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Rapid Isolation of Red Wine Polymeric Polyphenols by Solid-Phase Extraction

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A rapid technique for the isolation of polymeric polyphenols from red wine has been developed and validated. A copolymer reversed-phase SPE cartridge was utilized in conjunction with predominantly organic eluents to provide three phenolic fractions from red wine without the need for sample pretreatment. The first fraction contained the bulk of the monomeric and oligomeric phenolic material, while the second and third fractions contained the polymeric polyphenolic compounds, as determined by HPLC analysis. The two polymeric polyphenolic fractions differed in their solubility and extent of pigmentation, and the differences appeared to be related to wine age. This method contrasted with other available fractionation techniques because the interfering, nonpolymeric material can be removed in a single wash fraction, while the polymeric material is separated into two distinct fractions based on their diverse physicochemical properties. It is anticipated that the rapid access to discrete polymeric fractions afforded by this method will be of benefit in furthering the understanding of red wine polymeric polyphenols.

KEYWORDS: Red wine; polymeric polyphenols; solid-phase extraction; physicochemical properties; wine age

INTRODUCTION

A myriad of phenolic compounds are found in red wine which impart color, taste, flavor, and mouthfeel properties. The types of phenolics present impact the sensory qualities of a wine and contribute to the level of liking of a particular wine style. Additionally, phenolic compounds present in red wine have important roles in wine aging and contribute to the beneficial health properties associated with red wine consumption. Of particular interest in these regards are the polymeric polyphenols, which are the dominant class of phenolic compounds encountered in red wine. Polymeric polyphenols, a term synonymous with wine tannins and which includes polymeric pigments, encompass a diverse and complex range of structures and are derived from grape proanthocyanidins, with the incorporation of reactive components, including other phenolics, encountered in the wine medium (*1, 2*).

The analysis of red wine polymeric polyphenols attracts a high level of attention, but much remains to be understood regarding wine polymeric polyphenol composition and structure. Factors which complicate the analysis include the large number and variety of phenolic components present, which makes direct analysis of a red wine, whether by HPLC or other means, a difficult task. The separation of nonpolymeric and polymeric polyphenolic material is therefore desirable and has been addressed in recent years. Several techniques are available to separate red wine into fractions that are more easily analyzed, and these typically utilize low-pressure chromatography sorbents such as LH-20 (*3–6*), Toyopearl (*7–11*) and to a lesser extent polyamide resin (*12, 13*), and C18 mini-column (*11*) or solid-phase extraction (SPE) cartridges (*13–16*). Isolation of phenolics and other organic components from red wine has also been undertaken using SPE on C18 (*17–20*) or other reversed-phase polymer cartridges (*21–25*), but these reports tend to focus on obtaining certain classes of compounds without accounting for all the phenolics present.

Improvements are continually being sought for the separation of polymeric and nonpolymeric polyphenolic material from red wine. There is still no ideal method, and the difficulty is perhaps due to attempting to extract and fractionate components from such a complex matrix with only one technique. As observed by Pinelo et al. (*16*), while LH-20 is a useful fractionation medium, the technique is neither rapid nor straightforward, and the same applies to separations using Toyopearl. These sorbents are perhaps best left to fractionation of phenolic material after it has been isolated from a wine using a simpler technique. Chromatography on polyamide resin is less commonly used and is more appropriate for the isolation of monomers and oligomers, as the polymeric polyphenolic material binds irreversibly to the sorbent (*13*).

Solid-phase extraction (SPE) cartridges appear to be more suited to the isolation of classes of components from a red wine compared to the low-pressure chromatographic sorbents mentioned above. Most often, however, SPE is used for isolation

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of a single compound or a small class of compounds from a matrix and not for fractionation of a multitude of components. Nonetheless, the use of C18 SPE with red wine has proved effective, but the technique requires dealcoholization and usually pH adjustment of the wine prior to loading onto the cartridge. Polymer SPE material is an attractive alternative, as it allows for direct loading of a wine sample and has a higher loading capacity than silica-based C18 due to the larger surface area of the polymer sorbent. Interestingly, little attention has been devoted to the use of polymer SPE sorbents to separate polymeric from nonpolymeric red wine components. The focus of this work, therefore, was to investigate the use of a polymer SPE sorbent to provide a rapid technique for the isolation of polymeric polyphenolic components directly from red wine, which could then be analyzed as isolated or further fractionated as required.

MATERIALS AND METHODS

Chemicals. All chromatographic solvents were high-performance liquid chromatography (HPLC) grade. All chemicals were analytical reagent grade unless otherwise stated, and water was obtained from a Milli-Q purification system. Acetone (Merck), acetonitrile (Merck), ethanol (Merck), ethyl acetate (Merck), methanol (Merck), propan-2-ol (Merck), formic acid (98–100%, Merck), acetic acid (100%, Merck), orthophosphoric acid (85%, Ajax Fine Chemicals), and hydrochloric acid (HCl, 32%, Ajax Fine Chemicals) were all purchased from Rowe Scientific (Lonsdale, SA, Australia), and cysteamine hydrochloride (≥98%, titration) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All prepared solutions were % v/v with the balance made up with Milli-Q water, unless otherwise specified.

Wine Samples. Thirty commercially available Australian red wines were assessed over the course of the study, the majority being Cabernet Sauvignon and Shiraz, with vintages ranging from 1995 to 2006. Wine samples were centrifuged in a Thermo Electron Corp. IEX Micromax microcentrifuge (Biolab, Mulgrave, Vic, Australia) at 4000 rpm (1500 rcf) for 5 min prior to any extraction or analysis.

Solid-Phase Extraction: Preliminary Assessments.

Hydroalcoholic Eluents. Methanol and water mixtures were screened to determine the retention characteristics of various phenolics on the following polymer cartridges: Oasis HLB, MAX, MCX, WAX, and WCX (3 mL, 60 mg, 30 μm) (Waters, Rydalmere, NSW, Australia), and Strata-X (3 mL, 200 mg) (Phenomenex, Lane Cove, NSW, Australia). The cartridges were conditioned with methanol followed by water, using the volumes specified by the supplier for the given bed mass. The wine sample (1 mL) was diluted with 0.1 M HCl (1 mL) and applied to the cartridge under gravity. All cartridges were eluted sequentially (keeping the fractions separate) with 5 mL each of water (F1aq), 25% methanol (F2aq), 50% methanol (F3aq), 75% methanol (F4aq), and 99% methanol (F5aq), with each eluent containing 1% formic acid. The solvent was removed in vacuo on a rotary evaporator at 40 mbar with a 30 °C water bath. The samples were dissolved in 1 mL of 10% aqueous ethanol containing 0.1% formic acid for HPLC analysis.

Organic Eluents. Acetonitrile (or ethyl acetate) and methanol mixtures were screened to determine the retention characteristics of various phenolics on the following polymer cartridges: Oasis HLB (3 mL, 60 mg, 30 μm) (Waters, Rydalmere, NSW, Australia), Bond Elut Plexa (3 mL, 30 mg), Focus (3 mL, 20 mg), and Nexus (6 mL, 200 mg) (Varian, Mulgrave, VIC, Australia). The cartridges were conditioned with methanol followed by water, using the volumes specified by the supplier for the given bed mass. The wine sample (1 mL) was diluted with 0.1 M HCl (1 mL) and applied to the cartridge under gravity. The cartridge was dried with a gentle stream of nitrogen gas for 5 min. All cartridges were eluted sequentially (keeping the fractions separate) with 5 mL each of acetonitrile (or ethyl acetate) (F1org), 95% acetonitrile (or ethyl acetate)/5% methanol (F2org), and methanol (F3org), while the remainder of the phenolic material was eluted with 300 μL of neat formic acid followed by 2.7 mL of 95% methanol (F4org). The solvent was removed in vacuo on a rotary evaporator at 40 mbar with a 30 °C water bath, and the fraction containing formic acid (F4org) was dried further with a gentle stream of nitrogen gas. The samples were dissolved in 1 mL of 10% ethanol/0.1% formic acid for HPLC analysis, with the exception of F4org. Once dried, F4org had 10 μL of formic acid added, followed by a similar amount of 10% ethanol/0.1% formic acid. This small volume of solvent was used to dissolve the sample prior to the addition of the remainder of the 10% ethanol/0.1% formic acid to give a final volume of 1 mL.

Solid-Phase Extraction: Optimized Method. An Oasis HLB cartridge (3 mL, 60 mg, 30 μm) (Waters, Rydalmere, NSW, Australia) was utilized as follows: The cartridge was conditioned with 2 mL of methanol followed by 2 mL of water, with the water being left level with the top frit of the cartridge (*important*, otherwise successful loading may be impeded). The wine sample (1 mL) was applied to the cartridge under gravity. When the wine volume was completely adsorbed, the cartridge was dried with a gentle stream of nitrogen gas (*important*, as any residual liquid left at this stage will affect the separation; usually required 5 min of drying). The cartridge was washed with 40 mL of 95% acetonitrile/5% 0.01 M hydrochloric acid (F1 - contained phenolic acids, nonpolymeric flavanols, flavonols, anthocyanins, and pigmented monomers; ranged from bright pink to orange in color, depending on wine age) and eluted with 5 mL of methanol containing 0.1% formic acid (F2 - polymeric polyphenols; deep red in color), followed by 300 μL of neat formic acid prior to 2.7 mL of 95% methanol (F3 - polymeric polyphenols; deep red to red/brown in color). The solvent was removed in vacuo on a rotary evaporator at 40 mbar with a 30 °C water bath, and the fraction containing formic acid (F3) was dried further with a gentle stream of nitrogen gas. The samples were dissolved in 1 mL of 10% ethanol/0.1% formic acid for HPLC analysis, with the exception of F3. Once dried, F3 was treated in the same manner as when organic eluents were preliminarily assessed, as detailed for F4org.

Cartridge Reuse. After F3 was eluted, the cartridge was washed with 2 mL of water, conditioned, and loaded in the usual manner, noting the important points. Care must be taken, however, to ensure the sorbent does not dry out completely (such as from overnight storage) or the sorbent bed will compact and the SPE method will not be successful.

HPLC Analysis of SPE Fractions. To assess the phenolic compounds present in each fraction, HPLC analyses were performed on an Agilent 1100 instrument (Agilent, Forest Hill, VIC, Australia) equipped with a quaternary pump and diode array detector (DAD), using gradient elution based on the method described by Cozzolino et al. (*26*) with a slight modification as detailed by Mercurio et al. (*27*). Data acquisition and processing were performed using Agilent ChemStation software (version A.09.03). A 20 μL injection volume was used for each sample, and DAD signals were recorded at 280, 320, 353, 370, and 520 nm. Compounds in each sample were identified by comparison of their retention times and UV/vis spectra with those of standards.

LC-MS Analysis of SPE Fractions. Identification of phenolic compounds derived from the SPE procedure was aided by LC-MS analyses performed on an Agilent 1200 instrument (Agilent, Forest Hill, VIC, Australia) equipped with a binary pump and Agilent 1100 diode array detector (DAD) connected in series to a 4000 Q Trap hybrid tandem mass spectrometer with a TurboIonSpray source (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Data acquisition and processing were performed using Applied Biosystems/MDS Sciex Analyst software (version 1.4.2). The column was a 150 × 2 mm i.d., 4 μm, 80 Å, Synergi Hydro-RP operated at 25 °C and protected by a 4 × 2 mm i.d. guard column (Phenomenex, Lane Cove, NSW, Australia). The solvents were formic acid/water (5:95 v/v, Solvent A) and formic acid/water/acetonitrile (5:15:80 v/v/v, Solvent B) with a flow rate of 0.200 mL/min. The linear gradient for solvent B was as follows: 0 min, 10%; 35 min, 35%; 50 min, 60%; 55 min, 90%, 60 min, 90%. The column was equilibrated with 10% B for 10 min prior to an injection. A 10 μL injection volume was used for each sample, and DAD signals were recorded at 280, 370, and 520 nm. Mass spectra were recorded between *m/z* 200 and 1500 with a scan time of 2 s in positive (IonSpray 5500 V) and negative (IonSpray −4500 V) ion modes during separate analyses. Other MS parameters were as follows: source temperature, 500 °C; nitrogen as curtain gas (CUR), 103.4 kPa; nebulizing gas (GS1), 344.7 kPa;

and drying gas (GS2), 344.7 kPa; entrance potential, 10 V (−10 V in negative mode); declustering potential, 60 V (−60 V in negative mode).

Nonretained Material from the Optimized SPE Procedure. The material which eluted upon loading an Oasis HLB cartridge was analyzed in positive and negative modes by LC-MS using the same apparatus and column with a flow rate of 0.300 mL/min and the following changes to the gradient and MS parameters. The linear gradient for solvent B was as follows: 0 min, 0%; 5 min, 0%; 30 min, 90%; 40 min, 90%, and mass spectra were recorded between *m/z* 50 and 1500. All other parameters remained the same.

Thiolysis and LC-MS Analysis of SPE Fractions.

Thiolysis in the Presence of Hydrochloric Acid. Fractions F1, F2, and F3 from a 2006 Shiraz wine were subjected to acid-catalyzed cleavage in the presence of cysteamine hydrochloride (*28*) under various conditions. Solvent-free fraction F2 was dissolved in methanol (1 mL), and solvent-free fraction F3 was dissolved in 1:1 formic acid/methanol (20 μL) and further diluted with methanol (980 μL). An aliquot (200 μL) of each solution was added to a screw-cap vial containing the thiolysis mixture (200 μL), which was prepared with cysteamine hydrochloride (50 mg), 32% hydrochloric acid (25 μL), and methanol (to give a total volume of 1 mL). The vials were sealed, heated at 65 °C for 30 min, and quenched with 0.1% TFA (600 μL). The samples were centrifuged at 4000 rpm for 5 min prior to LC-MS analysis using the same apparatus and column as described for the LC-MS analysis of SPE fractions. The solvents were formic acid/water (5:95 v/v, Solvent A) and formic acid/water/acetonitrile (5:15:80 v/v/v, Solvent B) with a flow rate of 0.300 mL/min. The linear gradient for solvent B was as follows: 0 min, 5%; 45 min, 25%; 46 min, 90%; 50 min, 90%. The column was equilibrated with 5% B for 10 min prior to an injection. A 10 μL injection volume was used for each sample, and DAD spectra were recorded from 200 to 650 nm. Mass spectra were recorded between *m/z* 200 and 1000 with a scan time of 2 s in positive ion mode (IonSpray 5300 V). Other MS parameters were as detailed previously, except for curtain gas, which was 96.5 kPa. The presence of depolymerization adducts was determined by detecting the protonated 2-aminoethylthio derivatives of catechin (*m/z* 366), epicatechin (*m/z* 366), epigallocatechin (*m/z* 382), and epicatechin-3-*O*-gallate (*m/z* 518).

Thiolysis in the Presence of Formic Acid. The thiolysis technique was repeated with the following alterations: solvent-free fractions F2 and F3 from a 2006 Shiraz wine were dissolved in formic acid (100 μL) and further diluted with methanol (900 μL). An aliquot of each solution (200 μL) was added to a screw-cap vial containing the thiolysis mixture (200 μL), which was prepared with cysteamine hydrochloride (50 mg) and methanol (to give a total volume of 1 mL). Three such vials were prepared from each fraction, with one vial of each fraction being heated, quenched, and centrifuged as above, prior to LC-MS analysis. The two remaining vials of each fraction were heated at 35 °C (to approximate the rotary evaporator water bath temperature). One vial of each fraction was heated for 15 min, and the remaining vials were heated for 30 min. The reactions were quenched, centrifuged, and analyzed by LC-MS.

Validation of the Optimized SPE Procedure. All samples were analyzed by HPLC to determine the components present in each fraction. Repeatability was determined by a single operator performing the optimized SPE method on a 2004 Shiraz wine sample for seven replicates spread over the course of several days. For ease of quantification of the phenolic material, the 280 nm HPLC chromatogram of each fraction was integrated as one large integral (0.5 to 32 min) for comparison with other replicates. Reproducibility was determined using an independent single operator performing the optimized SPE method on a separate 2004 Shiraz wine for three replicates spread over the course of several days. For ease of quantification of the phenolic material, the 280 nm HPLC chromatogram of each fraction was again integrated as one large integral for comparison with other replicates. Recovery of total phenolics was determined for 30 red wines, mainly Cabernet Sauvignon and Shiraz with vintages ranging from 1995 to 2006, by a single operator performing the optimized SPE method. Recovery was calculated by integrating chromatograms at 280 nm as one large integral, summing the chromatogram areas for F1, F2, and F3, dividing by the chromatogram area at 280 nm of the whole wine direct injection, and expressing the result as a percentage. Separation efficiency was determined in the same manner as Pinelo et al. (*16*) using HPLC areas at 280 nm for the three fractions F1, F2, and F3. Polymeric polyphenol separation efficiency (PPSE = polymeric polyphenols/{polymeric + nonpolymeric phenols}) was assessed for polymeric fractions F2 and F3, while nonpolymeric phenol separation efficiency (NPSE = nonpolymeric phenols/{nonpolymeric + polymeric phenols}) was assessed for nonpolymeric fraction F1.

Statistical Analysis. The results reported for repeatability were obtained from the average of seven replicate measurements; results for reproducibility were the average of three replicate measurements; and results for recovery were the average of single measurements for 30 different wine samples. The coefficients of variation, which were ≤6% in each case, were determined by dividing standard deviations by mean values of the replicates and expressing the result as a percentage. Statistical analyses were performed on Microsoft Excel 2003.

RESULTS AND DISCUSSION

Assessment of Polymer SPE Cartridges. Polymer SPE sorbents were identified as potential alternatives to typical C18-based SPE material for the isolation of polymeric polyphenols from red wine, due to the ability to directly load wine samples without the need for dealcoholization or pH adjustment. A range of polymer SPE sorbents were evaluated to identify the most suitable sorbent and conditions for optimum red wine polymeric polyphenol isolation. Brief screening exercises were undertaken with the various SPE sorbents to determine which combination of sorbent and eluents provided the most promising results.

HPLC Method for Compositional Analysis. Fractions obtained from the various SPE cartridges were analyzed by RP-HPLC (*27*) to determine the phenolic compounds present. **Figure 1** shows the 280 nm (all phenolics including polymerics), 520 nm (red-colored phenolics, e.g., anthocyanins and pigmented polymers) and 370 nm (flavonols) chromatograms of a direct injection of a commercial 2003 Shiraz wine. Direct wine injections such as this were used for decision-making by comparison with the fractions obtained from SPE of the corresponding wine on the various cartridges. The chromatograms in **Figure 1** are representative of direct injections of many other red wines of a similar vintage analyzed by this RP-HPLC method. The polymeric polyphenols are detected throughout the elution range as a broad "hump", causing the raised baseline (*8, 29–33*), and are compressed into a sharper, unresolved peak at the end of the gradient as the acetonitrile content is increased (*34*). Although this RP-HPLC method was developed primarily to resolve anthocyanins, there is reasonable resolution of many other wine components, and this HPLC method was deemed to be suitable for screening the fractions obtained by SPE.

Polymer Cartridges with Hydroalcoholic Eluents. Initial investigations were performed using a commercial Australian 2003 Shiraz wine on a variety of reversed-phase (Oasis HLB, Strata X) and mixed-mode (reversed-phase ion exchange; Oasis MCX, MAX, WCX, and WAX) polymer SPE cartridges, with eluents containing various amounts of water and methanol, acidified with 1% formic acid. These cartridges were chosen in an attempt to exploit some of the more diverse physicochemical properties of polymeric polyphenols, which are amphiphilic molecules (i.e., possess both hydrophobic and hydrophilic properties).

Sorbents such as Oasis HLB (divinylbenzene-*N*-vinylpyrrolidone copolymer) and Strata-X (styrene-divinylbenzene (SDVB) polymer, surface modified with *N*-methyl-2-piperidone moieties) possess both hydrophobic and hydrophilic properties and were deemed to be suited to the enhanced retention of polymeric polyphenolic material, when compared to typical SDVB (reversed-

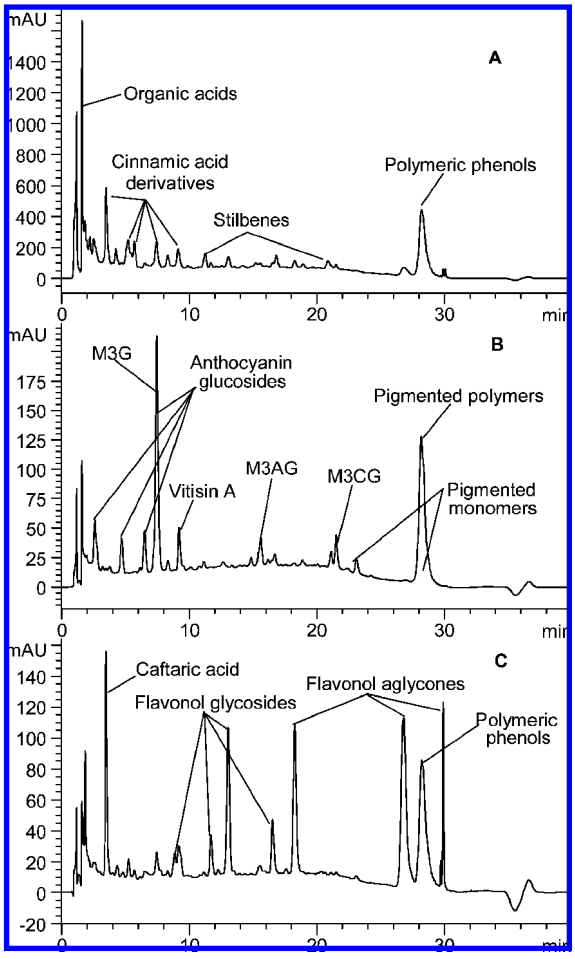

Figure 1. Chromatograms recorded at (**A**) 280 nm, (**B**) 520 nm, and (**C**) 370 nm of the direct injection of a commercial 2003 Australian Shiraz wine. M3G = malvidin-3-glucoside, M3AG = malvidin-3-acetylglucoside, and M3CG = malvidin-3-coumaroylglucoside.

phase) polymer sorbents. The various Oasis mixed-mode (reversed-phase ion exchange) polymer sorbents were assessed for their ability to retain the polymeric polyphenols while potentially allowing for the selective removal of the other classes of phenolics present in red wine. The wine sample was acidified with 0.1 M HCl prior to loading onto a conditioned cartridge, primarily to ensure that the flavylium forms of the anthocyanins were favored to facilitate their elution within a discrete fraction. Naturally, phenolic acids would also remain protonated (and potentially retained) due to the acidification.

Elution was performed with increasing percentages of methanol to provide the hydroalcoholic fractions. Analysis of the wine fractions by HPLC revealed that with all the mixed-mode cartridges polymeric polyphenols eluted with nonpolymeric components when the methanol content was as low as 25%, and material remained irreversibly adsorbed to the cartridges. Oasis HLB and Strata-X performed better, but polymeric polyphenols were eluted with nonpolymeric components when the methanol content was between 25 and 50%. Flavonols such as myricetin and quercetin continued to elute with polymeric polyphenols at higher methanol percentages (data not shown). Nonetheless, sorbents such as Oasis HLB and Strata-X were superior to the mixed-mode Oasis sorbents, and further method development focused on similar polymer sorbents with hydrophilic properties, in conjunction with a shift from hydroalcoholic to organic eluents.

Polymer Cartridges with Organic Eluents. Polymer sorbents with hydrophilic properties showed the most promise, but due to the limited success using aqueous methanol, the focus switched to using organic eluents in a manner similar to that used with C18 SPE. Oasis HLB was assessed, and several Varian polymer cartridges (Plexa, Focus, Nexus) were added to the investigation with organic eluents due to their ability to retain polar and nonpolar analytes. Strata-X, however, was omitted from this series of experiments as it retained some colored material that could not be eluted with various organic solvents and acid or base, regardless of eluent volume. A commercially available Australian 2003 Cabernet Sauvignon wine was used for an assessment of the retention characteristics of wine phenolics on the different sorbents with various organic solvents.

Variability of Sorbents During Cartridge Loading. Differences between the sorbents were observed when loading red wine and drying the various cartridges. A breakthrough of polymeric polyphenolic material occurred on the Focus cartridge during loading, while Nexus required slow drying so the sorbent bed did not crack and affect the separation. Furthermore, as Nexus can be used as a nonconditioned SPE (NC-SPE) sorbent, it was evaluated both with and without conditioning. Nexus performed better when it was conditioned, as the sample adsorbed in a substantially thinner colored band. In contrast, Oasis HLB was not adversely affected by drying, and the band formed upon loading was the thinnest of the cartridges tested. In addition, loading of a sample onto Oasis HLB was the most reproducible. After loading and drying the cartridges, elution with organic eluents such as acetonitrile, ethyl acetate, and methanol was evaluated.

Variability of Sorbents as a Function of Solvent Polarity. The range of sorbents differed in their retention characteristics for the various phenolic analytes depending on the solvent used. If the cartridges were not dried adequately, the water present from the initial loading caused unwanted elution of polymeric polyphenols when acetonitrile was applied. In the absence of any water, acetonitrile only caused elution of a portion of the nonpolymeric, noncolored phenolics, including aroma compounds (detected by the odor of solvent-free F1org). Marked differences were observed in relation to the elution of flavonols, particularly aglycones such as quercetin and myricetin. Plexa (hydrophilic surface with a polarity gradient to hydrophobic interior) and Nexus (SDVB-methyl methacrylate copolymer) performed similarly, with the majority of flavonols eluting with acetonitrile (or ethyl acetate with Nexus), while Focus (polar-enhanced SDVB) and Oasis HLB (DVB-*N*-vinylpyrrolidone) retained flavonols more strongly (**Figure 2**). Interestingly, acetonitrile caused some elution of polymeric polyphenols (ca. 29 min) from Nexus, but ethyl acetate did not.

First Observation of Obtaining Two Polymeric Polyphenol Fractions. Elution of anthocyanins and nonpolymeric pigments required acetonitrile and methanol, while any remaining phenolics and some polymeric polyphenols were eluted with methanol. Interestingly, a substantial portion of polymeric polyphenols could only be eluted from HLB and the Varian sorbents with formic acid followed by methanol, with the exception of the Nexus cartridge, which generally only gave one polymeric fraction. This outcome seemed to be partially due to the insolubility of some portion of the polymeric polyphenols once they were isolated from the wine matrix. Experimentation with eluent volume and composition was undertaken for elution of the two polymeric fractions. We established that 5 mL of methanol was sufficient to give the first polymeric fraction, which also contained some nonpolymeric species at this stage, and 0.3 mL of neat formic acid followed by 2.7 mL of 95% methanol yielded a distinctly

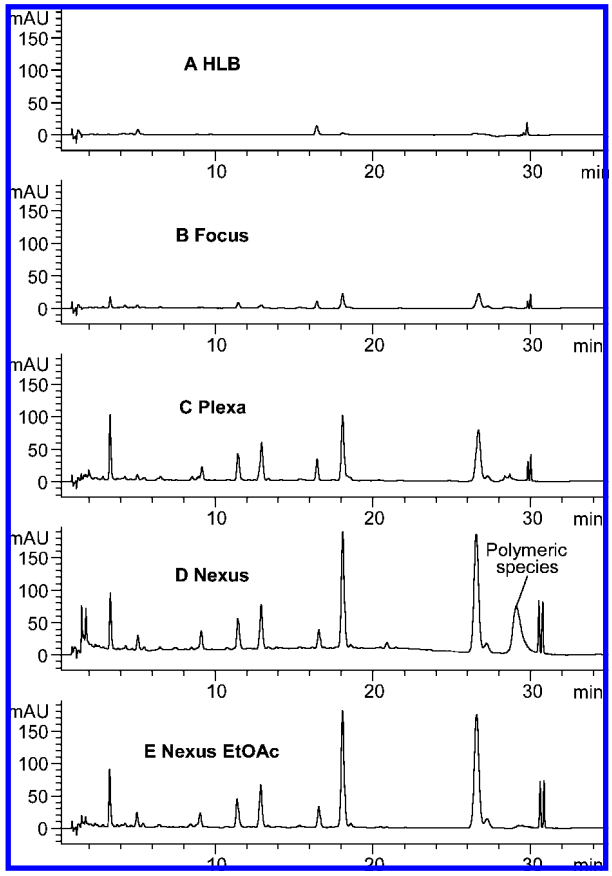

Figure 2. Chromatograms recorded at 370 nm displaying fraction F1org of a commercial 2003 Cabernet Sauvignon wine obtained from (**A**) Oasis HLB, (**B**) Focus, (**C**) Plexa, (**D**) Nexus, and (**E**) Nexus using ethyl acetate (EtOAc), showing the extent of flavonol retention by each cartridge. Note the elution of polymeric phenols (ca. 29 min) from Nexus in (**D**) but not (**E**).

different, second polymeric fraction. From this series of experiments, we were satisfied with the eluents used for the recovery of polymeric polyphenols from the cartridges, but further work was required to optimize the wash eluent.

Optimization of Polymeric Polyphenol Isolation on Oasis HLB. The results highlighted the differences in the chemical (e.g., functionalization) and physical (e.g., surface area) properties of the sorbents and led to the decision to pursue Oasis HLB for further method refinement. Improvement focused on the removal of all nonpolymeric phenols in the first (wash) fraction while maintaining retention of the polymeric polyphenols, which could then be eluted in two separate fractions. Furthermore, nonretained material was collected during sample loading to determine its composition. The potential to reuse an Oasis HLB cartridge after reconditioning was assessed, and the effect of formic acid on the polymeric polyphenolic species was examined. The volume of wine used throughout the experiments was deemed appropriate and maintained during the method optimization.

Elution of Material during Cartridge Loading. It was evident from LC analyses during the experimentation that some early eluting, polar components were not accounted for (data not shown). It was envisaged that these nonretained compounds would be similar to those reported by del Alamo et al. (*25*), who found that gallic and protocatechuic acids were not retained on a 60 mg Oasis HLB cartridge. Analysis of the nonretained material from our work by LC-MS revealed molecular ions, most notably in the negative mode, that were consistent with the presence of various hydroxybenzoic acids, along with non-UV absorbing material such as tartaric and malic acids (data not shown). The nonretention of these acids was of little consequence in the current work as they were not analytes of interest, but using a larger bed mass could overcome this lack of retention (*25*).

Cartridge Reuse after Reconditioning. Although reuse of Oasis HLB cartridges for subsequent separations led to slight differences in reproducibility between fractions, there was some success in using a cartridge up to three times for consecutive separations. However, cartridge reuse was implicated in the formation of artifacts, so reusing a cartridge would not be recommended.

Optimization of Wash Eluent Composition. The goal at this stage was to maximize polymeric polyphenol retention while ensuring all nonpolymeric phenols eluted in one wash fraction. It was clear from previous experimentation this would require fine balancing of the solvent polarity and an increase in volume of the eluent. Acetonitrile (a water-miscible, polar aprotic solvent) was a good starting point but not polar enough on its own. An additional study, using red wines of various ages, focused on combinations of acetonitrile with up to 10% water or methanol and 1% formic acid; this solvent system approached the desired eluent polarity but increased elution of polymeric polyphenolic material in the wash fraction (data not shown). Interestingly, an analogous observation was made by Pinelo et al. (*16*), where water with a methanol content greater than 10% led to undesired elution of polymeric material from a C18 Sep-Pak. Therefore, although the wash eluent required a polar modifier such as water or methanol, the polar component had to be less than 10%.

Effect of Solvent Polarity on Retention of Phenolics. As expected, water rather than methanol had the greatest effect in causing unwanted elution of polymeric material in the wash fraction, but some percentage of water or methanol was required to ensure complete elution of nonpolymeric material, especially flavonols. Water performed better than methanol in this regard, whereby a smaller percentage of water (5%) could be used in the wash eluent for a given volume. Furthermore, although some acid in the eluent was desirable to ensure that anthocyanins eluted together in their flavylium form, formic acid caused unacceptable elution of polymeric polyphenols in the wash fraction. This resulted in the decision to replace formic acid with HCl.

Artifact Formation Due to Wine Acidification with 0.1 M HCl. Acidification of a wine prior to loading onto HLB caused the formation of anthocyanin artifacts. This was particularly evident when assessing young wines (2005 and 2006 vintages), due to the higher levels of anthocyanins encountered in these younger wines. Artifact formation was readily apparent from HPLC analysis, with the 520 nm peak of the most abundant anthocyanin, malvidin-3-glucoside (M3G, T_R 8.5 min), being reduced as a new peak appeared at a longer retention time (T_R 15.3 min) (**Figure 3**). Other 520 nm absorbing material also began appearing on the baseline, presumably from derivatives of the other anthocyanins present.

The most prominent newly formed anthocyanin derivative was clearly less polar than the parent anthocyanin malvidin-3-glucoside but had an identical UV/vis spectrum (**Figure 3**, inset). Analysis by LC-MS (data not shown) gave a molecular ion with *m*/*z* 521, with a fragment at *m*/*z* 331, consistent with malvidin after the neutral loss of a derivatized glucose molecule (*m*/*z* 190). While not conclusive, these data are consistent with the anthocyanin derivative being malvidin-3-glucoside that has been ethoxylated on the glucose moiety by the action of HCl in the

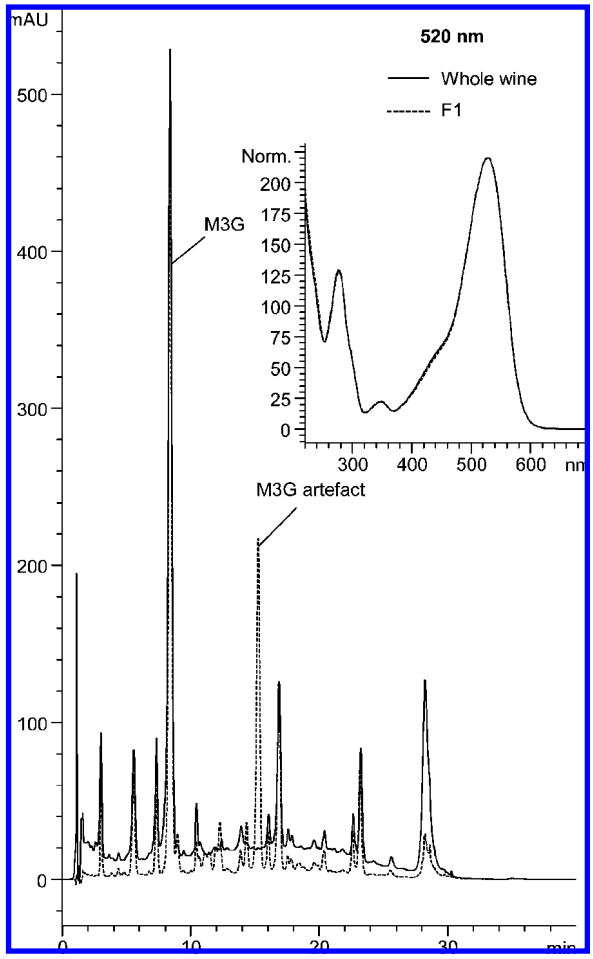

Figure 3. Overlaid chromatograms recorded at 520 nm of a direct injection of a commercial 2006 Shiraz wine and the corresponding wash fraction (F1), showing anthocyanin artifact. Note the flatter baseline for F1, where the polymeric material has not eluted in this fraction. Inset: overlay (normalized) of UV–vis spectra of malvidin-3-glucoside (M3G) and artifact.

presence of the ethanol inherent in the wine sample. Although the HCl concentration was implicated as a factor, the length of time the sample was left to dry on the cartridge appeared to be the main contributor to the formation of anthocyanin derivatives. Reusing a cartridge by first washing with water and conditioning and loading as usual seemed to enhance the formation of anthocyanin derivatives.

Method Improvement with Reduced HCl Concentration. Artifact formation was prevented by using a dilute solution of HCl and omitting the wine acidification prior to loading. This formed the basis of the optimized SPE procedure whereby the wash eluent was composed of 95% acetonitrile and 5% 0.01 M HCl. The use of dilute HCl to acidify the wash eluent alleviated some of the undesired elution of polymeric species caused by formic acid. Furthermore, although acidifying a wine meant the sample loaded in a thinner band, this step seemed to have little influence on the efficient elution of anthocyanins.

Optimal Wash Eluent Composition Achieved. Optimizing the wash eluent composition was a complex task. A compromise had to be made between preventing elution of polymeric polyphenols in the wash fraction and removing all nonpolymeric material from the fractions containing polymeric polyphenols; both of these factors were dependent on the aqueous content of the eluent. This compromise was easier to achieve with older wines (>2 years old) for the same reasons as those described by Pinelo et al. (*16*), namely, that aging affects polymeric polyphenol composition, and monomers and oligomers undergo changes such that they pose less interference in older wines due to their lower levels. This had a dual influence, whereby in younger wines a greater proportion (up to 10%) of polymeric species eluted in the wash fraction, and monomeric and oligomeric species would remain in the two polymeric fractions. Nonetheless, the wash eluent composition was optimized, but an adequate eluent volume was still to be addressed.

Wash Eluent Volume for Adequate Removal of Nonpolymeric Phenols. The wash eluent volume was investigated to ensure adequate elution of nonpolymeric material. In the end, a wash eluent volume of 40 mL was chosen for the range of red wines assessed. This volume was necessary to ensure complete elution of hydrophobic material, in particular flavonols such as myricetin and quercetin. Indeed, elution of most components was primarily complete after using 20 mL of this eluent, with the remaining 20 mL needed to remove residual traces of some compounds.

Efficiency of the Wash Eluent. The effectiveness of the wash eluent is highlighted by observing the relevant chromatograms. **Figure 4A** provides chromatogram overlays recorded at 280 nm of a direct wine injection, and the respective wash fraction of a commercial 1995 Shiraz. **Figure 4B** displays the relevant 520 nm chromatogram overlays, and **Figure 4C** displays the relevant 370 nm chromatogram overlays. These chromatograms show that removal of nonpolymeric species in one wash fraction was achievable. Additionally, the flat baseline of the wash fraction compared to the whole wine is noteworthy, where the polymeric polyphenols causing the raised baseline in the whole wine have been retained by the cartridge and subsequently recovered (**Figure 5**).

Pyranoanthocyanins Elute in the Wash Fraction. Based on the RP-HPLC method in use, the broad peak found at 29 min in a whole wine chromatogram would ordinarily be presumed to contain polymeric polyphenols. However, with the development of the SPE method, some of this material was found to exist in the wash fraction (**Figure 4**). Once separated from the bulk of the polymeric species, the peaks around 29 min in the wash fractions indicated the presence of discrete compounds and, in particular, red-colored components (520 nm chromatogram, **Figure 4B**). Analysis of the wash fractions by LC-MS revealed the occurrence of *m*/*z* values typical for molecular ions of pyranoanthocyanins (*32, 35, 36*) in this 29 min region, consisting mainly of 4-vinyl derivatives of malvidin-3-glucoside (data not shown). This was in accord with the orange-red color (*32, 35*) observed for the wash fractions for older wines, where anthocyanins were not dominating the perceived color of the wash fractions as they do with younger wines.

Obtaining Polymeric Polyphenol Fractions F2 and F3. Two fractions of polymeric material could be obtained after removing nonpolymeric material with the wash eluent. The first polymeric fraction was eluted with methanol (F2), whereas the second polymeric fraction could only be eluted with the use of formic acid followed by methanol (F3). Various other solvents such as acetone, tetrahydrofuran, and acetic acid, either alone or in combination with water or methanol, failed to elute this second fraction. **Figures 5A** and **B** show chromatogram overlays recorded at 280 and 520 nm, respectively, of the two polymeric fractions from the commercial 1995 Shiraz wine. These chromatograms are representative of the separations achieved using this SPE method and are in accord with results reported by many other researchers, whereby polymeric polyphenols lead to a broad baseline "hump" in RP-HPLC chromatograms (*8, 29–34*). Despite the differences in physicochemical properties between

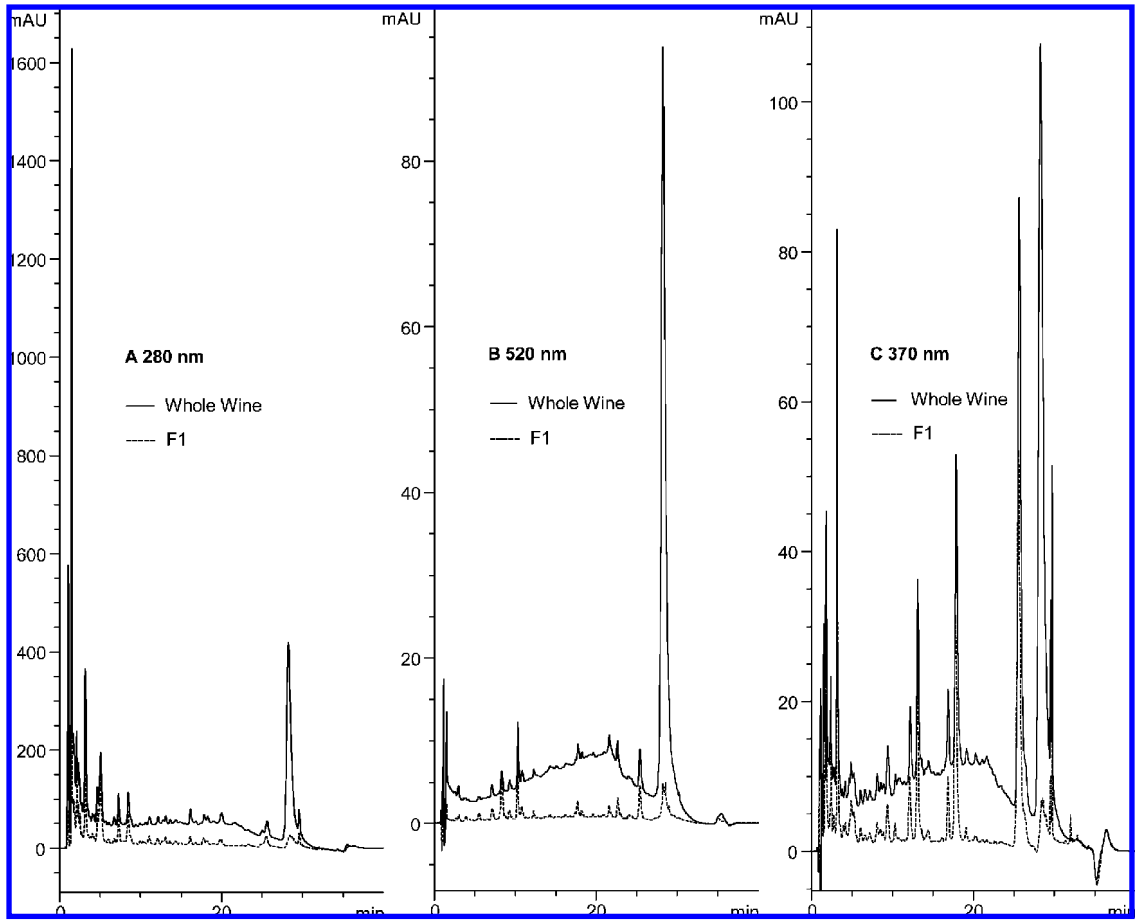

Figure 4. Overlaid chromatograms of a direct wine injection and the respective wash fraction (F1) of a commercial 1995 Shiraz wine recorded at (**A**) 280 nm, (**B**) 520 nm, and (**C**) 370 nm, showing the flat baseline of the wash fraction and absence of polymeric species.

the two polymeric fractions being responsible for their elution in two different SPE fractions, the polymeric polyphenols in F2 and F3 gave similar HPLC chromatograms. However, it appears that wine age played some role in the observed physicochemical differences, as discussed later.

Effect of Formic Acid on Polymeric Polyphenols. While obtaining two different polymeric polyphenolic fractions from Oasis HLB was encouraging, the need to use neat formic acid in the initial elution of F3 was somewhat concerning with regard to polymeric polyphenol integrity. The time F3 spent in contact with formic acid was limited to no more than 10 min, which was the time taken to elute the fraction from the SPE cartridge and concentrate it in vacuo. Nonetheless, to assess the effect formic acid was having with respect to potential depolymerization of this polymeric fraction, several thiolysis experiments were conducted with cysteamine as the nucleophile (*28*). This method was chosen for reasons including that the reagent itself is not UV-active, depolymerization adducts give abundant molecular ions in positive mode using ESI-MS detection, and the protonated, N-containing molecular ions clearly differentiate thioadducts from other phenolic material.

A 2006 commercial Australian Shiraz wine was fractionated using Oasis HLB, and the fractions (F1−F3) were subjected to thiolysis with cysteamine under various conditions. The wash fraction (F1) was included to identify the presence of depolymerizable material in that fraction. Thiolysis was conducted using HCl (*28*) or formic acid at 65 °C, with formic acid also being assessed at 35 °C (to approximate the rotary evaporator water bath temperature) for 15 and 30 min. The samples were analyzed by LC-MS after quenching, which revealed that depolymerization adducts were not present in the 35 °C samples heated for 15 min but were present in F3 heated at 35 °C for 30 min (data not shown). Depolymerization adducts were present in the samples heated at 65 °C for F2 and F3, but no apparent adducts were identified in F1, leading to the conclusion that the compounds present in F1 were not polymeric in nature. Furthermore, HCl was much more effective at catalyzing the depolymerization than formic acid, more than likely due to the presence of water in the HCl reaction. It was concluded from these experiments that formic acid had a limited effect on the polymeric material in F3, particularly as the time and temperature at which the two were in contact were restricted.

Validation of the Optimized Method. Having addressed the many factors required in developing the optimized SPE method, validation was undertaken to determine its repeatability and reproducibility. The coefficients of variation were no greater than 3% for repeatability of any fraction, whereas the coefficients of variation for reproducibility were no greater than 6%. These figures fall within an acceptable range, showing that the method is repeatable and, perhaps more importantly, reproducible by an independent operator performing the method.

Recovery of Phenolics for the Optimized Method. Recovery of phenolics in each fraction was also determined by performing the SPE procedure on over two dozen commercial Australian red wines of various ages. **Table 1** shows the recovery figures for these wines based on integration of the HPLC 280 nm chromatograms of the SPE fractions compared to a direct injection of the corresponding wine. Recoveries of greater than 85% for wines of various ages and styles were achieved, affording an average recovery of 88 ± 3%. If material

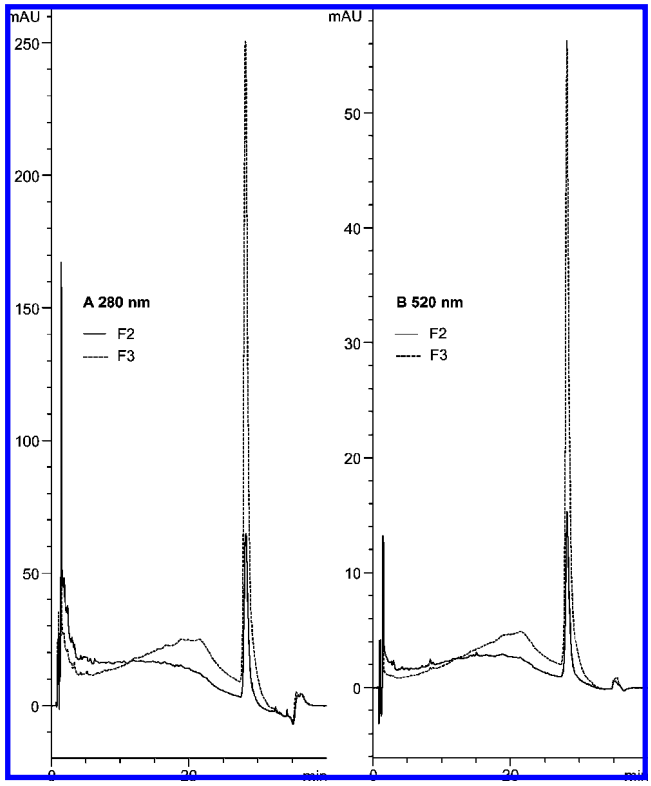

Figure 5. Overlaid chromatograms of polymeric fractions (F2 and F3) from a commercial 1995 Shiraz wine recorded at (**A**) 280 nm and (**B**) 520 nm, showing the absence of nonpolymeric material, which was found in the wash fraction.

not retained by the SPE cartridge upon loading a wine sample was accounted for, the recovery figure was closer to 95% (data not shown).

Separation Efficiency of the Optimized Method. Separation efficiencies as determined using HPLC areas at 280 nm for the three fractions derived from the SPE procedure are shown in **Table 1**. The values reported for F1 are calculated assuming the peak at 29 min is composed of polymeric material, although as discussed previously much of this peak in F1 actually consists of monomeric pigments such as pyranoanthocyanins. Regardless, the separation efficiencies are all high and approach 90% or greater for all fractions across different wine styles of various vintages. These efficiencies are comparable to those reported for red wine recently by Pinelo et al. (*16*) and show that the wash fraction (F1) contains no polymeric material, while polymeric fractions F2 and F3 are essentially free of nonpolymeric species.

Relationship of Wine Age to F2 and F3. Wine age was identified as being related to the relative amounts of material found in the two polymeric fractions. **Table 2** shows the amount of 520 nm absorbing material in F2 and F3 as a percentage of the combined 520 nm HPLC area of these fractions, for four different vintages of a commercial Australian Cabernet Sauvignon wine from a single winery. This highlights the effect of wine age on the relative proportions of colored polymeric species eluting in each fraction, whereby in younger wines there is a greater amount eluting in the first polymeric fraction (F2). **Table 2** also displays the ratio of HPLC areas at 520 and 280 nm for each fraction as a percentage, generally showing F2 to have a higher proportion of colored compared to noncolored material relative to F3, for a given wine. These two fractions appeared to be related to the changes polymeric polyphenols undergo during aging and are associated with the extent of pigmentation.

Table 1. Recovery and Separation Efficiency Values for a Range of Commercial Australian Red Wines Based on Integration of the HPLC 280 nm Chromatograms of Fractions Obtained by SPE

			separation efficiency		
wine	year	recovery	F1	F2	F3
Shiraz Winery 1	1995	85%	97%	93%	96%
Shiraz Winery 1	1999	87%	95%	95%	97%
Shiraz Winery 1	2000	90%	96%	93%	94%
Shiraz Winery 1	2003	87%	96%	91%	93%
Cab Sauvignon Winery 2	1997	89%	96%	94%	96%
Cab Sauvignon Winery 2	2001	87%	97%	91%	95%
Cab Sauvignon Winery 2	2003	86%	96%	89%	92%
Cab Sauvignon Winery 2	2004	92%	96%	91%	93%
Shiraz Winery 3	2003	89%	94%	90%	92%
Shiraz Winery 4	2003	92%	96%	91%	94%
Shiraz Winery 5	2003	89%	95%	89%	93%
Shiraz Winery 6	2003	89%	96%	89%	92%
Shiraz Winery 7	2002	93%	96%	88%	92%
Shiraz Winery 8	2002	88%	96%	89%	91%
Shiraz Winery 9	2003	88%	97%	89%	94%
Shiraz Winery 10	2003	85%	97%	90%	94%
Shiraz Winery 11	2002	80%	96%	89%	91%
Shiraz Winery 12	2002	87%	96%	93%	95%
Shiraz Winery 13	2006	91%	97%	88%	91%
Shiraz Cab Sauv Winery 14	2006	89%	95%	88%	90%
Cab Sauvignon Winery 15	2002	89%	97%	89%	94%
Cab Sauvignon Winery 16	2002	88%	96%	90%	94%
Cab Sauvignon Winery 17	2003	88%	97%	89%	94%
Cab Sauvignon Winery 18	2002	88%	97%	89%	91%
Cab Sauvignon Winery 19	2003	86%	96%	91%	94%
Cab Sauvignon Winery 20	2003	86%	96%	91%	93%
Cab Sauvignon Winery 21	2003	91%	96%	90%	93%
Cab Sauvignon Winery 22	2003	89%	97%	91%	91%
Cab Sauvignon[a] Winery 23	2002	87%	97%	90%	94%
Cab Sauvignon[b] Winery 24	2002	87%	97%	90%	93%
Average ± SD		88 ± 3%	96 ± 1%	90 ± 2%	93 ± 2%

[a] Blend containing Merlot, Petit Verdot, and Cab Franc. [b] Blend containing Merlot and Petit Verdot.

Table 2. Effect of Age on Colored Polymeric Material Present in F2 and F3 Obtained by SPE of Commercial Australian Cabernet Sauvignon Wines of Different Vintages From a Single Winery[a]

	HPLC area at 520 nm		HPLC area F2	HPLC area F3
vintage	% in F2	% in F3	520/280 (%)	520/280 (%)
1997	20	80	20	19
2001	24	76	29	22
2003	29	71	34	25
2004	41	59	42	27

[a] Analyses were conducted in June 2007.

Furthermore, there are potential implications for these fractions in relation to the mouthfeel properties of aged red wine, as the two fractions may have different sensory properties.

Retention and Solubility of F3. Kantz and Singleton (*4*) also obtained two polymeric fractions for aged red wines in their LH-20 fractionation study. While they attributed the difference of their fractions to polymer size and hydrogen bonding interactions with the sorbent, we also believe another factor is the insolubility of some components of red wine polymeric species once they are isolated from the wine matrix. Indeed, in our work the second polymeric fraction (F3) obtained was not soluble in water, methanol, or acetic acid but was soluble in acetone or formic acid. It is of interest that acetone, although able to solubilize polymeric species, could not elute F3 from the SPE cartridge. The fact that formic acid (a chaotrope) worked was thought due to a combination of its solubilizing properties and its ability to interrupt the hydrogen bonding between the polymeric species and the hydrophilic pyrrolidone

moieties of the SPE sorbent. The results indicated that the methanol eluting polymeric fraction (F2) was substantially more hydrophilic than the formic acid/methanol eluting fraction (F3), with the latter being predominantly hydrophobic (insoluble in water or methanol) but still possessing hydrogen bonding capabilities. This exemplifies both the amphiphilic nature of red wine polymeric polyphenols and the different retention mechanisms of the analytes on the sorbent and indicates that F3 is markedly different from F2 in its physicochemical properties.

Relationship of Subunit Composition to F2 and F3. Additional information regarding potential reasons for the difference in physicochemical properties of F2 and F3 was gleaned from the depolymerization experiments. Although the experiments were not designed to determine depolymerization yield or subunit composition, a difference was revealed between F2 and F3 samples depolymerized according to the method of Torres and Selga (*28*). While both fractions F2 and F3 contained thioadducts of catechin, epicatechin, and epigallocatechin, only F3 contained the adduct derived from epicatechin gallate, which is typically found in grape seeds. This may explain some of the physicochemical differences observed for the two polymeric fractions. The advent of this SPE method makes investigation of the detailed structure of these red wine polymeric polyphenols possible.

In summary, a new SPE method to isolate polymeric polyphenols, based on a copolymer Oasis HLB cartridge, has been developed and validated using commercial red wines. The method affords a wash fraction (F1) containing the majority of the nonpolymeric material present in a red wine and yields the polymeric species as two distinct fractions (F2 and F3) which possess different physicochemical properties. There appears to be a relationship between the amount of material found in each polymeric fraction and wine age. The method is ideally suited to aged (two years or older) red wines of different styles. Figures for reproducibility, repeatability, recovery, and separation efficiency were all highly acceptable and give confidence in using the method for polymeric polyphenol isolation. The differences observed between the two polymeric fractions obtained from red wines using this method require further investigation in terms of the structural characterization and sensory properties of these polymeric polyphenol fractions.

Supporting Information Available: Table containing repeatability and reproducibility values for SPE separations of commercial Australian red wine. This material is available free of charge via the Internet at http://pubs.acs.org.

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