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Study and quantification of monomeric flavan-3-ol and dimeric procyanidin quinonic forms by HPLC/ESI-MS. Application to red wine oxidation

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Abstract: Monomeric flavan-3-ols and dimeric procyanidins of red wine were oxidised by two processes, chemical and enzymatic. Unstable quinonic forms were stabilised by sulphonation, and the resulting sulphones were quantified by coupled HPLC/ESI-MS. The effects of temperature and UV light on the oxidation of phenolic compounds were also investigated. The concentration of quinones increased with increasing temperature, but the kinetics of the reaction was independent of temperature. Likewise, more quinones were formed in the presence of UV light than in its absence, but UV light also did not affect the kinetics of the reaction. Thus temperature and UV light affect the quantity of quinones formed but not the rate at which they appear.

Keywords: flavan-3-ol; procyanidin; quinone; oxidative reaction; red wine; HPLC/ESI-MS

INTRODUCTION

Phenolic compounds in wine come from grape seeds and skins, and also from wood if the wine has been matured in barrels. They can be roughly divided into two groups:

- tannins, composed of polymers of (+)-catechin and (−)-epicatechin, which are the structural units (procyanidins) of grapes, and of wood ellagitannins that have dissolved in the wine during aging in barrels;[1–3]
- anthocyanins, located exclusively in skin cell vacuoles.[4]

Phenolic compounds can be polymerised, can combine with polysaccharides, proteins and other substances and can be oxidised.[5–7] Oxidised and combined forms are essential to the quality of premium red wines: on the one hand, anthocyanin degradation by oxidation modifies the colour, with the wine taking on a brick-red hue; on the other hand, tannin polymerisation noticeably modifies the organoleptic characteristics.[8–10] Generally, the evolution of phenolic compounds, which tend to combine with other substances and become stable, is due to oxidation. Thus it is important to understand the significance of oxidation phenomena and to control their evolution in order to preserve the quality of wines. However, wine is subject to oxidation throughout the vinification process. First, during maturation, grape skins may deteriorate under the influence of *Botrytis cinerea* for instance, leading to enzymatic oxidation by laccase. Then, when the grapes are pressed, the must may be oxidised enzymatically through tyrosinase activity.[11] Finally, although protected against enzymatic oxidation by the presence of ethanol, the wine is subjected throughout vinification, maturation and aging to chemical oxidation because of the presence of iron, copper and peroxide radicals which can trigger and catalyse such reactions.

This study demonstrated the existence of oxidised procyanidins in wine. First, the quinonic forms of the pure phenolic compounds were studied. The results relate to monomeric flavan-3-ols ((+)-catechin and (−)-epicatechin) and dimeric procyanidins (B_1, B_2, etc). The oxidised forms of these compounds were then identified and quantified after sulphonation according to a method based on that of Cheynier *et al.*[12]

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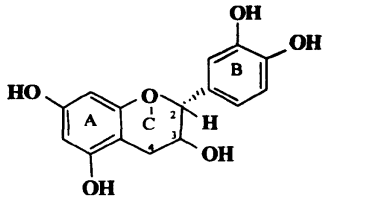
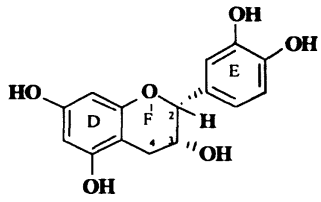
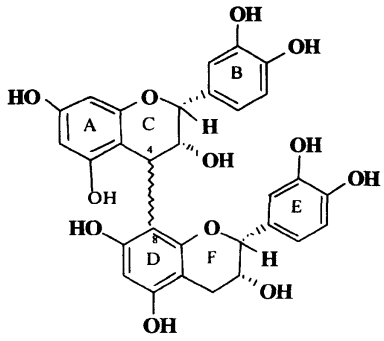


(+)-catechin



(-)-epicatechin



example of series B dimer: procyanidin B2

	natures of units and linkages	fractional purity
B1	epicatechin-(4β-8)-catechin	0.894
B2	epicatechin-(4β-8)-epicatechin	0.927
B3	catechin-(4α-8)-catechin	0.938
B4	catechin-(4α-8)-epicatechin	0.889

Figure 1. Chemical structures of phenolic compounds tested.

MATERIALS AND METHODS

Products and reagents

Monomeric flavan-3-ols ((+)-catechin and (−)-epicatechin; Fig 1) were supplied by Extrasynthése SA (Genay, France). Dimeric procyanidins were obtained by semisynthesis under the conditions described by Freitas.[13] The structure and purity of the semisynthesised products were verified by ^{1}H NMR (Bruker 400; Bruker, Wissembourg, France) and are shown in Fig 1. The two enzymes, tyrosinase and laccase, were supplied by Sigma (Saint-Quentin Fallavier, France), sodium hydrogen tartrate by Cofralab (Gradignan, France) and sodium benzenesulphinate by Fluka (Saint-Quentin Fallavier, France). Sodium hydrogen sulphite, hydrogen peroxide, copper sulphate crystals, iron sulphate crystals and the chromatographic solvents (acetonitrile and acetic acid) came from Cofralab.

Quinone formation

Quinones were obtained from the phenols by two different oxidation methods.

Enzymatic oxidation

Enzymatic oxidation was performed in the presence of air, using tyrosinase (PPO), which is present during fermentation, and laccase, which causes grape rot.

Enzymatic oxidation by PPO. PPO (1 g l^{-1}) and the phenolic compound (2 mM) were added to a solution of potassium hydrogen tartrate (2.5 g l ^{1}) at pH 3.2. The mixture was stirred for 10 min in air at ambient temperature using a magnetic stirrer.

Enzymatic oxidation by laccase. Laccase (34 mg l^{-1}) and the phenolic compound (2 mM) were added to a solution of sodium acetate (0.2 M) at pH 5.0 (adjusted with acetic acid 0.2 M). The mixture was stirred for 10 min in air at ambient temperature using a magnetic stirrer.

Chemical oxidation

The phenolic compound (2 mM) was added to a solution of potassium hydrogen tartrate (2.5 g l^{-1}) at pH 3.2 together with a few drops of hydrogen peroxide and a crystal of iron sulphate or copper sulphate. The mixture was stirred for 10 min in air at ambient temperature using a magnetic stirrer.

o-Quinone sulphonation

Sodium benzenesulphinate (2.5 g l^{-1}) was added to each of the enzymatically or chemically oxidised mixtures and mixed for about 15 s. A few drops of sodium hydrogen sulphite were then added to stop oxidation.

High-performance liquid chromatography (HPLC)

The analytical method used was similar to that described by Da Silva *et al.*[14] The column (Beckman Ultrasphere ODS® C_{18} with 5 μm packing, 250 mm × 4.6 mm id; Villepinte, Roissy CDG, France) was used in reverse phase, with a guard column of the same material (100 mm × 4.6 mm id). The eluant was a mixture of two solvents filtered through 0.45 μm membrane filters, solvent A (acetic acid/water, 1:40 v/v) and solvent B (acetonitrinile/ solvent A, 1:4 v/v), with the following elution profile:

time (min)	fraction of solvent A	fraction of solvent B
0	0.95	0.05
4	0.95	0.05
20	0.80	0.20
30	0.20	0.80
35	0.00	1.00
45	0.00	1.00
50	0.95	0.05

The programme included washing and reconditioning of the column. The flow rate was 1 ml min^{-1}. The injection volume was 20 µl (Rheodyne 7725; Thermoquest, Les Ulis, France) at a temperature of 20 ± 1 °C. Detection was performed with a variable-wavelength detector (Waters, Saint-Quentin en Yvelines, France) at 280 nm.

Mass spectrometry (MS)

A Platform II mass spectrometer (Micromass, Manchester, UK) with electrospray injection (ESI) was used, coupled to the HPLC apparatus. Polyphenols can easily shed a proton, generating intense negative ions $[M-H]^-$, detection was therefore performed in negative mode. A low voltage was used to avoid fragmentation; the products were identified by their molecular peaks.

Quantification method

The *o*-quinones formed and trapped as sulphones were quantified by integrating the chromatographic peaks corresponding to predefined masses. Because the reaction was incomplete, we took account of the unsulphonated compounds, since the initial concentrations of these reagents ((+)-catechin, (−)-epicatechin and dimeric procyanidins) were known.

The response coefficient K_r for each compound was determined as

$$K_r = C/A$$

with C the concentration (in catechin equivalents) and A the peak area. After oxidation and sulphonation the peak area of the unsulphonated compound was determined and that of the sulphonated compound was obtained by subtraction. After multiplying by the response coefficient K_r, the concentration of the sulphonated compound, ie the trapped *o*-quinone, was obtained.

RESULTS AND DISCUSSION

Principle of oxidised polyphenol quantification

To enable us to identify and quantify oxidised phenolic compounds in wine, we attempted to prepare reference quinones by oxidising pure polyphenols. However, this process led to the formation of very unstable quinones which polymerised very quickly and precipitated, making quantification impossible. To overcome this problem, the unstable quinonic forms were first stabilised by sulphonation for subsequent identification and quantification by coupled liquid chromatography/mass spectrometry (LC/MS). This involved a two-step reaction (Fig 2). First, the phenolic compounds were oxidised either chemically, in the presence of an iron or a copper salt and hydrogen peroxide, or enzymatically, by tyrosinase or laccase. The *o*-quinones thus formed were then stabilised by sulphonation with sodium benzenesulphinate, and the resulting stable sulphones were quantified.

Results obtained with monomeric flavan-3-ols

Fig 3 shows the results obtained before enzymatic oxidation of (+)-catechin, which, along with (−)-epi-

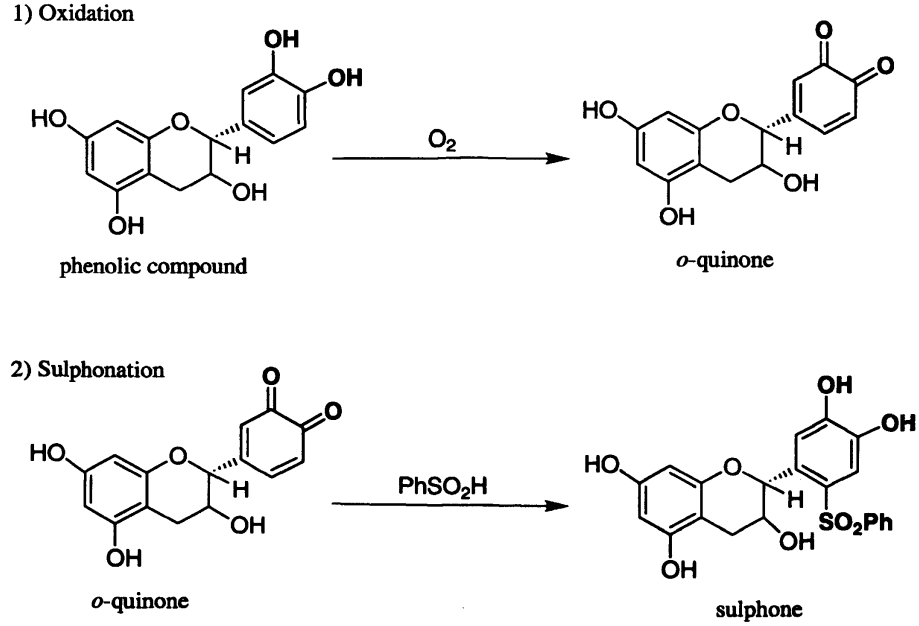


Figure 2. Principle of oxidised phenol characterisation: (1) *o*-quinone formation; (2) sulphone formation.

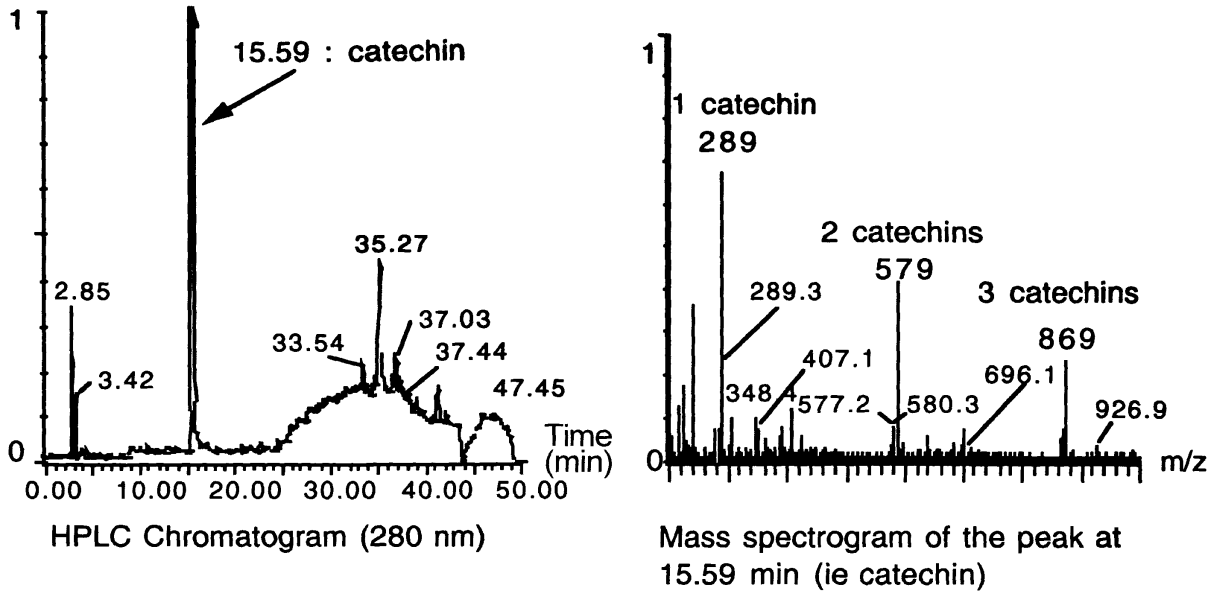


Figure 3. HPL chromatogram (280 nm) and mass spectrogram of (+)-catechin. The MS peak at *m/z* 289 represents monomeric catechin; the peaks at *m/z* 579 and 869 are due to polymers containing two and three catechin units respectively.

catechin, forms the structural units of polymerised procyanidins. Before oxidation the retention time for (+)-catechin was 15.59 min and its peak on the mass spectrogram was at *m/z* 289 as expected; additional peaks at *m/z* 579 and 869 were due to combinations of two and three catechin molecules respectively in ionic forms. After oxidation and sulphonation (Fig 4) the retention time for sulphonated (+)-catechin was 27.20 min. However, some unoxidised (+)-catechin was also found at 15.61 min, since the reaction was incomplete. Consequently, as previously, the mass spectrogram of the oxidised product showed a monomeric peak at *m/z* 429.2, but there was an additional peak at *m/z* 858.7 due to a combination of two

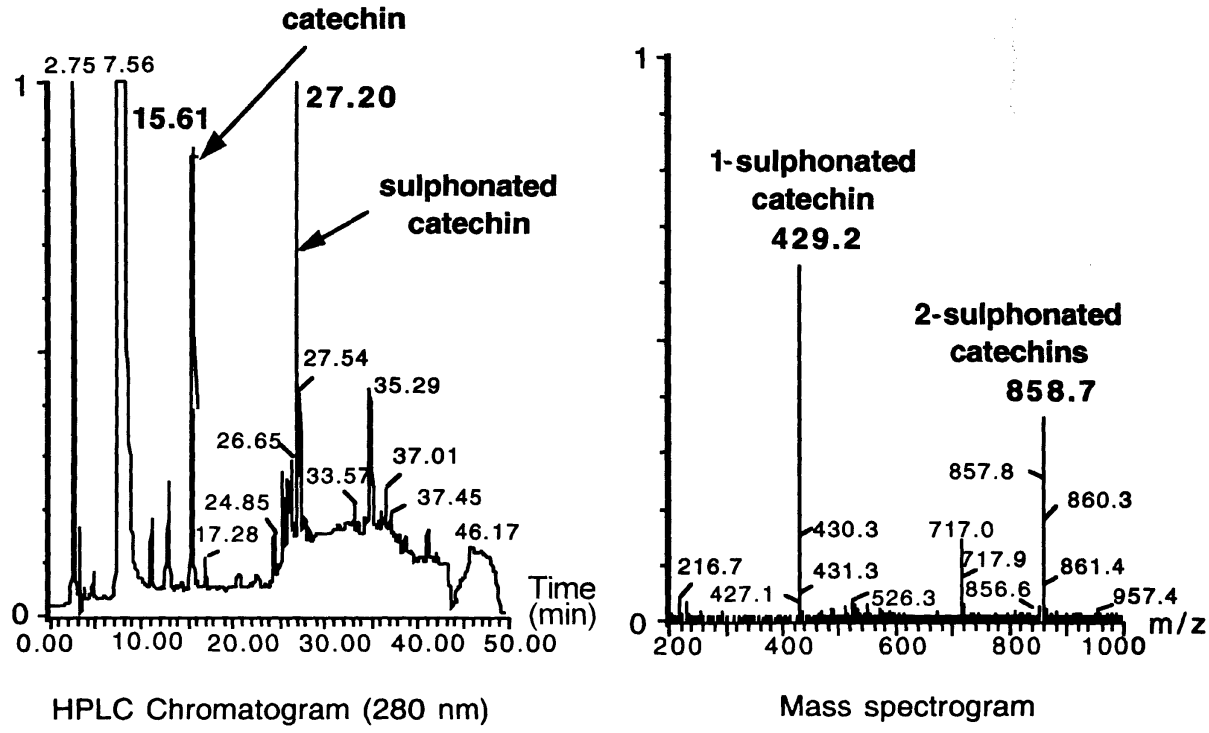


Figure 4. HPL chromatogram (280 nm) and mass spectrogram of (+)-catechin after oxidation and sulphonation. The HPLC peak at 27.20 min is due to oxidised and sulphonated catechin; the peak at 15.61 min shows the presence of unreacted catechin. The MS peaks are at *m/z* 429.2 (sulphonated monomeric quinone) and *m/z* 858.7 (sulphonated dimeric quinone).

	Enzymatic oxidation				Chemical oxidation[c]			
	Laccase		Tyrosinase		Copper		Iron	
	[q][a]	f_{ox}[b]	[q][a]	f_{ox}[b]	[q][a]	f_{ox}[b]	[q][a]	f_{ox}[b]
(+)-Catechin	2.63	0.63	2.87	0.72	3.87	0.97	3.52	0.74
(−)-Epicatechin	2.48	0.59	2.67	0.64	2.09	0.72	2.51	0.65
B_1	—	—	3.83	0.48	5.63	0.63	4.49	0.56
B_2	—	—	4.72	0.59	7.69	0.96	7.78	0.97
B_3	—	—	2.99	0.37	5.27	0.66	8.00	1.00
B_4	—	—	1.91	0.24	7.76	0.97	2.26	0.28

[a] Concentration of quinones (in catechin equivalents; 10^{-8} g l^{-1}).
[b] Oxidised fraction.
[c] By H_2O_2.

Table 1. Quantification of quinones obtained by oxidation of pure phenolic compounds

sulphonated catechin molecules. All the results for this monomer and the second structural unit, (−)-epicatechin, are summarised in Table 1. For these products, chemical oxidation catalysed by copper thus proved to be the most effective process, since the oxidised fraction of (+)-catechin was 0.97 and that of (−)-epicatechin was 0.72.

Results obtained with dimeric procyanidins

Semisynthesised dimeric procyanidins were also analysed in the same way. The results for procyanidin B_2, made up of two (−)-epicatechin units bound together by a C_4–C_8 linkage, are given below. The HPL chromatograms (detection at 280 nm) before and after chemical oxidation (Fig 5) showed that B_2 appeared at 17.7 min and sulphonated B_2 at 26.3 min. Here also the presence of unoxidised B_2 on the chromatogram after sulphonation showed that the reaction was incomplete. The mass spectrogram of B_2 after enzymatic oxidation and sulphonation is shown in Fig 6. A

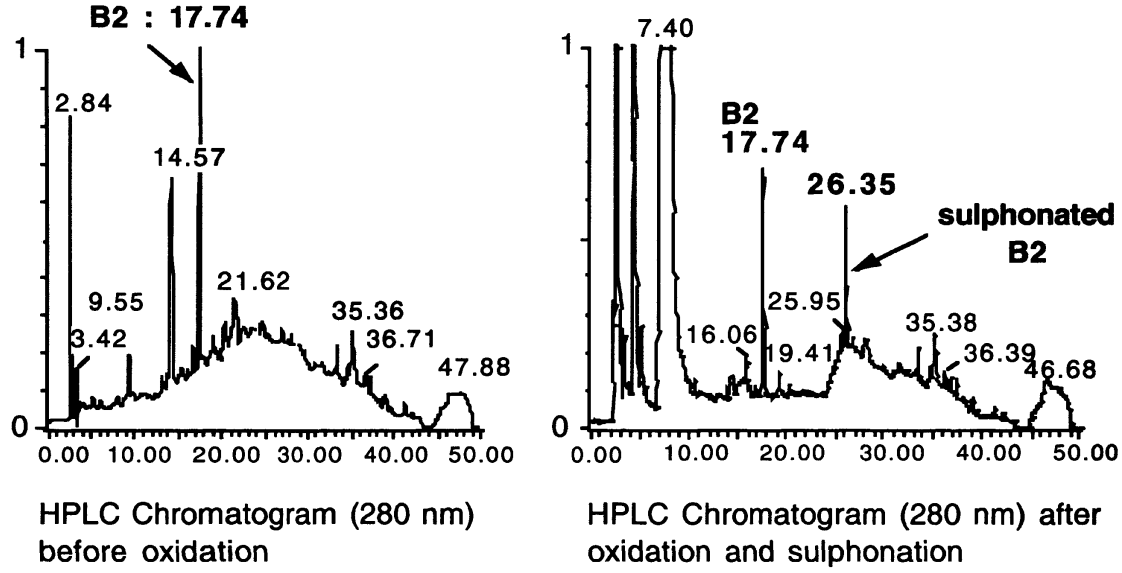


Figure 5. HPL chromatograms (280 nm) of dimeric procyanidin B_2 before and after chemical oxidation. The peak at 17.74 min (left-hand diagram) represents dimeric procyanidin B_2. The right-hand figure shows peaks at 17.74 min (unreacted B_2) and 26.35 min (sulphonated quinone).

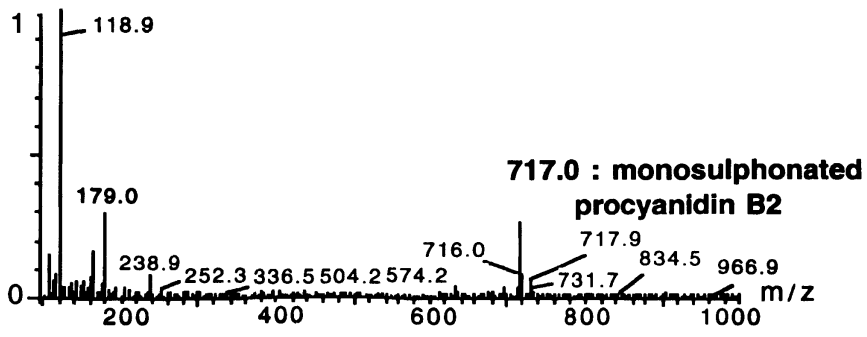


Figure 6. Mass spectrogram of dimeric procyanidin B_2 after enzymatic oxidation and sulphonation. The peak at *m/z* 717.0 shows that only one structural unit has been oxidised and sulphonated.