

Ethylidene-Bridged Flavan-3-ols in Red Wine and Correlation with Wine Age

JESSICA DRINKINE,[†] PAULO LOPES,[†] JAMES A. KENNEDY,[‡]
 PIERRE-LOUIS TEISSEDE,[†] AND CEDRIC SAUCIER*[†]

Faculté d'Oenologie de Bordeaux, Université Victor Segalen Bordeaux 2, UMR 1219 INRA ISVV,
 351 Cours de la Libération, 33405 Talence Cedex, France, and Department of Food Science and
 Technology, Oregon State University, 220A Wiegand Hall, Corvallis, Oregon 97331-6602

Condensed tannins are responsible for astringency and bitterness and participate in the color stability of red wines. During wine making and aging, they undergo chemical changes including, for example, acetaldehyde-induced polymerization. Following this study, the ethylidene-bridged flavan-3-ols were monitored in different vintage wines made from grapes collected in the same vineyard in three wineries in Bordeaux, Pauillac, and Saint Julien. Flavan-3-ol ethylidene bridges were quantified by wine 2,2'-ethylidenediphloroglucinol (EDP) phloroglucinolysis. This method was based upon the analysis of EDP, a product formed after acid-catalyzed cleavage of wine flavan-3-ols in the presence of excess phloroglucinol. The flavan-3-ol ethylidene bridges were then compared to flavan-3-ol contents (phloroglucinolysis), phenolic contents, and color measurements. Low amounts of flavan-3-ol ethylidene bridges (0.8–2.5 mg L⁻¹) were quantified in wines. Flavan-3-ol ethylidene bridges represent less than 4% of flavan-3-ol bonds, but the proportion of these linkages relative to native interflavan bonds increased with wine age. This proportion correlated with pigmented polymers.

KEYWORDS: Tannin; flavan-3-ol, ethylidene bridge; red wine; aging; EDP; phloroglucinolysis

INTRODUCTION

Phenolic compounds play an important role in wine quality because of their color and taste properties. Condensed tannins influence bitterness and astringency (1, 2) and are involved in wine colloidal and color stability (3–6). In the grape, they exist as polymers of flavan-3-ol units [(+)-catechin, (-)-epicatechin, (-)-epigallocatechin, and (-)-epicatechin-3-O-gallate] with C4–C6 or C4–C8 linkages (7).

During wine making and aging, condensed tannins undergo enzymatic or chemical modifications and many of these modifications result in the formation of new interflavanoid linkages. One such modification is the acid-catalyzed cleavage of the original plant-derived interflavanoid bond and subsequent condensation reaction (4, 8, 9). Another type of modification is a nucleophilic substitution reaction involving the C6 or C8 of the A ring of multiple flavan-3-ol units (10). Acetaldehyde has been identified as one of the most important electrophiles in this type of bridging reaction (3, 11, 12). It may be produced in two ways: production by yeasts (e.g., *Saccharomyces cerevisiae*) (13) and the oxidation of ethyl alcohol (14). To date, only dimers and trimers of ethylidene-bridged flavan-3-ols (FI) have been identified in wine (15) and a previous study has shown that it is possible to quantify them directly in wine (16).

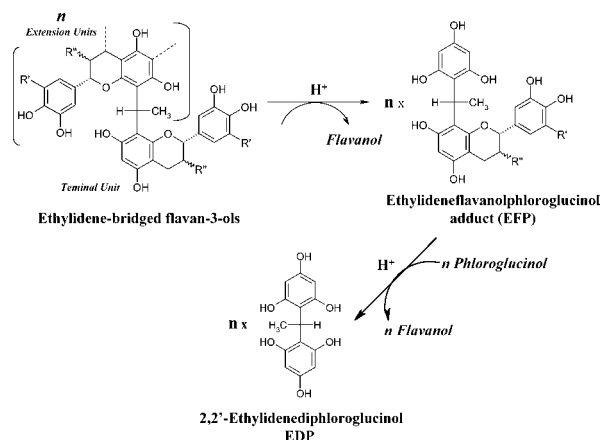


Figure 1. Reaction pathway of phloroglucinolysis of ethylidene-bridged FI leading to EDP. Procyanidins, R' = H and R'' = OH; prodelpinidins, R' = OH and R'' = OH or O-gallate.

The method was based upon the analysis of the ethylidene-diphloroglucinol (EDP), a product formed after acid-catalyzed cleavage of FI in the presence of excess phloroglucinol (Figure 1). To date, no study has determined the amounts of flavan-3-ol ethylidene bridges (CH–CH₃) as a function of wine age.

The purpose of this investigation was to quantify CH–CH₃ in several different aged red wines and compare them to native interflavanoid bonds of FI. To estimate the importance of

* To whom correspondence should be addressed. Fax: 33-5-40-00-6468.
 E-mail: saucier@oenologie.u-bordeaux2.fr.

[†] Université Victor Segalen Bordeaux 2.

[‡] Oregon State University.

acetaldehyde in wine aging, the amounts of CH—CH₃ were compared to phenolic quantities and color measurements.

MATERIALS AND METHODS

Chemicals. Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA). Acetonitrile [high-performance liquid chromatography (HPLC) grade] was obtained from Fischer Chemicals (Elancourt, France); ethyl alcohol (HPLC grade) was from Carlo Erba (Val de Reuil, France); acetaldehyde (RP) was from Riedel-De Haën (Val de Reuil, France); and methyl alcohol (HPLC grade), acetic acid (RP), L-ascorbic acid, L-tartaric acid, hydrochloric acid, and sodium acetate were from ProLabo-VWR (Fontenay s/Bois, France). (+)-Catechin, (–)-epicatechin, (–)-epigallocatechin, and (–)-epicatechin-3-*O*-gallate were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Phloroglucinol and 4-methylcatechol (4MC) were purchased from Fluka (Saint Quentin Fallavier, France).

Wines. This study was carried out with 21 Bordeaux red wines. The grapes were collected in the same vineyard from three different wineries from different labels: Bordeaux, Pauillac, and Saint Julien. The vintages used were as follows: Bordeaux 2004, 1999, 1995, 1991, 1990, 1988, and 1985; Pauillac 2001, 2000, 1999, 1998, 1994, 1992, and 1984; and Saint Julien 2002, 2000, 1999, 1996, 1993, 1983, and 1981.

Wine EDP Phloroglucinolysis Method. CH—CH₃ were quantified by liquid chromatography–mass spectrometry (LC-MS) as previously described (16). The extractions and analysis were realized in triplicate.

The wine was purified and concentrated using C₁₈ solid-phase extraction as follows: 5 mL of wine was diluted in 15 mL of water. By 10 mL, the sample was applied on the column after conditioning. The column was washed with 50 mL of water and eluted with 50 mL of methyl alcohol. The fraction was dried under reduced pressure and then dissolved in 2 mL of methyl alcohol. A solution of 0.2 N HCl in methyl alcohol, containing 100 g L⁻¹ phloroglucinol and 20 g L⁻¹ ascorbic acid, was prepared. One hundred microliters of wine sample dissolved in methyl alcohol was reacted with 100 μL of the phloroglucinol reagent at 50 °C for 20 min and then combined with 200 μL of 400 mM aqueous sodium acetate to stop the reaction. Twenty microliters of 500 mg L⁻¹ aqueous 4MC was then added as an internal standard.

Specific ionization masses, *m/z* 277 (EDP) and *m/z* 123 (4MC), were recorded. EDP quantification was accomplished using the EDP/4MC peak areas after calibration and considering the sample dilution (1.68). The column used was a reversed-phase C₁₈ Waters Xterra protected with a guard column of the same material (3.5 μm packing, 4.6 mm × 100 mm i.d.) (Agilent, Saint Quentin-en-Yvelines, France). Solvent A was water/acetic acid (95:5, v/v), and solvent B was acetonitrile. Elution was conducted at room temperature, and the sample loop was 20 μL. The elution gradient was as follows: 10% B for 2 min, from 10 to 50% B in 8 min, from 50 to 100% B in 1 min, 100% B for 4 min, from 100 to 10% B in 1 min, 10% B for 4 min with a 1 mL min⁻¹ flow for the column, and 0.1 mL min⁻¹ for the MS source. The recovery of the ethylidene-bridged FI was determined at 90% for concentrations up to 4.5 mg L⁻¹ of ethylidene-bridged linkages, and it decreased to 83% above and until the concentration reached 7.6 mg L⁻¹ (16).

Mean Degree of Polymerization (mDP) and FI Analyses. The proanthocyanidin mDP and FI concentrations were quantified by phloroglucinolysis (16). The analyses were realized in triplicate.

The wine was purified and concentrated similar to that for wine EDP phloroglucinolysis. A solution of 0.2 N HCl in methyl alcohol, containing 100 g L⁻¹ phloroglucinol and 20 g L⁻¹ ascorbic acid, was prepared. One hundred microliters of wine sample was reacted with 100 μL of the phloroglucinol reagent at 50 °C for 20 min and then combined with 1000 μL of 40 mM aqueous sodium acetate to stop the reaction.

The proanthocyanidin composition was determined by phloroglucinolysis and LC-MS analysis at 280 nm after peak identification and calibration. The same elution conditions were used as for the wine EDP phloroglucinolysis but using water:acetic acid (99:1; v/v) as solvent A, methyl alcohol as solvent B, and the following elution gradient: 5% B for 25 min, from 5 to 20% B in 20 min, from 20 to 40% B in

25 min, from 40 to 100% B in 1 min, 100% B for 9 min, from 100 to 5% B in 1 min, 10% B for 4 min with a 1 mL min⁻¹ flow for the column, and 0.1 mL min⁻¹ for the MS source.

Proportion of Ethylidene Bridges Relative to Native Interflavan Bonds. Considering that 1 mol of EDP represents 1 mol of ethylidene bridges and that 1 mol of extension unit represents 1 mol of native bond, the proportion of ethylidene bridges relative to native interflavan bonds was calculated as the ratio, in mol, [EDP]/[flavan-3-ol extension units, FI_{ext}].

Color Parameters. The absorbances at 420 (d420), 520 (d520), and 620 nm (d620) were measured under 10 mm optical way. The color intensity (CI, sum of the three absorbances), the tint (d420/d520), and the components yellow (d420%), red (d520%), and blue (d620%) were estimated using the method described by Glories (18).

Phenolic Compounds. The total polyphenols index (TPI) was determined by measuring the absorbance at 280 nm under 1 mm optical way after 1/10 wine dilution. The absorbance was multiplied by 100 to give TPI (19).

Total proanthocyanidins (Tan) were estimated according to Ribéreau-Gayon and Stonestreet (20). The wine was diluted to 1/50 in water. One milliliter of the previous sample, 0.5 mL of water, and 1.5 mL of 12 N HCl were mixed. One of them was heated for 30 min in boiled water and cooled (sample S), while the last one stayed at room temperature (sample B). A 0.25 mL amount of 95% ethyl alcohol was added. The absorbance at 550 nm was read under 10 mm optical way and Tan = 19.33(abs_{550nm}S - abs_{550nm}B). Analyses were realized in triplicate.

Anthocyanins (Anthos) were determined using the SO₂ bleaching method (21). A solution "A" was prepared as follows: 1 mL of wine, 1 mL of 0.1% HCl ethyl alcohol, and 20 mL of 2% HCl. All analyses were realized in triplicate. Blank (B) was realized as follows: 2 mL of solution "A" and 0.8 mL of water. Sample (S) was prepared as follows: 2 mL of solution "A", 0.4 mL of water, and 0.4 mL of HNaSO₃. After 20 min at room temperature, the absorbance at 520 nm was measured under 10 mm optical way. Anthos were calculated as 875(abs_{520nm}B - abs_{520nm}S). Analyses were realized in triplicate.

Pigmented polymers (PP) corresponded to pigments resistant to SO₂ bleaching and were estimated according to Glories (22). Two samples were prepared to calculate IC (SO₂) and IC (H₂O). The first one was prepared as follows: 0.5 mL of wine, 4.5 mL of model wine (12% ethyl alcohol, 5 g L⁻¹ tartaric acid, pH 3.2), and 20 μL of Na₂O₃S. After 5 min, absorbances at 420 and 520 nm were read under 10 mm optical way: IC(SO₂) = abs_{420nm} + abs_{520nm}. The second one was prepared as follows: 0.5 mL of wine, 4.5 mL of model wine, and 20 μL of water; IC(SO₂) = abs_{420nm} + abs_{520nm}. PP = IC(SO₂)/IC(H₂O).

LC-MS and HPLC Apparatus and Absorbance Measurements. LC-MS analyses were performed on a Micromass Platform II simple quadrupole mass spectrometer (Micromass-Beckman, Roissy Charles-de-Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in negative-ion mode. The source temperature was 120 °C, the capillary voltage was set at ±3.5 kV, and the cone voltage was -30 V. HPLC separations were performed on a Hewlett-Packard 1100 series (Agilent, Massy, France) including a pump module and a UV detector. Both systems were operated using Masslynx 3.4 software. The absorbance was recorded at 280 nm, and mass spectra were recorded from 50 to 1500 amu. Absorbance measurements were made with an Uvicon 922 spectrophotometer (Kontron Instrument, Milan, Italia) and were converted to absorbance.

Statistical Analyses. All data were treated by one-way analysis of variance (ANOVA) and principal component analysis (PCA) using the Statistica 6.0 software (StatSoft Inc., Tulsa, OK).

RESULTS

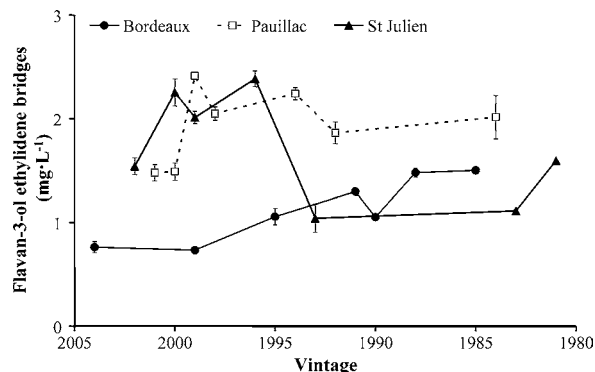
The amounts of CH—CH₃, the FI content, phenolic quantities, and color measurements were carried out in three series of seven red wines of different vintages and origins: Bordeaux, Pauillac, and Saint Julien.

CH—CH₃ and FI Content. The CH—CH₃ were quantified using the wine EDP phloroglucinolysis method (16). Low

Table 1. Statistical Results Carried out by One-Way ANOVA on CH-CH₃ and FI Contents in 21 Bordeaux Red Wines Issued from Three Different Origins (\pm SD, $N = 3$)^a

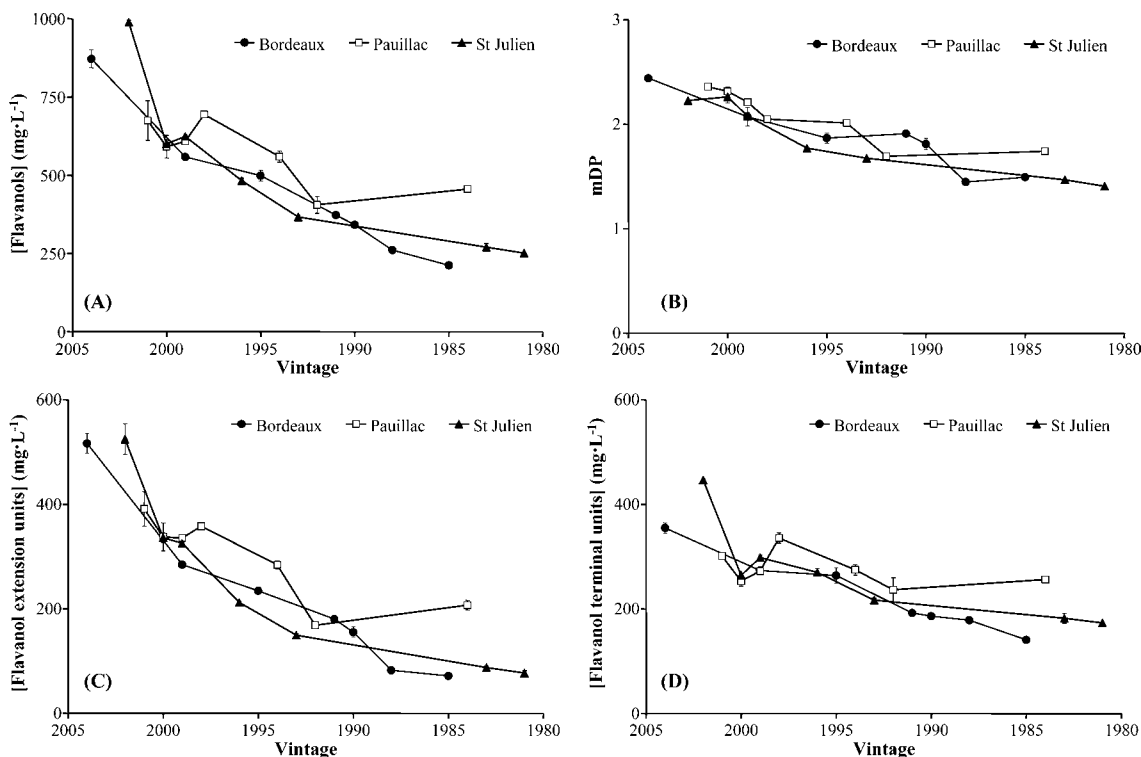
	Bordeaux	Pauillac	St. Julien	all
CH-CH ₃	***	***	***	***
FI _{term}	***	***	***	***
FI _{ext}	***	***	***	***
FI	***	***	***	***
mDP	***	***	***	***

^a NS, nonsignificant; * = 0.01 < p < 0.05; and *** = p < 0.01.

**Figure 2.** Evolution during wine age of the concentration of CH-CH₃, determinate by EDP phloroglucinolysis, in the 21 Bordeaux red wine series. CH-CH₃ = ethylidene-bridged linkages (\pm SD, $N = 3$).

amounts (0.8–2.5 mg L⁻¹) of CH-CH₃ were quantified in wines. Although the CH-CH₃ were significantly different ($p < 0.01$) for each wine series (Table 1), no statistical correlations existed between wine age and CH-CH₃ (Figure 2).

Independent of origin, FI concentrations declined with wine age ($p < 0.01$) (Figure 3A). FI concentrations were four times lower in the oldest Bordeaux (1985) and Saint Julien (1981) wines as compared to the youngest wines (2004 and 2002,

**Figure 3.** Evolution during wine age of mDP and FI contents in the 21 Bordeaux red wine series (\pm SD, $N = 3$).

respectively). This ratio was only 1.5 in the 1984 Pauillac wine as compared to the 2001 vintage. Following the FI decrease, the concentrations of flavan-3-ol terminal units (FI_{term}) and FI_{ext} were lower in the oldest wines (Figure 3C,D). The FI loss mainly affected the extension units and was correlated with a decrease in mDP (Figure 3B): mDP was between 2.2 and 2.4 for the youngest wines and between 1.4 and 1.7 for the oldest. The statistical results carried out by one-way ANOVA are summarized in Table 1.

The percentage of CH-CH₃ as compared to native interflavan bonds was also studied. It was calculated assuming that 1 mol of extension unit corresponded to 1 mol of native interflavan bonds. The proportion of ethylidene bridges generally increased with wine age (Figure 4).

Color Parameters and Phenolic Compounds. Classical color and phenolic analyses were performed on the wines. These results are summarized in Table 2, and statistical results were carried out by one-way ANOVA in Table 3. For the main parameters analyzed, bottle aging had the same effect on the wines of the three series.

The results showed that the total polyphenols index (TPI) and Tan were significantly lower in the oldest wines when compared to the youngest wines ($p < 0.01$). Nevertheless, these effects were limited to the TPI decrease in the Pauillac series and for the Tan decrease in the Bordeaux series. Moreover, the concentration of Anthos decreased while PP increased with wine age.

These chemical differences in the polyphenol contents implied color change; with an increase in wine age, and consistent with this, CIs were lower and the hue higher (d520% lower and d420% higher). The 620 nm color component did not seem to be affected by wine age.

PCA. PCA was applied to the 21 wines based on the 13 previously reported parameters as follows: CH-CH₃, FI, CH-CH₃/FI_{ext}, mDP, TPI, Tan, Anthos, PP, CI, Hue, d620%, d520%, and d420%. Seven components have eigenvalues higher

Table 2. Color Parameters and Phenolic Compounds in 21 Bordeaux Red Wines Issued from Three Different Origins (\pm SD, $N = 3$)

wine	CI	hue	d620 (%)	d520 (%)	d420 (%)	TPI	Tan (g L^{-1})	Anthos (mg L^{-1})	PP (%)
Bordeaux									
2004	0.9 \pm 0.1	0.7 \pm 0.1	12 \pm 1	51 \pm 2	37 \pm 2	49 \pm 2	1.4 \pm 0.1	344 \pm 3	56 \pm 1
1999	0.7 \pm 0.1	1.0 \pm 0.1	12 \pm 1	43 \pm 2	44 \pm 2	44 \pm 1	1.4 \pm 0.2	80 \pm 6	81 \pm 2
1995	0.7 \pm 0.1	1.1 \pm 0.1	12 \pm 1	42 \pm 2	46 \pm 2	48 \pm 1	1.5 \pm 0.1	55 \pm 6	86 \pm 4
1991	0.6 \pm 0.1	1.2 \pm 0.1	12 \pm 0	41 \pm 2	48 \pm 2	35 \pm 1	1.2 \pm 0.1	31 \pm 2	92 \pm 3
1990	0.8 \pm 0.1	1.2 \pm 0.1	12 \pm 0	41 \pm 2	47 \pm 2	41 \pm 1	1.4 \pm 0.1	48 \pm 16	87 \pm 8
1988	0.6 \pm 0.1	1.2 \pm 0.1	11 \pm 0	41 \pm 2	48 \pm 2	36 \pm 1	1.1 \pm 0.1	30 \pm 3	91 \pm 1
1985	0.6 \pm 0.1	1.3 \pm 0.1	12 \pm 0	38 \pm 2	50 \pm 2	36 \pm 1	1.2 \pm 0.1	30 \pm 2	94 \pm 3
Pauillac									
2001	0.9 \pm 0.1	0.8 \pm 0.1	13 \pm 1	48 \pm 2	39 \pm 2	52 \pm 1	4.7 \pm 0.1	167 \pm 1	77 \pm 2
2000	1.3 \pm 0.1	0.9 \pm 0.1	14 \pm 1	46 \pm 2	40 \pm 2	56 \pm 1	5.1 \pm 0.1	163 \pm 12	84 \pm 8
1999	0.9 \pm 0.1	0.9 \pm 0.1	12 \pm 1	46 \pm 2	42 \pm 2	52 \pm 0	5.1 \pm 0.1	110 \pm 1	81 \pm 2
1998	1.1 \pm 0.1	0.9 \pm 0.1	13 \pm 1	46 \pm 2	41 \pm 2	56 \pm 1	5.7 \pm 0.1	125 \pm 9	80 \pm 3
1994	1.0 \pm 0.1	1.0 \pm 0.1	12 \pm 1	45 \pm 2	44 \pm 2	54 \pm 1	3.9 \pm 0.2	82 \pm 1	88 \pm 3
1992	0.8 \pm 0.1	1.1 \pm 0.1	12 \pm 1	42 \pm 2	46 \pm 2	47 \pm 1	3.1 \pm 0.1	59 \pm 4	90 \pm 3
1984	0.7 \pm 0.1	1.2 \pm 0.1	11 \pm 0	41 \pm 2	48 \pm 2	51 \pm 1	3.2 \pm 0.2	34 \pm 2	90 \pm 2
St. Julien									
2002	1.3 \pm 0.1	0.8 \pm 0.1	12 \pm 1	49 \pm 2	39 \pm 2	71 \pm 1	4.2 \pm 0.1	328 \pm 2	72 \pm 1
2000	1.4 \pm 0.1	0.8 \pm 0.1	13 \pm 1	48 \pm 2	39 \pm 2	65 \pm 0	4.3 \pm 0.2	242 \pm 3	77 \pm 1
1999	1.4 \pm 0.1	0.8 \pm 0.1	12 \pm 1	48 \pm 2	40 \pm 2	65 \pm 1	4.5 \pm 0.1	152 \pm 4	83 \pm 1
1996	1.2 \pm 0.1	0.9 \pm 0.1	12 \pm 1	47 \pm 2	41 \pm 2	59 \pm 1	4.1 \pm 0.2	94 \pm 4	88 \pm 1
1993	1.1 \pm 0.1	1.0 \pm 0.1	11 \pm 1	45 \pm 2	43 \pm 2	53 \pm 1	3.6 \pm 0.2	60 \pm 2	89 \pm 1
1983	0.9 \pm 0.1	1.1 \pm 0.1	11 \pm 0	43 \pm 2	46 \pm 2	51 \pm 1	3.3 \pm 0.2	41 \pm 1	93 \pm 1
1981	0.8 \pm 0.1	1.1 \pm 0.1	10 \pm 0	43 \pm 2	47 \pm 2	49 \pm 1	3.4 \pm 0.2	35 \pm 1	90 \pm 1

than 1. The first two principal components explained 82.7% of the variance.

Figure 5A shows the contribution of the variable to the first two axes. The first axis (factor 1) represented 65.6% of the total variance. It contrasted hue, d420%, concentration of PP, and proportion of CH-CH₃ (CH-CH₃/Fl_{ext}) with concentrations of Anthos, Fl, mDP, d520%, TPI, and CI. Hue and d420% were both highly positively correlated and to a lesser extent to PP and CH-CH₃/Fl_{ext}. Fl, mDP, and Anthos were highly correlated between them and to a lesser extent to d520%, TPI, and CI. Tan and d620% did not really contribute to the first axis; their variable factor coordinates were only -0.61 and -0.63, respectively. Ethylidene bridges (CH-CH₃) and to a lesser extent Tan were positively correlated and contributed to the second axis (17.1%).

Figure 5B shows the distribution of wine samples in the plane defined by the two components. Three groups, distinguishing the wines as a function of their series, could be observed. Bordeaux wines were very scattered while the Pauillac wines were less so. Moreover, Pauillac wines were clustered within the Saint Julien wines while Bordeaux wines were distinct and separate. Within each group, wines were clearly separated as a

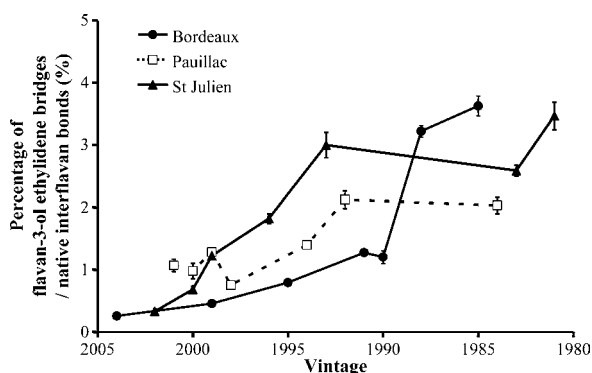


Figure 4. Evolution during wine age of the percentage (in %) of CH-CH₃ over native interflavan bonds in the three Bordeaux red wine series (\pm SD, $N = 3$).

Table 3. Statistical Results Carried out by One-Way ANOVA on the Color Parameters and Phenolic Compounds in 21 Bordeaux Red Wines Issued from Three Different Origins (\pm SD, $N = 3$)^a

	Bordeaux	Pauillac	St. Julien	all
CI	***	***	***	***
hue	***	***	*	***
d620 (%)	NS	***	***	***
d520 (%)	***	***	***	***
d420 (%)	***	***	***	***
TPI	***	***	***	***
Tan	***	***	***	***
Anthos	***	***	***	***
PP	***	***	***	***

^a NS, nonsignificant; * = 0.01 < p < 0.05; and *** = p < 0.01.

function of vintage along the first axis. This could be interpreted as a “wine age” axis, with an increase in age observed from left to right.

Young wines were characterized by higher concentrations of Anthos, Fl, mDP, d520%, TPI, and CI. Higher levels of hue, d420%, PP, and proportion of ethylidene bridges were associated with wine age. Bordeaux wines were separated from Pauillac and Saint Julien wines along the second axis. Higher concentrations of CH-CH₃ and Tan characterized both Pauillac and Saint Julien wines, while Bordeaux wines contained less of these components.

DISCUSSION

Ethylidene-bridged flavan-3-ol contents and their correlation with wine age were analyzed in wines made from the same winery and vineyard within Bordeaux, Pauillac, or Saint Julien and across vintage. The rationale for analyzing wines from a single vineyard was that grapes grown in the same vineyards should yield more consistent compositions year after year. Each sample would then represent a given moment in a wine's life, while knowing that there is likely to be some vintage variability. It was considered that this approach would provide reasonable information on the evolution of ethylidene-bridged Fl with wine age.

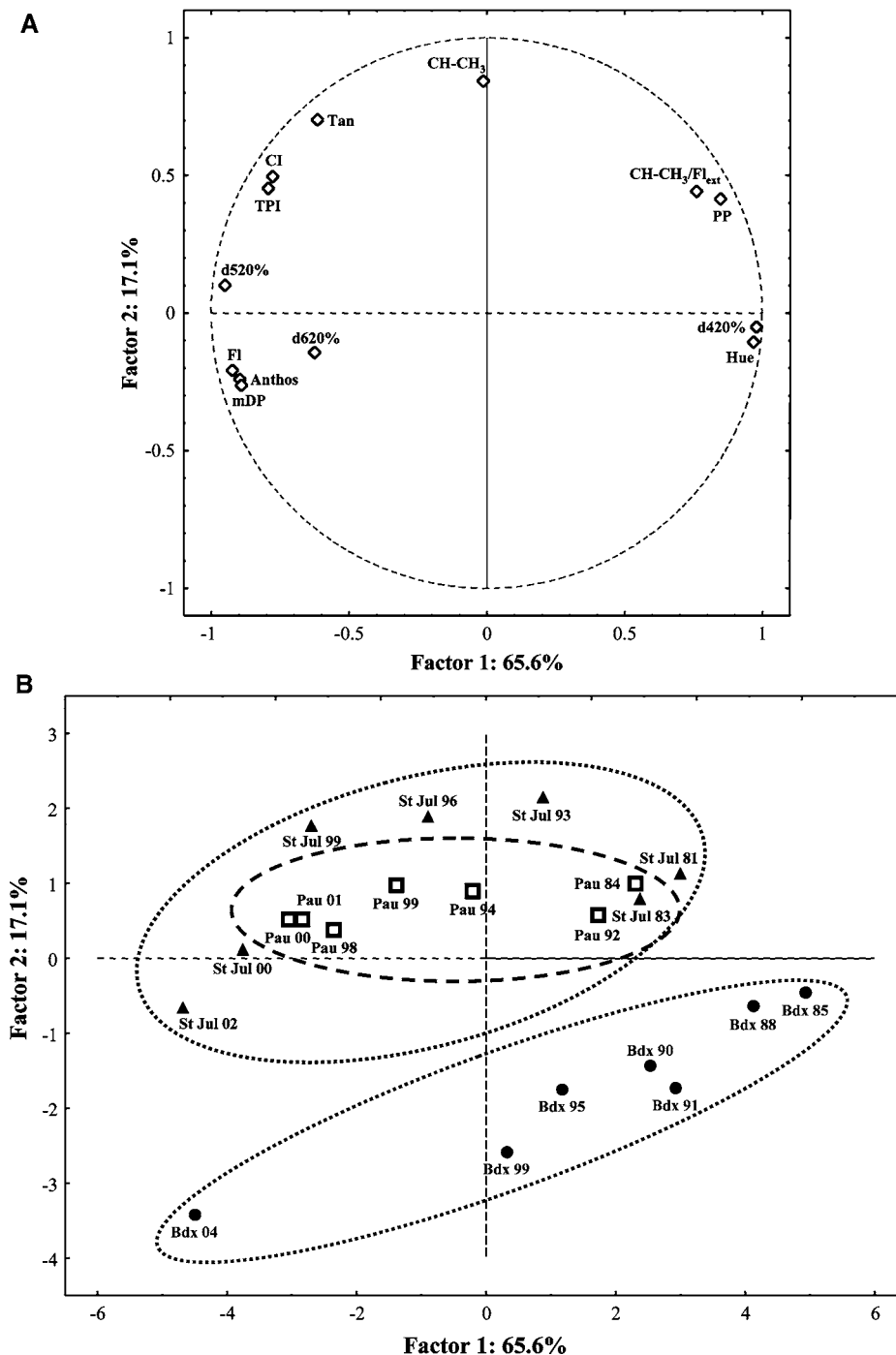


Figure 5. Contribution of the variables (A) and distribution of wine (B) in the two-dimensional coordinate system defined by the first two principal components. Bdx, Bordeaux; Pau, Pauillac; St. Jul, Saint Julien; 04, vintage; and CH-CH₃/FI_{ext}, percentage (in %) of CH-CH₃ over native interflavan bonds.

According to Ribéreau-Gayon et al. (19), once a wine is bottled, transformations are dominated by nonoxidative reactions. Nevertheless, wines are subject to oxidative reactions if the bottle closures allow oxygen ingress (23, 24). It was recently established that for natural cork from 0.1 to 8 μL of oxygen may pass into a well-sealed bottle per day (24). The presence of oxygen in wine will lead to the formation of acetaldehyde via the coupled oxidation of oxygen and phenolic compounds (14). In young wines, while free sulfur dioxide is present in the wine, formed acetaldehyde is generally considered to be present as the bisulfite adduct and therefore would not be available for reaction with phenolic compounds (23, 25) (Figure 6, reaction 1). While this may explain why the amounts of CH-CH₃ would

be stable in young wines, the amounts observed in this study remained relatively stable throughout the study. This suggests that sulfur dioxide does not influence ethylidene-bridged flavan-3-ol formation and avoids this hypothesis.

A second hypothesis is that the rate of ethylidene-bridged flavan-3-ol formation may be similar to its rate of breakdown [i.e., the ethylidene-bridged flavan-3-ol polymers could be depolymerized or undergo rearrangement reactions to form new compounds such as xanthenes or xanthylum salts (6, 9)] (Figure 6, reactions 2–4). Overall, the CH-CH₃ represented less than 4% of FI bonds. Nevertheless, the proportion of ethylidene bridge relative to native interflavan bonds showed an increased with wine age. This suggests that native bonds decrease with

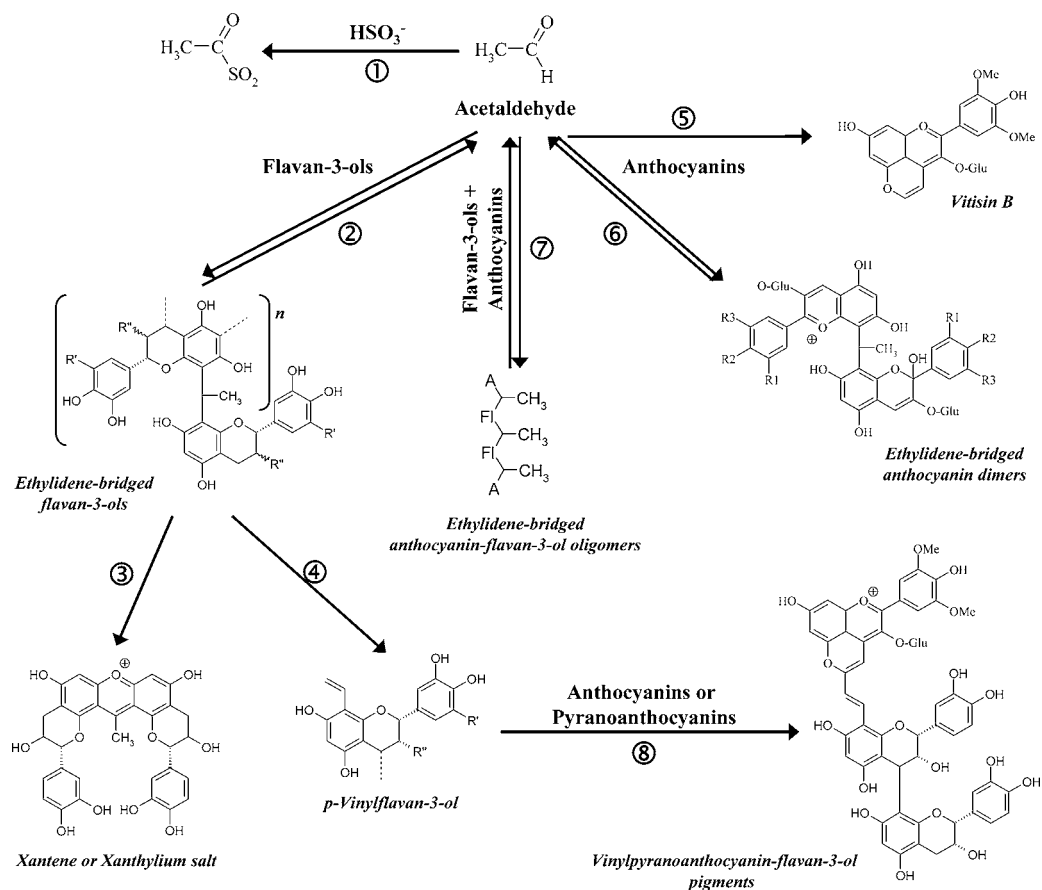


Figure 6. Reaction pathways of acetaldehyde with Fl and Anthos. Fl, R' = H or OH, R'' = OH, or O-gallate; O-glucose (O-Glu), R₁ = OH or OCH₃ and R₂ = H, OH, or OCH₃.

age. Interestingly, the amounts of ethylidene-bridged Fl were always very low (0.8–2.5 mg L⁻¹) while the quantity of free acetaldehyde have been reported to reach up to 50 mg L⁻¹ in red wines during wine making (26). Assuming that the observed amounts are accurate, previous work suggests that ethylidene-bridged Fl are unstable and are intermediate products formed during wine aging (27–29). The constant amounts observed therefore would then suggest that the rates of formation and degradation are similar.

Consistent with the above explanation for the overall small quantities of CH–CH₃ observed would be that the acetaldehyde formed during wine aging reacts with Anthos. It can react with Anthos either alone to form ethylidene-bridged anthocyanin dimers (27, 29–31) (Figure 6, reaction 5) and vitisin B (32, 33) (Figure 6, reaction 6) or in combination with Fl to form ethylidene-bridged copolymers (Figure 6, reaction 7). Previous studies have shown that acetaldehyde addition increased the rate of reaction anthocyanin-flavan-3-ol copolymerization (25, 30, 34). Ethylidene-bridged copolymers up to tetramers were synthesized in model wine containing malvidin-3-O-glucoside and (–)-epicatechin: The oligomers contained at most two Anthos, which were always in the terminal position (35). The copolymers formed are transient and may evolve into substances with higher degrees of condensation, which may finally precipitate (32, 35). The depolymerization of ethylidene-bridged Fl or the dehydration of ethyl alcohol-flavan-3-ol adduct formed during acetaldehyde-flavan-3-ol condensation may lead to the formation of *p*-vinylphenols (Figure 6, reaction 4). These compounds may react with Anthos and pyranoanthocyanins to form more complex compounds such as *p*-vinylpyranoantho-

cyanin-flavan-3-ol (35–40) in which the ethylidene bridges are rearranged and are no longer quantifiable as EDP with the EDP phloroglucinolysis (Figure 6, reaction 8).

The results of this study indicate that with wine age, the free anthocyanin amounts decreased, the polymerized pigments increased, and the flavan-3-ol amounts and mDP decreased. These results are in agreement with earlier work that showed that during aging Anthos and Fl were involved in nonoxidative acid-catalyzed reactions (8, 19, 39, 41, 42). The decrease in the anthocyanin concentrations may lead to the formation of reddish orange color-stable reaction products, consistent with the observed hue increase (d420% increase and d520% decrease) in the present study.

The observed decrease in TPI and CI with wine age may be explained by the precipitation of coloring matter as the wines age. The precipitated material may be polymerized Fl and flavan-3-ol-anthocyanin adducts that have become bulky and hydrophobic colloids (19, 43). One alternate explanation for such decrease is the change in extinction coefficients of the phenolic material as it evolves.

In conclusion, our study has shown that measurable ethylidene-bridged amounts are quite low (<2.5 mg L⁻¹) and that this quantity remained relatively stable during wine aging. The CH–CH₃ represent less than 4% of Fl bonds. Nevertheless, the percentage of ethylidene bridges over native interflavan bonds increases during wine aging may be an important component to follow. In future works, it would be interesting first to follow the effect of acetaldehyde on the ethylidene bridges amounts and to apply this method to explore the correlation between CH–CH₃ and wine aging in oak barrels.

ABBREVIATIONS USED

EDP, 2,2'-ethylidenediphloroglucinol; CH-CH₃, flavan-3-ol ethylidene bridges; Fl, flavan-3-ols; Fl_{term}, flavan-3-ol terminal units; Fl_{ext}, flavan-3-ol extension units; mDP, mean degree of polymerization; CI, color intensity; TPI, total polyphenol index; Tan, total proanthocyanidins; Anthos, anthocyanins; PP, pigmented polymers.

LITERATURE CITED

- Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds—Nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094–1117.
- Llaudy, M.; Canals, R.; Canals, J. M.; Rozéz, N.; Arola, L.; Zamora, F. New method for evaluating astringency in red wine. *J. Agric. Food Chem.* **2004**, *52*, 742–746.
- Saucier, C.; Bourgeois, G.; Vitry, C.; Roux, D.; Glories, Y. Characterization of (+)-catechin-acetaldehyde polymers: A model for colloidal state of wine polyphenols. *J. Agric. Food Chem.* **1997**, *45*, 1045–1049.
- Vidal, S.; Cartalade, D.; Souquet, J.-M.; Fulcrand, H.; Cheynier, V. Changes in procyanidin chain length in winelike model solutions. *J. Agric. Food Chem.* **2002**, *50*, 2261–2266.
- Boulton, R. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic.* **2001**, *52*, 67–82.
- Liao, H.; Cai, Y.; Haslam, E. Polyphenol interactions. Anthocyanins: Co-pigmentation and colour changes in red wines. *J. Sci. Food Agric.* **1992**, *59*, 299–305.
- Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781–784.
- Haslam, E. In vino veritas: Oligomeric procyanidins and the ageing of red wines. *Phytochemistry* **1980**, *19*, 2577–2582.
- Jurd, L. Review of polyphenols condensation reactions and their possible occurrence in the aging of wines. *Tetrahedron* **1969**, *25*, 2367–2380.
- Timberlake, C. F.; Bridle, P. Anthocyanins: Color augmentation with catechin and acetaldehyde. *J. Sci. Food Agric.* **1977**, *26*, 539–544.
- Es-Safi, N.; Fulcrand, H.; Cheynier, V.; Moutounet, M. Competition between (+)-catechin and (–)-epicatechin in acetaldehyde-induced polymerization of flavanols. *J. Agric. Food Chem.* **1999**, *47*, 2088–2095.
- Romano, P.; Suzzi, G.; Turbanti, L.; Polsinelli, M. Acetaldehyde production in *Saccharomyces cerevisiae* wine yeasts. *Microbiol. Lett.* **1994**, *118*, 213–218.
- Drinkine, J.; Saucier, C.; Glories, Y. (+)-Catechin-aldehyde condensations: Competition between acetaldehyde and glyoxylic acid. *J. Agric. Food Chem.* **2005**, *53*, 7552–7558.
- Singleton, V. L.; Kramling, T. E. Browning of white wines and an accelerated test for browning capacity. *Am. J. Enol. Vitic.* **1976**, *27*, 157–160.
- Saucier, C.; Guerra, C.; Laguerre, M.; Glories, Y. (+)-catechin-acetaldehyde condensation products in relation with wine-ageing. *Phytochemistry* **1997**, *46*, 229–234.
- Drinkine, J.; Lopes, P.; Kennedy, J. A.; Teissedre, P.-L.; Saucier, C. Analysis of ethylidene-bridged flavan-3-ols in wine. *J. Agric. Food Chem.* **2007**, *55*, 1109–1116.
- Peyrot des Gachons, C.; Kennedy, J. A. Direct method for determining seed and skin proanthocyanidin extraction into red wine. *J. Agric. Food Chem.* **2003**, *51*, 5877–5881.
- Glories, Y. La couleur des vins rouges. II. Mesure, origine et interprétation. *Conn. Vigne Vin* **1984**, *18*, 253–271.
- Ribéreau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. Les composés phénoliques. In *Traité d'oenologie. Tome 2. Chimie du vin Stabilisation et Traitements*; Dunod: Paris, France, 1998a; pp 163–237.
- Ribéreau-Gayon, P.; Stonestreet, E. Dosage des tanins du vin rouge et détermination de leur structure. *Chim. Anal.* **1966**, *48*, 188–196.
- Ribéreau-Gayon, P.; Stonestreet, E. Determination of anthocyanins in red wine. *Bull. Soc. Chim.* **1965**, *9*, 2649–2652.
- Glories, Y. La matière colorante du vin rouge. Thèse d'Etat, Université de Bordeaux 2, Bordeaux, France, 1978.
- Ribéreau-Gayon, J. Dissolution d'oxygène dans les vins. In *Contribution à l'étude des Oxydations et Réductions dans les vins. Application à l'étude du Vieillissement et des Casses*; Delmas: Bordeaux, France, 1933; pp 35.
- Lopes, P.; Saucier, C.; Teissedre, P.-L.; Glories, Y. Impact of storage position on oxygen ingress through different closures into wine bottles. *J. Agric. Food Chem.* **2006**, *54*, 6471–6746.
- Picinelli, A.; Bakker, J. P. B. Model wine solutions: Effect of sulphur dioxide on colour and composition during ageing. *Vitis* **1994**, *33*, 31–35.
- Flanzly, C. Tables de composition. In *Oenologie: Fondements Scientifiques et Technologiques*; Tec et Doc Lavoisier: Paris, 1998; pp 315–336.
- Rivas-Gonzalo, J.; Bravo-Haro, S.; Santos-Buelga, C. Detection of compounds formed through the reaction of malvidin 3-mono-glucoside and catechin in the presence of acetaldehyde. *J. Agric. Food Chem.* **1996**, *43*, 1444–1449.
- Francia-Aricha, E. M.; Guerra, M. T.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. New anthocyanin pigments formed after condensation with flavanols. *J. Agric. Food Chem.* **1997**, *45*, 2262–2266.
- Escribano-Bailon, T.; Alvarez-Garcia, M.; Rivas-Gonzalo, J.; 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.
- Timberlake, C. F.; Bridle, P. The effect of processing and other factors on the colour characteristics of some red wines. *Vitis* **1976**, *15*, 37–49.
- Dallas, C.; Ricardo-Da-Silva, M. J.; Laureano, O. Products formed in model wine solutions involving anthocyanins, procyanidin B2, and acetaldehyde. *J. Agric. Food Chem.* **1996**, *44*, 2402–2407.
- Bakker, J.; Picinelli, A.; Bridle, P. Model Wine solutions: Colour and composition changes during ageing. *Vitis* **1993**, *32*, 111–118.
- Morata, A.; Calderon, F.; Gonzalez, M. C.; Gomez-Cordoves, M. C.; Suarez, J. A. Formation of the highly stable pyranoanthocyanins (vitisins A and B) in red wines by the addition of pyruvic acid and acetaldehyde. *Food Chem.* **2007**, *100*, 1144–1152.
- Ribéreau-Gayon, P.; Pontallier, P.; Glories, Y. Some interpretations of colour changes in young red wines during their conservation. *J. Sci. Food Agric.* **1983**, *34*, 505–516.
- 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.
- Bakker, J.; Timberlake, C. F. Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *J. Agric. Food Chem.* **1997**, *45*, 35–43.
- Vivar-Quintana, A. M.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Anthocyanin-derived pigments and colour of red wines. *Anal. Chim. Acta* **2002**, *458*, 147–155.
- Mateus, N.; Carvalho, E.; Carvalho, R. F. A.; Melo, A.; Gonzales-Paramas, M. A.; Santos-Buelga, C.; De Freitas, A. P. V. Isolation and structural characterization of new acylated anthocyanin-vinyl-flavanol occurring in aging red wines. *J. Agric. Food Chem.* **2003**, *51*, 277–282.
- Mateus, N.; Oliveira, J.; Santos-Buelga, C.; Silva, A. M. S.; De Freitas, A. P. V. NMR structure characterization of a new vinylpyranoanthocyanin-catechin pigment (a portisin). *Tetrahedron Lett.* **2004**, *45*, 3455–3457.

- (40) He, J.; Santos Buelga, C.; Mateus, N.; De Freitas, A. P. V. Isolation and quantification of oligomeric pyranoanthocyanin-flavanol pigments from red wines by combination of column chromatographic techniques. *J. Chromatogr. A* **2006**, *1134*, 215–225.
- (41) Somers, T. C. The polymeric nature of wine pigments. *Phytochemistry* **1971**, *10*, 2175–2186.
- (42) Cheynier, V.; Fulcrand, H.; Brossaud, F.; Asselin, C.; Moutounet, M. 10. Phenolic composition as related to red wine flavor. In *Chemistry of Wine Flavor*; Waterhouse, A. L., Ebeler, S. E., Eds.; American Chemical Society: Washington, DC, 1999; p 256.
- (43) Saucier, C.; Bourgeois, G.; Vitry, C.; Roux, D.; Glories, Y. Characterization of (+)-catechin-acetaldehyde polymers: A model for colloidal state of wine polyphenols. *J. Agric. Food Chem.* **1997b**, *45*, 1045–1049.

Received for review January 5, 2007. Revised manuscript received March 27, 2007. Accepted April 11, 2007. We thank Yvon Mau Co. (Gironde sur Dropt, France) and ANRT (Association Nationale pour la Recherche Technologique, Paris, Cifre Grant 855/2002) for their financial support in this research.

JF070038W