, E.; Le Guernevé, C.; Fulcrand, H.; Poncet-Legrand, C.; Cheynier, V. Structure determination and color properties of a newly synthesized direct-linked flavanol-anthocyanin dimer. *Tetrahedron Lett.* **2004**, *45*, 8725−8729.

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(33) Atanosova, V.; Fulcrand, H.; Le Guernevé, C.; Dangles, O.; Cheynier, V. First evidence of acetaldehyde-induced anthocyanin polymerisation. *Polyphenol Commun.* **2002**, *2*, 417−418.

(34) Guyot, S.; Vercauteren, J.; Cheynier, V. Colourless and yellow dimers resulting from (+)-catechin oxidative coupling catalysed by grape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279−1288.

(35) Berké, B.; Chèze, C.; Vercauteren, J.; Deffieux, G. Bisulfite addition to anthocyanins: Revisited structures of colourless adducts. *Tetrahedron Lett.* **1998**, *39*, 5771−5774.

(36) Timberlake, C.; Bridle, P. Flavylium salts, anthocyanidins and anthocyanins. II. Reactions with sulphur dioxide. *J. Sci. Food Agric.* **1967**, *18*, 473−478.

(37) Escribano-Bailon, T.; Alvarez-Garcia, M.; Rivas-Gonzalo, J. C.; Heredia, F. J.; Santos-Buelga, C. Color and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin-3-O-glucoside and (+)-catechin. *J. Agric. Food Chem.* **2001**, *49*, 1213−1217.