Solid-State ^{13}C NMR Investigation into Insoluble Deposits Adhering to the Inner Glass Surface of Bottled Red Wine

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^{13}C nuclear magnetic resonance spectroscopy with cross polarization and magic angle spinning was used to analyze insoluble, lacquer-like pigmented deposits that adhere to the inner glass surface of bottled red wines. The deposits were found to be composed of a phenolic polymer of anthocyanins, procyanidins, and protein. Glucose was also present, probably as the glucoside moiety of the anthocyanidin units involved in the phenolic polymer. Formation of the deposit is likely to result from extensive cross-linking between the phenolic polymer and protein, rendering the complex insoluble.

Keywords: *NMR; red wine instability; tannin; protein; anthocyanin*

INTRODUCTION

In recent years a new type of red wine instability problem, evident as a lacquer-like pigmented deposit adhering to the inner bottle surface, has been increasingly observed in some Australian and other red wines (Figure 1). Deposition can begin in the first few months after bottling and may eventually cover the entire glass surface with which the wine is in contact. Wine quality appears to be unaffected by formation of the deposit. Nevertheless, such deposit formation cannot be regarded as a positive attribute commercially, and its presence in bottled wines is totally unacceptable in some markets. Response by Australian winemakers to a questionnaire on the subject in 1990 indicated that the problem affects, with considerable irregularity, about 5–10% of bottled red wines and has occurred in wines made from all major black grape varieties and produced in all winemaking regions (T. C. Somers, personal communication). The problem has been observed in New Zealand, French, Italian, and Portuguese red wines and is thus unconfined to any viticultural region or wine producer. This form of instability contrasts with the often common occurrence of sediments in aged red wines resulting from the precipitation of, predominantly, potassium hydrogen tartrate.

The deposit, which can occur in amounts of up to 50 mg/bottle, in insoluble in water and ethanol but soluble in alkali. The material appears to be polymeric and was assumed to have resulted from some form of phenolic instability, although the intractable nature of the deposit severely restricted earlier work on diagnosing the cause of deposition. However, the availability of solid-state high-resolution ^{13}C nuclear magnetic resonance (NMR) with cross polarization and magic angle spinning (CP/MAS) has allowed a new perspective on the problem. This paper describes the composition of the deposit, as elucidated by solid-state ^{13}C CP/MAS NMR spectroscopy together with other analytical techniques and discusses some possible mechanisms for its formation.

Figure 1. Empty commercial red wine bottle demonstrating the lacquer-like bottle deposit.

MATERIALS AND METHODS

Sample Preparation. The lacquer-like bottle deposit samples were obtained from commercial wines by carefully decanting most of the wine from the bottles and then detaching the deposit from the glass surface with the aid of a bottle brush. The deposit, suspended in wine, was transferred to a centrifuge bottle and centrifuged (1400*g*, 30 min, 4 °C), washed with water, and lyophilized.

Bottle deposit 1 was a combination of lacquer-like bottle deposits from three different commercial Australian wines from the 1990, 1988, and 1980 vintages. Bottle deposits 2 and 3 were each sourced from individual commercial Australian wines from the 1987 and 1988 vintages, respectively. Bottle

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deposit 3 (210 mg) was dissolved in DMSO (10 mL) and reprecipitated by the addition of water (500 mL). The reprecipitated deposit was collected by centrifugation (27000*g*, 60 min, 10 °C), washed with water, and lyophilized (yield 101 mg).

A crystalline tartrate sediment was obtained by carefully decanting and discarding most of the wine from a bottle of commercial Australian red wine which did not show the lacquer-like deposit and then centrifuging the turbid residue as above but omitting the water wash steps before lyophilization.

White wine protein haze was prepared by heating (80 °C, 6 h) a protein-unstable Muscat of Alexandria wine from the 1993 Australian vintage. The resultant haze was collected by centrifugation (18000*g*, 30 min, 4 °C), washed with water, and lyophilized.

Analytical Methods. Amino acid composition was determined, after hydrolysis with 6 M HCl (110 °C, 6 h under nitrogen) by reversed-phase HPLC using the PicoTag method (Cohen et al., 1986).

Neutral monosaccharide composition was determined, after hydrolysis with 2 M trifluoroacetic acid (120 °C, 75 min) (Albersheim et al., 1967), by gas chromatography [200 °C for 5 min, then 2 °C/min for 15 min; DB-1701 column (J&W Scientific Inc., Folsom, CA)] of the alditol acetate derivatives (Harris et al., 1984).

Uronic acid composition was determined, after hydrolysis with 2 M trifluoroacetic acid (120 °C, 75 min) (Albersheim et al., 1967), by gas chromatography [130 °C for 3 min, then 4 °C/min for 23 min; DB-5 column (J&W Scientific)] of the trimethylsilyl derivatives. The trimethylsilyl derivatives were prepared by derivatization of the dry hydrolysate with TriSil Z reagent (200 μL, 60 °C, 30 min, Pierce, Rockford, IL).

The presence of condensed tannin (procyanidin) in the deposit was implied from the formation of cyanidin after hydrolysis in methanol/6 M HCl (1:1 v/v, 100 °C, 30 min) (Porter et al., 1986; Scalbert, 1992). Cyanidin and other anthocyanidins were detected in the hydrolysate after separation by HPLC using a Spherisorb C_{18} ODS-2 column (5-μm particle size, 250 × 4.6 mm, ICI, Australia) eluted with a gradient of 0.6% perchloric acid (solvent A)/methanol (solvent B) at 1.5 mL/min and 35 °C with on-line detection at 520 nm. Gradient conditions were as follows: 20–35% B from 0 to 10 min, 35% B from 10 to 15 min, 35–70% B from 15 to 30 min, 70–100% B from 30 to 35 min.

Elemental analysis was performed by the Australian Microanalytical Service, Victoria.

^{13}C CP/MAS NMR Analyses. The 50.309-MHz CP/MAS spectra of samples (100–300 mg) were obtained on a Varian Unity 200 spectrometer with a 4.7-T wide-bore Oxford superconducting magnet. Samples were spun at 5 kHz in 7-mm-diameter zirconia rotors with Kel-F caps in a Doty Scientific MAS probe. All spectra were obtained with a 1-ms contact time and a 300-ms recycle time. The number of transients required for acceptable S/N ranged from 1000 to 10 000. Using the standard Varian pulse sequence, the free induction decays were acquired over a spectral width of 40 kHz with an acquisition time of 20 ms in a 1600-point database. All spectra were obtained with 32K zero filling, and between 10- and 20-Hz Lorentzian line broadening and 0.010–0.015-s Gaussian broadening were applied prior to transformation. Chemical shift assignments were externally referenced to the methyl resonance of hexamethylbenzene at 17.36 ppm. Protonated carbon suppression spectra were obtained using a dephasing delay of 41 μs (Alemany et al., 1983).

RESULTS

Solid-state ^{13}C CP/MAS NMR spectroscopy of a mixture of deposits harvested from three commercial red wines (deposit 1) gave a spectrum (Figure 2) that showed well-defined signals for at least three different structural categories, *i.e.*, protein, phenolic, and carbohydrate.

The presence of protein in the bottle deposit was implicit from the large amide carbonyl signal centered at a chemical shift of 174 ppm together with an envelope of aliphatic carbon signals consistent with methyl and methylene groups of valine, leucine, isoleucine, *etc.*, at 40–20 ppm in the spectrum (Baianu, 1989). Elemental analysis of deposit 1 showed that it contained 3.7% nitrogen (Table 1), and amino acid analysis after acid hydrolysis (Table 2) demonstrated that 22% of the weight of the deposit could be accounted for by amino acids, further confirming the presence of peptidic material. The major amino acids in the deposit were glycine, aspartic acid/asparagine, serine, and threonine.

To support the assignment of the protein signals in the spectrum of deposit 1, a solid-state ^{13}C CP/MAS NMR spectrum of a white wine protein haze was obtained (Figure 3). This spectrum showed a large carbonyl signal at 173 ppm and an envelope of aliphatic carbon signals at 40–20 ppm corresponding to the aliphatic amino acid side chains. There were also signals in the *O*-aryl and aryl region of the spectrum (117–157 ppm) assignable to the carbons of aromatic amino acids including histidine (Baianu, 1989).

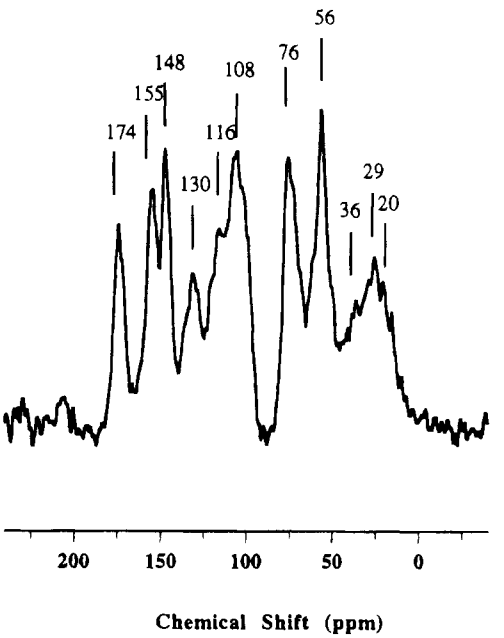

Figure 2. Solid-state ^{13}C CP/MAS NMR spectrum of commercial red wine bottle deposit 1.

Table 1. Elemental Analysis of Red Wine Bottle Deposit 1

element	% by mass	element	% by mass
carbon	52.4	sulfur	1.1
oxygen	36.1	ash	0.8
hydrogen	5.4	phosphorus	0.5
nitrogen	3.7		

Table 2. Amino Acid Composition of Red Wine Bottle Deposit 1

amino acid	molar %	% by mass	amino acid	molar %	% by mass
Asx	15.5	3.9	Val	5.0	1.1
Glx	9.1	2.6	Met	1.1	0.3
Ser	13.0	2.5	Cys	0.4	0.1
Gly	21.0	2.7	Ile	2.3	0.6
His	0.6	0.3	Leu	3.5	0.9
Arg	2.9	1.0	Phe	4.0	1.3
Thr	10.0	2.2	Lys	0.3	0.1
Ala[a]	8.3	1.3	total	100.0	22.0
Tyr	3.2	1.1			

[a] Coelution with proline.

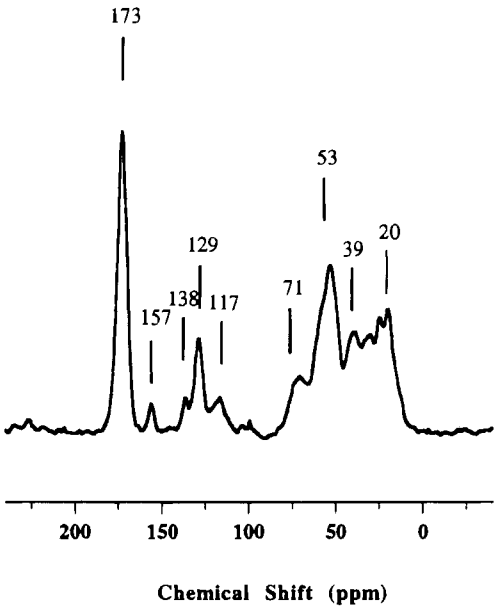

Figure 3. Solid-state ^{13}C CP/MAS NMR spectrum of a white wine protein haze.

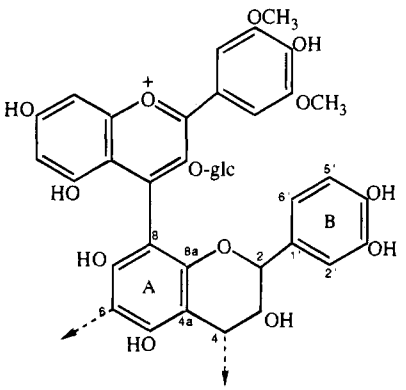

Figure 4. Representation of a red wine tannin pigment complex made up of malvidin 3-glucoside and catechin units, after Somers (1971).

The spectrum of red wine bottle deposit 1 (Figure 2) also revealed the presence of flavonoids. Clearly evident were signals centered at 155 and 148 ppm and in the region 130–108 ppm corresponding to *O*-aryl and aryl carbons, respectively, such as are present in condensed tannins (Newman and Porter, 1992). In particular, the signals at 155 ppm were assigned to the O-substituted carbons at positions 5, 7, and 8a of procyanidins. Similarly, the signals at 148 ppm were assigned to the O-substituted carbons at positions 3′ and 4′, those at 130 ppm were assigned to C1′, at 116 ppm to C2′, C5′, and C6′, and at 108 ppm to C4a (and C6 and C8 when these carbons are involved in interflavin linkages, refer to numbering system in Figure 4).

The presence of anthocyanin units incorporated into the phenolic polymer (see Figure 4), as suggested by the highly pigmented nature of the deposit, was supported by the strong methoxyl signal at 56 ppm assignable to malvidin, peonidin, and petunidin units. Other signals contributed by anthocyanins were combined with those of condensed tannin, *i.e.*, C5 and C8a at 155 ppm, C3 at 148 ppm, C4 at 130 ppm, C4a at 116 ppm, and C6 and C8 at 108 ppm (Rüedi and Hutter-Beda, 1990; Pedersen et al., 1993).

A protonated carbon suppressed spectrum of deposit 1 (data not shown) clearly indicated that the signals at 155, 148, and 174 ppm were unsuppressed and thus

Table 3. Sugar Composition of Red Wine Bottle Deposit 1

sugar[a]	% by mass	sugar[a]	% by mass
glucose	6.9	galactose	0.3
mannose	0.6	galacturonic acid[b]	<0.1
arabinose	0.3		

[a] Determined as alditol acetate derivatives. [b] Determined as trimethylsilyl derivatives.

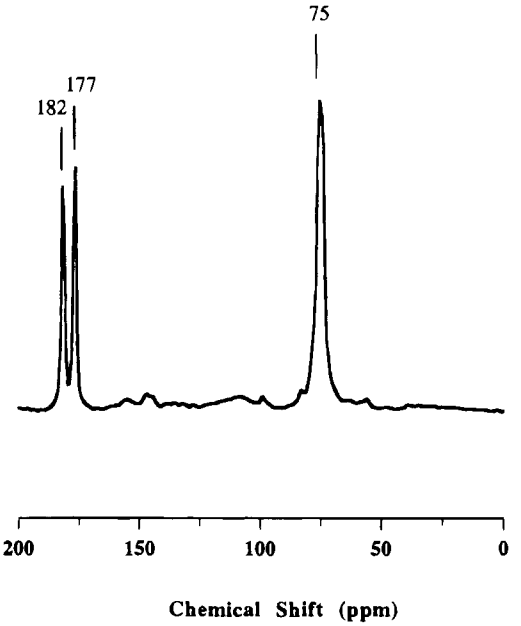

Figure 5. Solid-state ^{13}C CP/MAS NMR spectrum of a crystalline sediment from a commercial red wine.

from carbon atoms not bearing a proton substituent, as assigned above. The partial suppression of the signal at 56 ppm was consistent with the presence of methoxyl moieties in deposit 1 (Alemany et al., 1983).

Nonanomeric carbohydrate carbons were also present in deposit 1 as indicated by the signal at 76 ppm in Figure 2. (The signals for anomeric carbons were at *ca.* 100 ppm.) Analysis of the carbohydrates liberated by hydrolysis of bottle deposit 1 with trifluoroacetic acid showed that it contained glucose and traces of other sugars (Table 3).

Since bottled red wines often contain crystalline sediments of tartrate salts, a solid-state ^{13}C CP/MAS NMR spectrum of such a crystalline sediment was investigated. This experiment allowed a determination of any possible contribution that the presence of tartrate may make to the signal at 76 ppm in the spectrum of deposit 1. The solid-state spectrum of a crystalline sediment from a commercial red wine is given in Figure 5. This sample showed two signals corresponding to the carbonyl groups of the hydrogen tartrate anion at 177 and 182 ppm as well as the signal at 75 ppm corresponding to the two O-alkylated carbons. Although the spectrum of the red wine bottle deposit 1 (Figure 2) showed a signal at 76 ppm, the absence of the characteristic pair of low-field carbonyl signals for tartrate indicated that tartrate made no significant contribution to the 76 ppm signal in this spectrum.

A second bottle deposit sample was obtained from another commercial red wine (bottle deposit 2), and this was studied by solid-state ^{13}C CP/MAS NMR spectroscopy (Figure 6) for comparison with the data given above. The spectrum of this sample showed three carbonyl signals, *i.e.*, at 182 ppm, at 177 ppm for

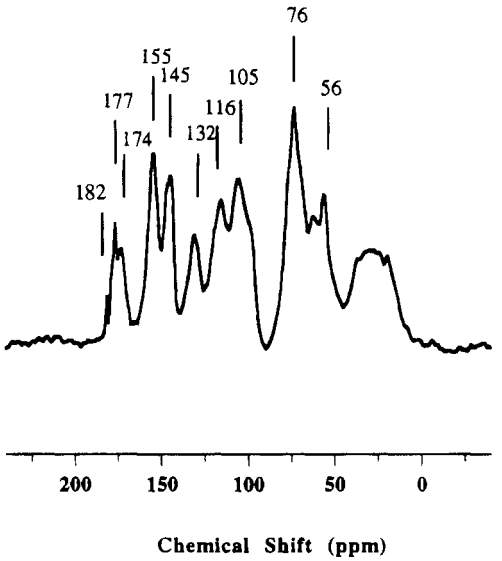

Figure 6. Solid-state ^{13}C CP/MAS NMR spectrum of commercial red wine bottle deposit 2, comprising both crystalline and lacquer-like material.

tartrate, and at 174 ppm for protein, together with other signals similar to those of deposit 1. This established the presence in deposit 2 of tartrate as well as the protein/polyphenolic/carbohydrate complex found in deposit 1.

A third bottle deposit sample was also studied (bottle deposit 3). The ^{13}C CP/MAS NMR spectrum of this sample (data not shown) was very similar to that of bottle deposit 1 and showed signals at 174, 155, 145, 131, 116, 106, 76, 56, 31, and 21 ppm, again indicating that bottle deposit 3 was composed of protein, procyanidins, anthocyanins, and carbohydrate. The presence of a tannin pigment complex in the deposit was further confirmed by the finding that the deposit yielded predominantly cyanidin, after hydrolysis in dilute mineral acid, and also malvidin and dephinidin (Somers, 1966).

Bottle deposit 3 was soluble in DMSO but reprecipitated as a pigmented species on addition of water. The ^{13}C CP/MAS NMR spectrum (data not shown) of this reprecipitated material was identical to that of the original deposit.

DISCUSSION

The nature of red wine polyphenolic polymers has been a subject of interest for many years, but due to the complexity and large size of the polymers, their structures have remained essentially conceptual and speculative. Recent experiments (Singleton and Trousdale, 1992) have demonstrated that anthocyanins quickly complex with, and are incorporated into, the condensed tannins that originate from the seeds and skins of grapes. These results further support data from earlier experiments showing that the pigmented polymeric phenolics of red wines were composed of procyanidins and anthocyanins (Somers, 1966, 1971). The use of solid-state ^{13}C CP/MAS NMR to analyze insoluble pigmented bottle deposits in this work has confirmed that such hybrid polymers are present in red wines. This is because the spectra of deposits 1 and 2 shown here, and of other similar red wine bottle deposits that we have examined, displayed signals characteristic of both procyanidins and anthocyanins. Furthermore, hydrolysis of deposit 3 in dilute mineral acid yielded cyanidin, indicative of procyanidins, in addition to the anthocyanin aglycons, delphinidin and malvidin. The presence of delphinidin in the hydrolysate could also be due to prodelphinidin units in the condensed tannin (Haslam, 1980).

The strength of the 56 ppm signals seen in the solid-state ^{13}C CP/MAS NMR spectra, and assigned to methoxyls on ring B of anthocyanin units in the polymer, indicated that the amount of pigment in the deposit was significant. This assignment follows from the recognition that one of the features distinguishing the major anthocyanins (malvidin, petunidin, and peonidin derivatives) as a group from other flavonoid derivatives of black *Vitis vinifera* grapes is that they contain methoxylated B rings (Singleton, 1988, and references therein). Although oak-derived methoxylated lignins would also contribute to the 56 ppm signal, their presence in the deposit seemed improbable. This is because other work has shown no evidence of de- or repolymerization or chemical alteration of oak lignin oligomers over 30 years of aging of cognacs and brandies (Viriot et al., 1993). The possibility that the methoxyl signals were contributed by other grape or wine components was unlikely. The lack of uronic acids in the deposit after acid hydrolysis demonstrated that methoxylated pectins were not present in the deposit. Ferulic acid is a minor compound of grapes and is not known to be involved in polymers. Methoxylated lignans have been observed as constituents of grapes but only in trace quantities (Marinos et al., 1992). Furthermore, the presence of malvidin after hydrolysis of the deposit further supports the assignment of the 56 ppm methoxyl signals to anthocyanin units in the deposit.

Although the mechanism of the polyphenolic polymerization in red wines is still uncertain, it is generally believed to be due to an acid-catalyzed condensation of procyanidins and anthocyanins in addition to some contribution from the Baeyer reaction in which $-CH(CH_3)-$ bridges between phloroglucinol rings are formed from acetaldehyde (Haslam, 1980; Bakker et al., 1993). However, in the spectra of the red wine bottle deposits examined here, the absence of a signal at 43 ppm for a bisbenzylic carbon atom indicated that acetaldehyde had a small involvement, if any, in the precipitates.

The relative proportion of the *O*-aryl signals at 155 and 145 ppm characteristic of condensed tannins, the methoxyl signal at 56 ppm of anthocyanins, and the amide carbonyl signal at 173 ppm corresponding to protein were altered between the red wine bottle deposits 1 and 2 (Figures 2 and 6) and also among other, similar, red wine bottle deposits that we have examined. It is thus apparent that red wine bottle deposits can have differing proportions of procyanidins, anthocyanin units, and protein.

It is well-known that condensed tannins react with proteins (Haslam, 1989), and it is commonly assumed that in red wines such interaction is rapid and that the high levels of phenolic compounds present quickly react with, and precipitate, any grape and yeast protein (Singleton and Trousdale, 1992). Nevertheless, protein has been measured, albeit at a low level, in red wine (Yokotsuka et al., 1977). The prospect of protein complexed with phenolic polymers as being the components of insoluble and lacquer-like bottle deposits, which form months or even years after the red wine is made, has not been previously reported. Since reprecipitation of the bottle deposit did not alter its composition, it

appears that the protein is strongly associated with, and probably covalently bound to, the phenolic polymer.

The general mechanism of irreversible complexation of polyphenolic compounds with proteins has been discussed by Haslam (1989). Under acid conditions, the interflavan bond of proanthocyanidins is readily cleaved and a carbocation intermediate is formed at the point of rupture (Beart et al., 1985). These electrophilic species react readily with nucleophilic groups on proteins such as the thiol group of cysteine and the hydroxyl groups of serine and threonine to give proanthocyanidins covalently linked to protein. At higher pH, the ϵ-amino group of lysine, the N-terminal amino groups, and the imidazole group of histidine could also be involved. It has been postulated that this mechanism is responsible for the formation of protein hazes in beer (Beart et al., 1985). It is likely that the same process also operates in red wine with pigmented copolymers of procyanidins and anthocyanins providing the electrophilic species. In this way, an extensive cross-linking of the protein, via its numerous reactive side chains, with the pigmented tannin polymer could occur. Such cross-linking would ultimately render the complex insoluble and would ensure that dissolution and reprecipitation, as shown here, would not change the composition of the deposit.

That bottle deposit 1 contained protein was indicated by ^{13}C CP/MAS NMR and elemental analysis (Figure 2; Table 1) and confirmed by amino acid analysis after acid hydrolysis of the deposit (Table 2). The amino acid composition of the deposit was similar to that of white grape proteins in having aspartic acid/asparagine, glycine, serine, and threonine as major components (Waters et al., 1992), indicating that the protein was of grape origin. Of the amino acids likely to be involved in cross-linking to polyphenolic polymers, serine and threonine were present in significant amounts. Cysteine was not detected in large amounts, a result not inconsistent with its involvement in cross-linking of the protein to the polyphenolic polymer, since thioether bonds would be resistant to acid hydrolysis (Beart et al., 1985). The elemental analysis also showed that the sulfur content of the deposit, at 1.1%, was high. This may indicate an extensive involvement of sulfur-containing amino acids in the copolymer, or alternatively, the sulfur content in the deposit could be due to SO_2 substituted into the anthocyanin components of the deposit (Jurd, 1964).

The deposit contained carbohydrate, but it seems unlikely that this was due to the involvement of wine polysaccharides. This is because the sugar composition of the deposit was dominated by glucose with only traces of the sugars that are the components of the major wine polysaccharides, *e.g.*, arabinose, galactose, and mannose (Llaubères et al., 1987; Brillouet et al., 1990; Schmitt et al., 1992). The glucose found seems most likely to be the glucose moiety of the anthocyanin units in the polymer (see Figure 4). As discussed above, pectins were not involved.

Red wine bottle deposits of the type investigated here have been noticed with increasing frequency in Australian wines since the late 1970s, and while detailed records of incidence are not available, it seems that this particular instability problem was not a feature of earlier red table wine production. As discussed above, red wine tannin–protein complexation would have always occurred, and probably rapidly, but contemporary winemaking practices could have made that interaction much slower. Deposits that would have precipitated while the wine was still in the barrel may now be occurring after the wine has been bottled. Over the past 20 years there have been numerous changes in Australian winemaking practice and technology, and any one of these could contribute to the problem. The more commonly used techniques in the past of aerobic handling, which favors the formation of reactive quinones from phenolic compounds, and fermentation without temperature control are both expected to accelerate cross-linking and thus cause deposition before bottling. These practices have now been largely replaced by more anaerobic handling practices and temperature-controlled fermentation and storage. In addition, the use of processes such as mechanical harvesting, which have been shown to retain grape protein in white must (Paetzold et al., 1990), may be responsible for the increasing presence of protein in red wines at bottling and thus the formation of deposits in the bottle.

Our success in understanding how these many processes affect red wine tannin–protein interaction will allow us to devise methods to prevent such deposition in commercial bottled wines.

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