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Analysis of Ethylidene-Bridged Flavan-3-ols in Wine

JESSICA DRINKINE,[†] PAULO LOPES,[†] JAMES A. KENNEDY,[‡] PIERRE-LOUIS TEISSEDRE,[†] 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 Weigand Hall, Corvallis, Oregon 97331-6602

A method was developed to determine the amount of ethylidene-bridged flavan-3-ols in wine. The method was based upon the analysis of 2,2′-ethylidenediphloroglucinol (EDP), a product formed after acid-catalyzed cleavage of wine flavan-3-ols in the presence of excess phloroglucinol. In the developed analytical method, the wine was purified and concentrated using C_{18} solid-phase extraction before the phloroglucinolysis reaction was carried out. This procedure was used to quantify ethylidene-bridged flavan-3-ols in wine and the molar ratio between ethylidene-bridged linkages and native interflavan linkages. The method validation showed 9.2% repeatability. The recovery of the ethylidene-bridged flavanols in wine was 90% for concentrations up to 4.5 mg·L^{-1} of ethylidene-bridged linkages, and it decreases to 83% above and until the concentration reached 7.6 mg·L^{-1}. Initial results showed that the concentration of ethylidene-bridged flavan-3-ols measured in wines was very low (less than 1.3 mg·L^{-1}) and that they represented less than 1.3% of the total interflavonoid linkages on a molar ratio.

KEYWORDS: Tannins; flavan-3-ol; ethylidene bridge; red wine; aging; 2,2′-ethylidenediphloroglucinol; 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 winemaking and aging, condensed tannins undergo enzymatic or chemical modifications, and many of these modifications result in the formation of new interflavonoid linkages. One such modification is the acid-catalyzed cleavage of the original plant-derived interflavanoid bond and a subsequent condensation reaction (*4*, *8*, *9*). Another type of modification is nucleophilic substitution reactions 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*). The ethylidene-bridged flavan-3-ol products of this reaction have also been referred to as ethyl-bridged and methyl−methine-bridged polymers. To date, only dimers and trimers of ethylidene-bridged flavan-3-ols have been identified in wine (*12*). The acetaldehyde involved in this reaction may be produced in two ways: by yeast production (e.g., *Saccharomyces cerevisiae*) (*13*) and from the oxidation of ethanol (*14*). In order to determine the importance of acetaldehyde in wine aging, it would be useful to have information on the quantity of ethylidene-bridged flavan-3-ols in wine.

Condensed tannins are complex flavan-3-ol polymers, and in general, only methods based upon their cleavage provide compositional information. Several methods such as thiolysis (*15*) or phloroglucinolysis (*16*, *17*) have historically been used for compositional analysis. Phloroglucinolysis is based upon the acid-catalyzed cleavage of flavan-3-ol interflavanoid bonds in the presence of phloroglucinol. The reaction releases the terminal unit as a flavan-3-ol monomer and forms C4 phloroglucinol adducts of the extension units (**Figure 1**). The products are then analyzed by reversed-phase HPLC. It is thus possible insofar as the cleavage is complete, to determine flavan-3-ol polymer composition and mean average degree of polymerization (mDP).

The purpose of the present work was to develop a method for the detection of ethylidene-bridged flavan-3-ols by conducting phloroglucinolysis on the flavan-3-ols to form an ethylidene-bridged phloroglucinol adduct.

MATERIALS AND METHODS

Materials. Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA). Acetonitrile (HPLC grade) was obtained from Fisher 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 methanol (HPLC

* Corresponding author. Fax: 33-5-40-00-6468. E-mail: saucier@oenologie.u-bordeaux2.fr.
[†] Université Victor Segalen Bordeaux 2.
[‡] Oregon State University.

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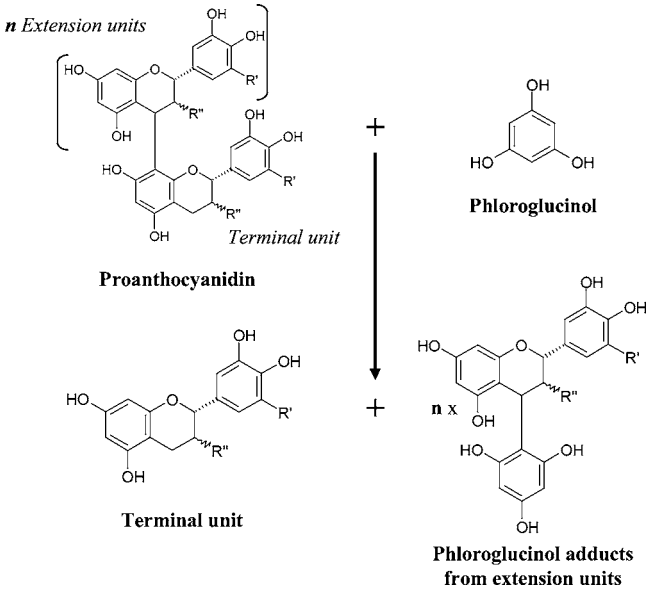

Figure 1. Reaction pathway of phloroglucinolysis. Procyanidins, R′ = H and R″ = OH; prodelphinidins, R′ = OH and R″ = OH.

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 were purchased from Fluka (Saint Quentin Fallavier, France).

MS Apparatus and LC−MS Analysis. LC−MS analyses were performed on a Micromass Platform II simple quadruple mass spectrometer (Micromass-Beckman, Roissy Charles-de-Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in negative-ion mode. Source temperature was 120 °C, capillary voltage was set at ± 3.5 kV, and 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.

^{1}H NMR Analysis. A Bruker Avance-300 NMR spectrometer (Bruker, Wissembourg, France), operating at 300.13 MHz for ^{1}H, using the UXNMR software package, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referred to the solvent peaks δ_H 3.31 for CD_3OD; coupling constants, *J*, are in hertz. Compounds were dissolved in deuterated methanol.

Synthesis and Characterization of (+)-Catechin Ethylidene-Bridged Oligomers. (+)-Catechin ethylidene-bridged oligomers were synthesized in model wine (12% v/v ethanol, 5 g·L^{-1} tartaric acid and pH 3.2) as follows: (+)-Catechin (100 mg) was dissolved in model wine (100 mL), and the solution (100 mL) was mixed with acetaldehyde (5 mL) and model wine (95 mL). The mixture was incubated at 40 °C for 8 and 24 h. To stop the reaction, a solid-phase extraction (SPE) step was used. Each sample was purified on a C_{18} cartridge (Supelco, St Quentin Fallavier, France) as follows: the column (5 g) was conditioned (50 mL MeOH followed by 50 mL H_20) and the sample (5 mL) applied. The column was washed with 15 mL of water to remove tartaric acid and excess acetaldehyde. The polymers were then eluted with 5 mL of methanol. The methanol fraction was dried under reduced pressure and lyophilized to a dry powder.

Oligomers were analyzed by LC−MS. The column used was a reversed-phase C_{18} Interchrom UP3 ODB-10QS (3 μm packing, 100 × 4.6 mm i.d.) protected with a guard column of the same material (Interchim, Montluçon, France). Solvent A was water:acetic acid (99:1 by volume), and solvent B was acetonitrile:water (4:1 by volume). Separation was conducted at room temperature, flow rate was 1 mL·min^{-1} for the column and 0.1 mL·min^{-1} for the MS source, and the sample loop was 20 μL. The elution gradient was as follows: from 0 to 20% B in 1 min, from 20 to 30% B in 5.5 min, from 30 to 50% B in 12.5 min, from 50 to 100% B in 0.5 min, 100% B for 3 min, from 100 to 0% B in 0.5 min, 0% B for 5 min. Peak identities were determined by mass spectrometry. (+)-Catechin ethylidene-bridged oligomers were quantified from the amount of (+)-catechin monomer that disappeared during the condensation.

Synthesis and Characterization of 2,2′-Ethylidenediphloroglucinol (EDP). EDP was prepared as follows: Phloroglucinol (2 g) and ascorbic acid (200 mg) were dissolved in 10 mL of 0.1 N HCl methanol, and 15 μL of acetaldehyde was combined with 2 mL of the phloroglucinol mixture and then the mixture stirred for 30 min at 50 °C. The reaction was stopped by adding 10 mL of 40 mM aqueous sodium acetate. EDP was purified on a C_{18} cartridge as follows: the column (5 g) was conditioned and the sample applied (10 mL). The column was washed with 50 mL of water followed by 50 mL of methanol/water (25:75, v/v). EDP was then eluted with 50 mL of methanol:water (1:1 by volume). The fraction was dried under reduced pressure and lyophilized to a dry powder.

EDP purity was determined by LC−MS. It was quantified at 280 nm and corresponded to the percentage of the EDP peak area compared to the total peak area. The column used was a reversed-phase C_{18} Waters Xterra protected with a guard column of the same material (3.5 μm packing, 4.6 × 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: from 5 to 15% B in 6 min, from 15 to 40% B in 26 min, from 40 to 100% B in 1 min, 100% B for 3 min, from 100 to 5% B in 1 min, 5% B for 4 min with a 1.5 mL·min^{-1} flow for the column and 0.15 mL·min^{-1} for the MS source. EDP characterization was also determined by ^{1}H NMR analysis. The compound was dissolved in deuterated methanol.

Phloroglucinolysis of (+)-Catechin Ethylidene-Bridged Oligomers. A solution of 0.1 N HCl in methanol containing 50 g·L^{-1} phloroglucinol and 10 g·L^{-1} ascorbic acid was prepared. A 5 g·L^{-1} solution of (+)-catechin ethylidene-bridged oligomers (8 h) was prepared in methanol, and 200 μL of the previous solution reacted in 200 μL of this solution at 50 °C for 10, 20, 40, 60, and 90 min and then combined with 1 mL of 40 mM aqueous sodium acetate to stop the reaction.

(+)-Catechin ethylidene-bridged oligomers cleavage products were monitored by LC−MS using the same elution conditions used for monitoring the **s**ynthesis and characterization EDP.

Wine EDP Phloroglucinolysis Method. The phloroglucinolysis protocol was divided into two steps. The first step consisted of purification and concentration of the wine and was carried out using C_{18} solid-phase extraction (SPE). Water (15 mL) was added to 5 mL of wine. The mixture was purified on a C_{18} cartridge as follows: the column was conditioned and the sample applied (10 mL). The column was washed with 50 mL of water and eluted with 50 mL of methanol. The fraction was dried under reduced pressure and then dissolved in 2 mL of methanol. The second step was the phloroglucinolysis reaction. A solution of 0.2 N HCl in methanol, containing 100 g·L^{-1} phloroglucinol and 20 g·L^{-1} ascorbic acid, was prepared, and 100 μL of wine sample was reacted with 100 μL of the phloroglucinol reagent at 50 °C for 20 min and then combined with 200 μL 400 mM aqueous sodium acetate to stop the reaction. Aqueous 4-methylcatechol (4MC; 20 μL of 500 mg·L^{-1}) was then added as 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 same elution conditions were used than for the **s**ynthesis and characterization of EDP but using the following elution gradient: 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.

Wine EDP Phloroglucinolysis Calibration. Calibration was done using an EDP extract (60% w/w purity, accounted for in the calibration). Solutions containing increasing EDP concentrations and fixed 4MC concentration (internal standard) were prepared. EDP concentrations were 100, 50, 20, 10, 5, and 2 mg·L^{-1}. The 4MC concentration in all samples was fixed at 23.8 mg·L^{-1}. Samples were separated and

monitored by LC−MS with molecular ion masses of *m*/*z* 277 (EDP) and *m*/*z* 123 (4MC). The calibration curve was obtained by fitting EDP/4MC peak area as a function of EDP concentration.

Wine EDP Phloroglucinolysis Repeatability. Method repeatability was carried out using one wine. Wine EDP phloroglucinolysis was determined as described above. SPE purification was conducted in triplicate. Phloroglucinolysis was then conducted in triplicate for each replicate. Each replicate was injected into the LC−MS. For one replicate, the injection was replicated three times. Repeatability was defined as the percent variation of the EDP concentration measured.

Wine EDP Phloroglucinolysis Recovery Study. (+)-Catechin ethylidene-bridged oligomers (24 h reaction) at three concentrations were prepared in methanol (100, 50, and 25 mg·L^{-1}). These solutions corresponded to 1.5, 3.0, and 5.9 mg·L^{-1} ethylidene units within the oligomers. Considering that 1 mol of combined acetaldehyde yields 1 mol of EDP after phloroglucinolysis, the expected EDP concentrations were calculated. The phloroglucinolysis reaction was carried out, and the EDP concentrations were measured by LC−MS. Measured EDP concentrations were then compared to expected EDP concentrations, and the method recovery was determined as follows:

$$\text{recovery (\%)} = \frac{[\text{EDP}]_{\text{measured}}}{[\text{EDP}]_{\text{calculated}}} \times 100$$

Recovery was also determined in model wine (12% ethanol, 5 g·L^{-1} tartaric acid, pH 3.2) and wine. Solutions of (+)-catechin ethylidene-bridged oligomers were then isolated. The EDP phloroglucinolysis method was applied (SPE + phloroglucinolysis), and EDP concentrations were measured by LC−MS. Measured EDP concentrations were compared to expected EDP concentrations, and the method recovery was determined.

Wine Phloroglucinolysis. The proanthocyanidin mDP and the flavan-3-ols analyses were measured by phloroglucinolysis, which was adapted from a previous study (*17*). The wine was purified and concentrated similar to that for wine EDP phloroglucinolysis. A solution of 0.2 N HCl in methanol, containing 100 g·L^{-1} phloroglucinol and 20 g·L^{-1} ascorbic acid, was prepared, and 100 μL 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 synthesis and characterization of EDP but using water:acetic acid (99:1, v/v) as solvent A, methanol 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.

RESULTS AND DISCUSSION

This study was conducted in order to develop a method to quantify ethylidene-bridged flavan-3-ols in wine by phloroglucinolysis. As the interflavanoid bonds of native flavan-3-ols are broken in acid with heat, we hypothesized that ethylidene bridges would undergo cleavage. Considering this hypothesis, phloroglucinolysis of ethylidene-bridged flavan-3-ols would release flavan-3-ol monomers from terminal units and their corresponding ethylideneflavan-3-ol−phloroglucinol adducts from extension units (**Figure 2**). Ethylidene-bridged flavan-3-ol phloroglucinolysis was first investigated using synthesized (+)-catechin ethylidene-bridged oligomers.

Synthesized (+)-Catechin Ethylidene-Bridged Oligomers. The synthesized extracts of (+)-catechin ethylidene-bridged oligomers consisted primarily of (+)-catechin monomer and dimer and trimer of ethylidene-bridged oligomers (**Figure 3**). The quantity of ethylidene-bridged oligomers was estimated by determining the (+)-catechin monomer disappearance during the polymerization reaction. Previous work has shown that during the reaction only ethylidene-bridged oligomers are formed (*18*). Upon analysis, 61% and 97% of the (+)-catechin existed as ethylidene-bridged oligomers, for the 8 and 24 h reaction, respectively. Considering the ethylidene-bridged (+)-catechin unit molecular weight (461 g·mol^{-1}) and the molecular weight of the ethylidene bridge (28 g·mol^{-1}), ethylidene-bridged (+)-catechin oligomers contained 6.07% ethylidene by weight. By extension, the reaction mixtures after 8 and 24 h contained, respectively, 3.7 and 5.9 mg of ethylidene per 100 mg of material.

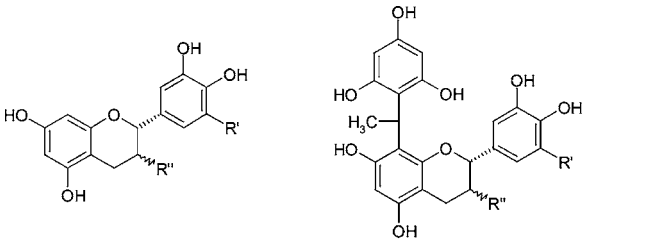

Figure 2. Proposed compounds released by the phloroglucinolysis of ethylidene-bridged flavan-3-ols.

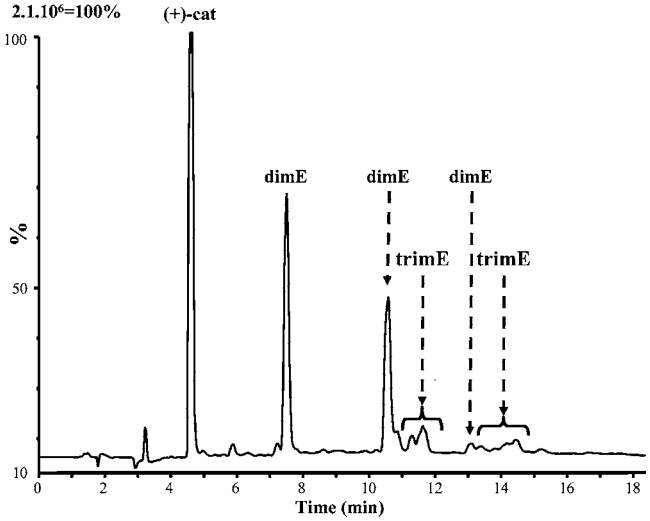

Figure 3. HPLC chromatogram of synthesized (+)-catechin ethylidene-bridged oligomers. (+)-cat = (+)-catechin, dimE = (+)-catechin ethylidene-bridged dimers, trimE = (+)-catechin ethylidene-bridged trimers.

Phloroglucinolysis of Ethylidene-Bridged (+)-Catechin Oligomers. Ethylidene-bridged (+)-catechin oligomers (8 h) and predicted cleavage products were observed by their molecular ion masses in the negative-ion mode: (+)-catechin (*m*/*z* 289), (+)-catechin ethylidene-bridged dimer (*m*/*z* 605), (+)-catechin ethylidene-bridged trimer (*m*/*z* 921), and ethylidene-flavan-3-ol−phloroglucinol adduct (*m*/*z* 441). After a phloroglucinolysis reaction time of 10 min (**Figure 4**), no (+)-catechin ethylidene-bridged trimers were detected and very little (+)-catechin ethylidene-bridged dimers remained. Ethylideneflavan-3-ol−phloroglucinol adducts (two stereoisomers) were found in small quantities.

The two major compounds, **1** and **2**, formed during phloroglucinolysis were unknown (**Figure 5**). MS analysis showed molecular ion masses of *m*/*z* 277 and 415, respectively. The proposed structure for **1** is the phloroglucinol ethylidene-bridged dimer, 2,2′-ethylidenediphloroglucinol (EDP), and **2** was pro-

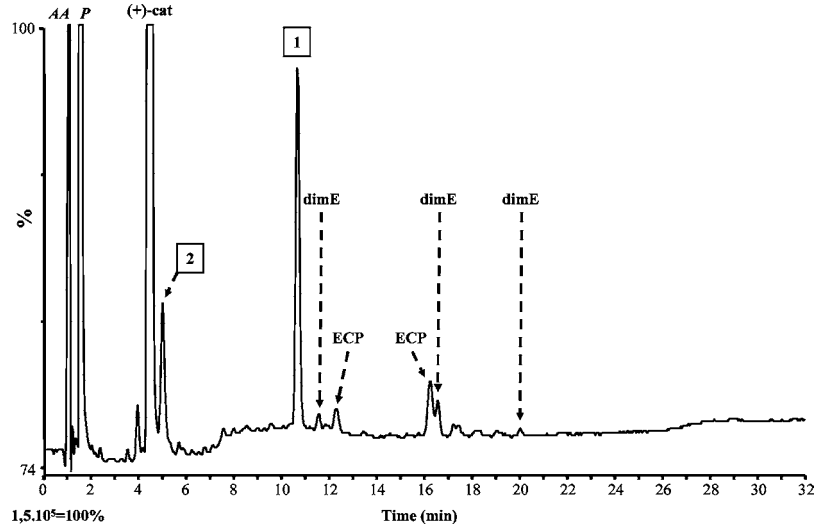

Figure 4. HPLC chromatogram of cleavage products from (+)-catechin ethylidene-bridged oligomers (8 h) following 10 min acid-catalysis in the presence of phloroglucinol. AA = ascorbic acid, P = phloroglucinol, (+)-cat = (+)-catechin, dimE = (+)-catechin ethylidene-bridged dimer, ECP = ethylidene-(+)-catechin–phloroglucinol adduct, **1** and **2** = unknown compounds.

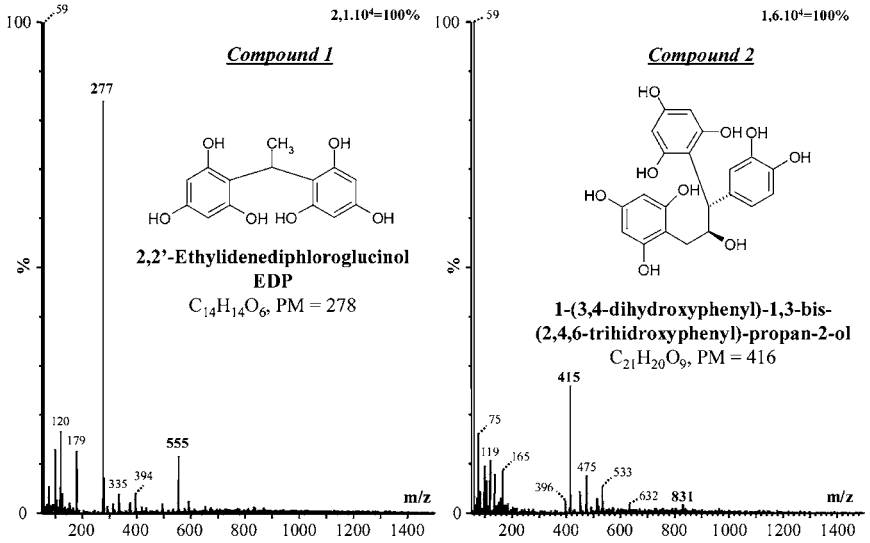

Figure 5. Mass spectra in negative-ion mode and proposed structures for unknown compounds following phloroglucinolysis of (+)-catechin ethylidene-bridged oligomers.

posed to be the 1-(3,4-dihydroxyphenyl)-1,3-bis(2,4,6-trihydroxyphenyl)propan-2-ol. This product has been previously described (*16*) and results from the reaction of phloroglucinol with the (+)-catechin B-ring quinone-methide following acid-catalyzed C-ring cleavage.

(+)-catechin and EDP were the major products of (+)-catechin ethylidene-bridged oligomer phloroglucinolysis. This led to the following hypothesis: ethylidene-bridged flavan-3-ol polymers should release EDP upon phloroglucinolysis. The pathway proposed for the ethylidene-bridged flavan-3-ol phloroglucinolysis is an acid-catalyzed nucleophilic substitution (**Figure 6**). The first step would consist of the acidic cleavage of one part of the ethylidene bridge, releasing one flavan-3-ol unit, followed by nucleophilic attack by phloroglucinol, leading to an ethylideneflavan-3-ol−phloroglucinol adduct. The second step would consist of the acidic cleavage of the second part of the ethylidene bridge, releasing the second flavan-3-ol unit, followed by phloroglucinol addition, leading to EDP formation. EDP would be the only product of ethylidene-bridged flavan-3-ol phloroglucinolysis.

In order to optimize EDP formation and reduce ethylidene-flavan-3-ol−phloroglucinol adducts, the phloroglucinolysis of (+)-catechin ethylidene-bridged oligomers was monitored as a function of time (data not shown). The optimum time found was 20 min. The reaction was not complete and low amounts of ethylidene-(+)-catechin−phloroglucinol adducts were found. The EDP peak area represented 77% of the sum of EDP and ethylidene-(+)-catechin−phloroglucinol adduct peak areas at 280 nm.

2,2′-Ethylidenediphloroglucinol Compound (EDP). As EDP was the major compound formed by phloroglucinolysis, it was synthesized to calibrate the method. The EDP extract, analyzed at 280 nm and by LC−MS, was 60% EDP purity (**Figure 7**). On the chromatogram, an additional peak other than EDP appeared with *m*/*z* 429 molecular ion mass corresponding to the phloroglucinol ethylidene-bridged dimer, consistent with stepwise polymerization kinetics.

The structure of EDP was confirmed by ^{1}H NMR. The spectrum (**Figure 8**) is readily interpretable: The 4.72 ppm quartet is consistent with the H_7 proton, and the 1.69 ppm doublet is consistent with the ethylidene methyl group (CH_3). H_4, H_6, $H_{4'}$, and $H_{6'}$ are equivalent and resonated as a singlet at 5.89 ppm (**Figure 8**).

Quantification of Ethylidene-Bridged Flavan-3-ols in Wine. The phloroglucinolysis method was adapted from previ-

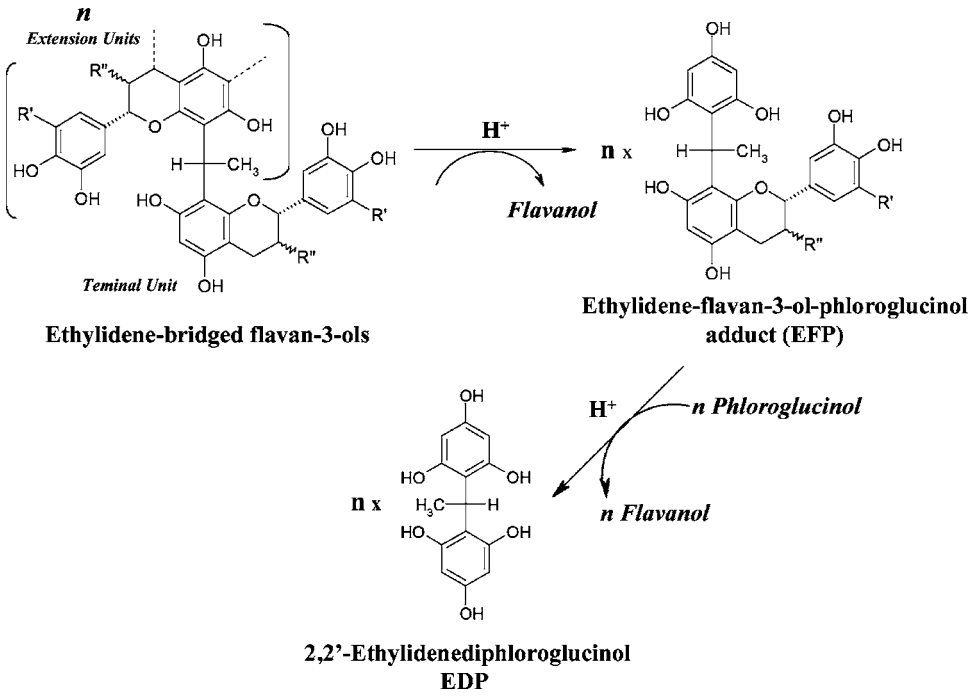

Figure 6. Hypothetical phloroglucinolysis reaction pathway for the formation of EDP from ethylidene-bridged flavan-3-ols. Procyanidins, R′ = H and R″ = OH; prodelphinidins, R′ = OH and R″ = OH.

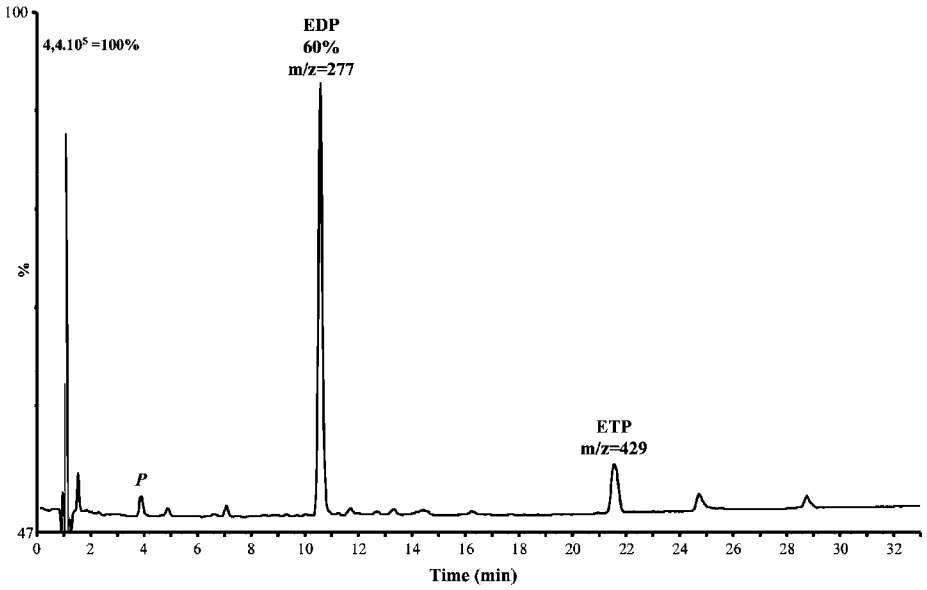

Figure 7. HPLC chromatogram of purified EDP extract. P = phloroglucinol, EDP = phloroglucinol ethylidene-bridged dimer, ETP = phloroglucinol ethylidene-bridged trimer.

ous work (*17*) and was conducted as follows: wine dilution in water, isolation, and concentration using SPE, followed by phloroglucinolysis. Wine samples were diluted 1.68 times before injection as a result of the SPE and phloroglucinolysis steps.

EDP quantification was carried out by mass spectrometry in the negative-ion mode with an internal standard, 4-methylcatechol (4MC), added at the end of the phloroglucinolysis. EDP quantification was calculated from the peak area ratio EDP/4MC after calibration. The calibration followed a linear regression ($R^2 = 0.997$):

$$\text{EDP (mg·L}^{-1}) = \frac{\text{EDP/4MC}}{0.037} \times 1.68$$

where the 1.68 coefficient corresponds with the wine dilution. Method repeatability was determined to be 9.2%, which is in agreement with criteria for intralaboratory analysis (*19*) (**Table 1**). The EDP concentration was then converted into ethylidene bridge concentration considering the molecular weight of EDP and ethylidene.

Known amounts of ethylidene-bridged oligomer (24 h) were dissolved in methanol and model wine to determine the method recovery. The recovery of ethylidene was determined in methanol samples, and the recovery of the entire method was determined in model wine samples. Expected EDP concentrations were then calculated and compared to measured EDP concentrations. The results showed that the measured concentra-

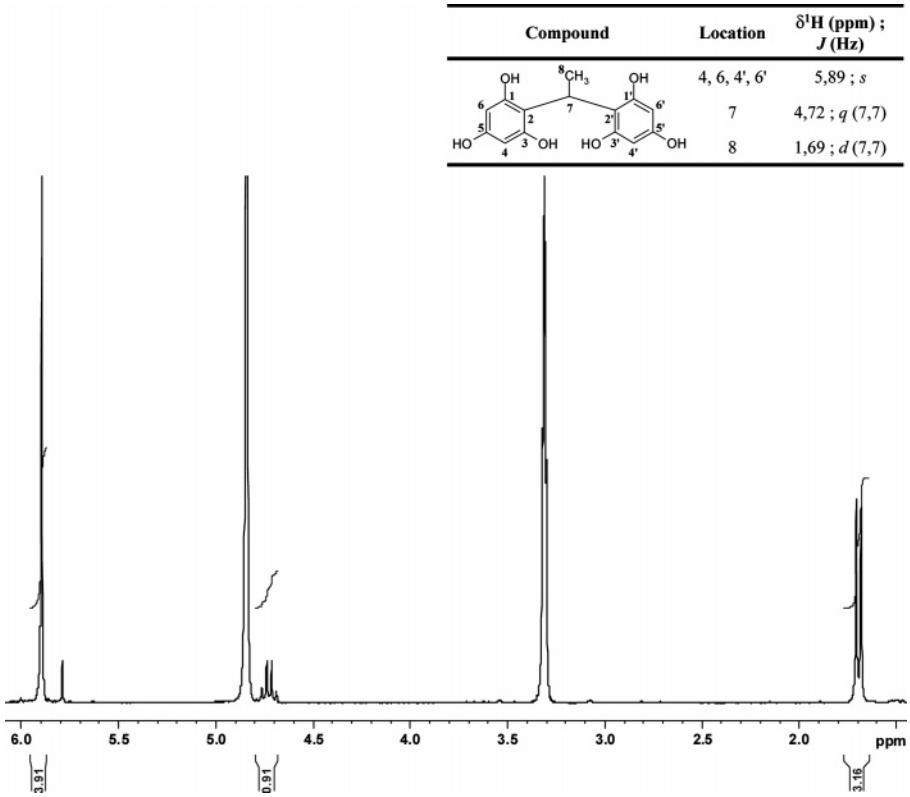

Figure 8. ^{1}H NMR spectrum and spectroscopic data (300 MHz) of EDP in deuterated methanol.

Table 1. EDP Mean Concentrations of Repeatability Assay and Corresponding Percentage Errors (±SD, $N = 3$)

	[EDP] (mg·L^{-1})	coefficient of variation (%)
injection	6.1 ± 0.3	5.4
phloroglucinolysis and SPE	6.2 ± 0.2	4.8
	5.1 ± 0.3	3.2
	5.7 ± 0.3	4.9
mean	5.8 ± 0.5	9.2

tions are close to expected concentrations (**Figure 9A**). The ethylidene recovery in methanol was 97% and the recovery of the entire method was 90%. The same known amounts were added to a wine, and the recovery was calculated again. The results showed that the measured concentrations are close to expected concentrations until an EDP concentration of 18 mg·L^{-1}(e.g., 4.5 mg·L^{-1} ethylidene) is reached. The recovery was then 90%. Above an EDP concentration of 30 mg·L^{-1} (e.g., 7.6 mg·L^{-1} ethylidene), the recovery decreased to 83%.

Application to Wine. The EDP phloroglucinolysis and flavan-3-ol determination were applied to three Bordeaux red wines, produced by the same winery but from different vintages: 2004, 1999, and 1991. Results showed that ethylidene bridges were measurable and comparable to flavan-3-ol amounts (**Table 2**). Nevertheless, their amounts were very low (0.7−1.3 mg·L^{-1}) compared to flavan-3-ol amounts. Ethylidene-bridged linkages represented less than 1.3% of the interflavanoid linkages by mol (**Figure 10**). The analyses of more wines are necessary to draw meaningful conclusions.

Specific questions remain about the quantity of ethylidene-bridged flavan-3-ols in wines. The surprisingly low amounts of EDP suggest that ethylidene-bridged flavan-3-ols are unstable and undergo further reaction. Given the relative ease with which EDP is formed under the phloroglucinolysis conditions, it is likely that ethylidene-bridged flavan-3-ols are easily hydrolyzed under wine conditions. Given that acetaldehyde reacts with other nucleophiles in wine such as anthocyanins (*20*−*28*) and the

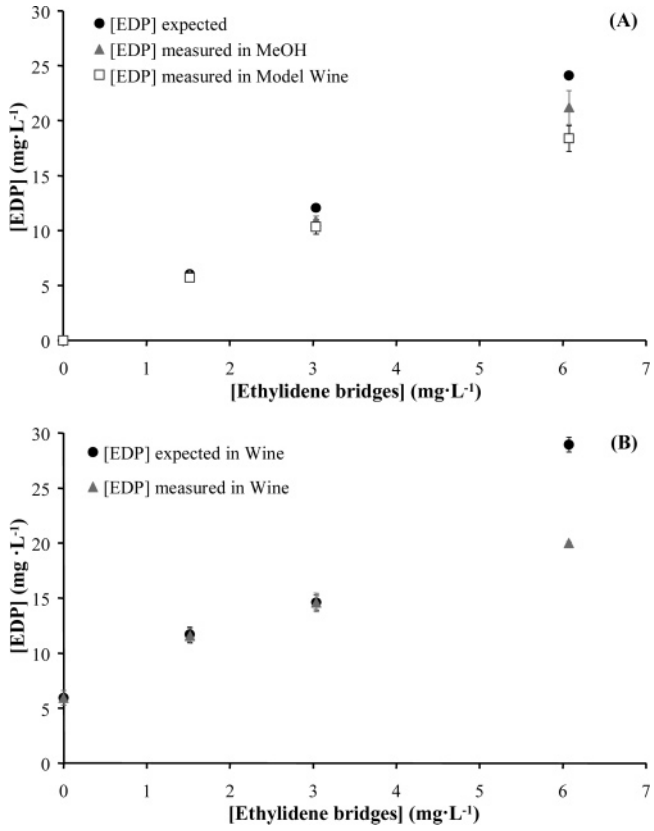

Figure 9. Recovery and linearity studies of the EDP phloroglucinolysis: (**A**) in methanol and in model wine and (**B**) in wine (±SD, $N = 3$).

Table 2. Ethylidene Bridge Amounts and Flavan-3-ol Composition in $mg \cdot L^{-1}$ for Three Bordeaux Wines (±SD, $N = 3$)[a]

vintage	$CHCH_3$	Fl_{term}	Fl_{ext}	Fl	mDP
2004	0.8 ± 0.1	355 ± 10	517 ± 17	871 ± 22	2.4 ± 0.1
1999	0.7 ± 0.1	260 ± 25	282 ± 5	541 ± 30	2.1 ± 0.1
1991	1.3 ± 0.1	192 ± 6	181 ± 3	373 ± 5	1.9 ± 0.1

[a] $CHCH_3$ = 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.

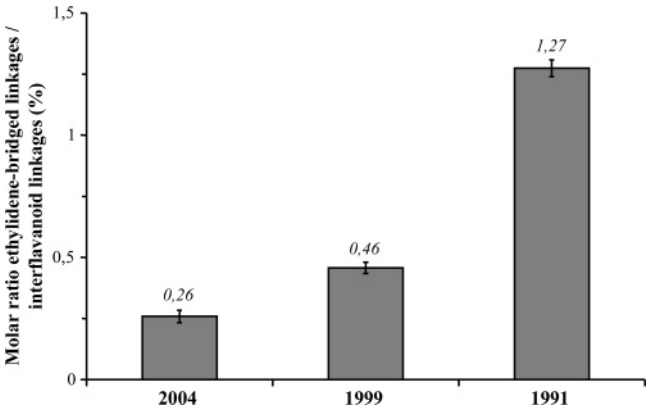

Figure 10. Molar ratio in percentage of ethylidene-bridged linkages compared to interflavonoid linkages in three Bordeaux wines (±SD, $N = 3$).

reactivity of these products to phloroglucinolysis has not been determined, it is important to elucidate this chemistry in order to fully understand the role of acetaldehyde in wine aging. Determining this is the subject of ongoing studies. Nevertheless, the results of our work shows that it should be possible to measure ethylidene-bridged flavan-3-ols directly in wines by quantifying the unique major cleavage product of phloroglucinolysis, EDP.

ABBREVIATIONS USED

EDP, 2,2′-ethylidenediphloroglucinol; 4MC, 4-methylcatechol.

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